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Characterization and structural analysis of the Neisseria gonorrhoeae outer membrane protein II and its functional role in attachment of the gonococcus to eukaryotic cells

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Characterization and structural analysis of the Neisseria gonorrhoeae outer membrane protein II and its functional role in attachment of the gonococcus to eukaryotic cells.

by

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DISSERTATION

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DISSERTATION ABSTRACT

This dissertation is about the N. gonorrhoeae outer membrane protein called PII. PII is identified by the heat modifiability of its apparent molecular weight (M_r). A strain can express none, one, or several different PIIs. PII variants a, b, c, and d from strain F62 and PII a, b, c, d, and e from strain FA1090 were selected in vitro. The variants from both strains had similar biochemical characteristics. The PIIs had a substantial content of hydrophic amino acids, very basic pIs (greater than 8.5), and M_rs ranging from 30 to 32 Kdaltons at 100°C. Peptide mapping studies showed that the PIIs contained conserved as well as unique cleavage sites, suggesting that the PII variants contained variable and constant regions. Antigenic analysis of PII variants by the Western blot technique, combined with peptide mapping, demonstrated that the PIIs contined antigenically conserved as well as unique peptides.

Studies were performed to analyze the role of PIIs in gonococcal attachment. A technique was developed to measure the binding of ^{125}I-labelled outer membrane (OM) vesicles from PII$^-$ and PII$^+$ organisms to HeLa 229 cells on coverslips. The binding of PII$^-$ and PII$^+$ OMs was pH dependent, reached equilibrium at 60 to 90 minutes, and was not fully reversible. Increased binding was highly associated with PII content. Binding did not always reach satruation and Scatchard analysis suggested a complex binding system. Attachment of OMs in the presence of immune, PII specific, rabbit IgG was only slightly inhibited (20%). Results from attachment experiments using ^{125}I-labelled PII$^+$ OMs in the presence of 100 fold, competing, unlabelled PII$^-$ OMs suggested that PII may not be directly involved in attachment. The nature of the HeLa receptor was partially characterized as a 15,000 dalton protein. In conclusion, the

surface of the gonococcus appears to be a dynamic mosaic which alters in composition and antigenic characteristics. PII contributes to this antigenic diversity and yet, the protein also contains antigenically conserved regions. The expression of PII by the gonococcus is highly associated with enhanced binding to eukaryotic cells, but it may not be involved as the ligand.

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DEDICATION

To the members of my family:

Barbara, Doug, Mary, Emily, and my father, Benjamin,

who taught persistence and commitment.

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LIST OF ABBREVIATIONS

OM	Outermembrane
CM	Cytoplasmic membrane
PG	Peptidoglycan
PI	Protein I
PII	Protein II
PIII	Protein III
LPS	Lipopolysaccharide
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
PMNs	Polymorphonuclear leukocytes
RBCs	Red blood cells
Mdalton	Megadalton
Kdalton	Kilodalton
M_r	Molecular weight
CNBr	Cyanogen bromide
McAbs	Monoclonal antibodies
NHS	Normal human serum
CFU	Colony forming unit
ml	Milliliter
ug	Microliter
ELISA	Enzyme linked immunoabsorbent assay
Op	Opaque
Tr	Transparent
TLC	Thin layer chromatography
EDTA	Ethylenediamine tetraacetic acid
NaHc	Sodium acetate
HAC	Acetic acid

2-ME	Beta-2-mercaptoethanol
PPT	Proteose peptone thiotione
DOC	Deoxycholate
PMSF	Phenylmethylsulfonyl flouride
HBSS	Hank's balanced salt solution
BSA	Bovine serum albumin
TEM	Transmission electron microscopy

Chapter 1

Introduction

Neisseria gonorrhoeae (the gonococcus) causes gonorrhea, the most common of the reported bacterial diseases in the United States. It is estimated that about 2 million infections occur each year although only one-half of these cases are reported. This large number of infections and the consequences of untreated infection, especially pelvic inflammatory disease and infertility, make gonorrhea a major health problem.

The major areas of research on gonococcal infection include the study of the organism's antigenic diversity, and its ability to attach to and invade the host's mucosa. These areas of research are important in order to better understand the pathogenesis of infection and determine whether or not effective immunoprophylatic measures are possible. One area of research has focused on the study of the components of the gonococcal outer membrane which are involved in interaction with the host cell. This dissertation is about N. gonorrhoeae and one of its variable surface proteins, protein II. The studies were aimed at characterizing the structure of protein II (PII) and its multiple forms, and defining the role of PII in gonococcal attachment to human cells.

The dissertation is organized into five chapters. Chapter 1 is an introduction to the immunobiology of N. gonorrhoeae and gonococcal

infections. The purpose of this section is to acquaint readers with the information they will need to review this work. Gonococcal outer membrane components and what is known about their structure and function in disease are reviewed; also provided is a discussion of possible attachment mechanisms. Data on PII and its unusual structural diversity are presented in Chapter 2. Studies on the attachment function of PII are in Chapter 3 and studies on the antigenic diversity of PII are in Chapter 4. Chapter 5 is an assessment of the results and a discussion of future prospects. Chapter 2, 3, and 4 are organized in the manner of journal articles in order to facilitate the flow of information presented.

Taxonomy and pathogenicity of N. gonorrhoeae

The members of the family Neisseriaceae include the genera Neisseria, Branhamella, Moraxella, and Acinetobacter. All species are Gram stain negative and possess the typical gram-negative cell envelope. The organisms are short rods to coccoid forms and occur in short chains or pairs.

The two species of the genus Neisseria, pathogenic for man, are the gram-negative diplococci N. gonorrhoeae and N. meningitidis. Both species are mucosal pathogens. N. gonorrhoeae most frequently causes infections of the human genital tract. The gonococcus has several characteristics which contribute to its success as a pathogen including variable composition of its outer membrane and marked antigenic diversity of the outer membrane components. Several variable components appear to have multiple forms, and these can vary in abundance. The antigenic diversity presumably helps to promote repeated infections because the immune system does not recognize the antigenic determinants of the second infecting strain. It is now thought that a gonococcal

infection may stimulate a protective immune response to repeated challenge with the homologous organism; however, the response will not prevent infection with another strain (1,2,3). It is not known whether or not the immune response provides site-specific protection.

Gonococcal colony phenotypes and disease.

Variation in gonococcal colony phenotypes was described by Kellogg and co-workers in 1963 (4). They observed cultures of urethral exudate with a dissecting microscope and described four colony types, i.e., T1, T2, T3, T4 (Table 1.1). The colony morphologies were correlated with infectivity. Urethral inoculations of human volunteers with gonococci from T1 and T2 colonies caused typical gonorrhea, while inoculation with gonococci from T3 or T4 colonies did not. Subsequent studies from Kellogg's group showed that the colony types could be subcultured daily, their phenotypes maintained and infectivity preserved for as long as 17 months in vitro (5).

Swanson (6) and Jephcott (7) independently discovered that organisms from T1 and T2 colonies had pili while gonococci from T3 and T4 colonies did not. Pili or fimbriae are the hair-like appendages that extend from the surface of the organism. (Figure 1.1).

Several investigators also have noted color differences in gonococcal colonies and have expanded the Kellogg typing scheme to include the colonial characteristics of opacity and transparency (8). (Table 1.1) In reality, the opacity/transparency colony variants are a continuum from very opaque through subtly opaque to very transparent colonies. Each strain is apparently unique in the extent of this variation. Colonial opacity has been correlated with the presence of

one or more similar proteins in the outer membrane, the PIIs (8,9).

Colonial characteristics of isolates have been correlated with infections at certain anatomic sites and with some clinical forms of gonococcal infection (10,11,12, Draper and Brooks, unpublished observations). In men, the organisms cultured from the urethra and pharynx tend to form opaque colonies (11). In women, isolates from the urethra, pharynx, and cervix (at midcycle) also form opaque colonies (11,13). However, at menses, the endocervical isolates usually form colonies which are more transparent than at other times during the cycle. Isolates from Fallopian tubes form more transparent appearing colonies than matching endocervical isolates (12). In disseminated gonococcal infection (DGI), blood and joint isolates yield very transparent colonies (Draper, James, and Brooks, unpublished observations). Isolates from the male rectum tend to form opaque colonies unless the patient has symptomatic proctitis, then the isolates tend to form transparent colonies (Draper and James, unpublished observations). Interestingly, asymptomatic male consorts of women with salpingitis or DGI tend to be infected with gonococci which form transparent colonies. These correlations between site of infection and colony phenotype have led to the hypothesis that N. gonorrhoeae from transparent colonies may represent the form of the organism present after tissue invasion. The mechanism of invasion is not known. It probably is a complex process involving changes in the surface of the gonococcus and mechanisms which allow the organism to avoid the host's defense systems.

It is important to recognize that each gonococcal strain has the ability to shift from phenotype to phenotype and because the rate of

change is so high, any population of organisms will be mixed (Table 1.1). In vitro, upon primary isolation, the isolate tends to have multiple colony phenotypes (Draper, unpublished observations). With selective subculture, the organisms usually can be stabilized into a uniform colony phenotype with greater than or equal to 98% homogeneity. Upon nonselective subculture, the organism will tend to form a transparent, nonpiliated phenotype, and the phenotype will rarely be uniform. The molecular basis of this phenotypic shift has not been described. It could involve chromosomal rearrangement or movable genetic elements (plasmids). However, it is not thought to be a classic, spontaneous mutational event because the rates of occurrence are very high. The only clue to the control mechanism is that the phenotype shift is "environment dependent." The organism apparently responds to changes in the environment, whether it be a medium change *in vitro*, or menstrual cycle changes *in vivo*. This flexibility assures high adaptability and survival because there are always subpopulations of different phenotypes available to respond to selective pressures.

Mayer has conducted extensive tests to determine what conditions could modify or promote the phenotype shift from opaque (Op) to transparent (Tr) colonies and vice versa (14). He found transition rates for Op to Tr and back to Op colonies to be about $1\text{-}3 \times 10^{-3}$ per colony forming unit (cfu) per generation. The rate of shift from Op to Tr was only slightly higher than the rate of Tr to Op. He tested the effect of numerous compounds and none of them affected transition rates. (see genetics section for more information) Thus, we are left with the notion that within a population there are several and perhaps many phenotypes, switching on and off, and that it is the phenotype which

best survives the environmental conditions that emerges as the predominant phenotype. Results of recent animal studies by McBride and Heckles suggest that this may also occur in human infection (15).

The cell envelope

N. gonorrhoeae possesses a typical gram-negative cell envelope consisting of an inner cytoplasmic membrane (CM), a peptidoglycan layer (PG), and an outer membrane (OM), (Figure 1.1).

The CM contains numerous proteins, phospholipids, the electron transport chain and the active transport functions (16,17). The CM is rich in phosphatidylethanolamine and has a buoyant density of 1.141 gm/cm^3. It does not contain lipopolysaccharide and the distribution of lipids differs from the outer membrane. The CM contains a lower proportion of cardiolipin and increased proportions of phosphatidylglycerol when compared to the OM (18,176).

The PG of N. gonorrhoeae has a similar composition of amino acids and amino sugars as other gram-negative bacteria. It contains muramic acid, glutamic acid, alanine, meso-diaminopimelic acid and glucosamine in molar ratios of 1:1:2:1:1, respectively (18,19). The PG is responsible for the structural integrity of the organism.

Gonococcal peptidoglycan has several unique characteristics. N. gonorrhoeae, unlike other gram-negative organisms, rapidly turnover their PG during exponential growth, (19) and shed soluble fragments into the growth medium (20). In addition, the extent of the crosslinking of the glycan strands is slightly higher than most other gram-negative bacteria and appears to be strain variable (21). Recently, interest in gonococcal PG has increased as several important biological effects have

been attributed to it. Intact PG or its soluble derivatives may the augment the activities of cells and molecules involved in the immune response and inflammation (22). PG activities include mitogenicity (23), adjuvanicity (24), and the consumption and activation of complement in the presense of antibody (25). PG has also been implicated in the process of arthritis (26) and in direct, toxic damage to human Fallopian tubes (25).

Numerous studies have been performed for the characterization of the gonococcal OM. By sucrose density gradient centrifugation, the OM exhibits a buoyant density of 1.22 g/cm^3 (27,28). Its increased density compared to the CM is thought to be due in part to its unique content of lipopolysaccharide.

The protein composition of the OM is more simple than the CM and contains several, prominant proteins species (29). Some of these proteins have recently been named and a nomenclature has been proposed for the gonococcal OM proteins (30).

Gonococcal outer membrane proteins

Protein I

Protein I (PI) is the predominant OM protein species. It comprises up to 60% of the total OM protein (29); it is a transmembrane protein (31), and is believed to complex with protein III (PIII) (see below) to form channels in the lipid bilayer (32,33,34). These channels are known as porins. The PI channel has been estimated as 25 angstroms (internal diameter) by passive diffusion studies. It is anion selective as opposed to the cation selective porins of other gram-negative bacteria. (34) This selectivity may be responsible for the sensitivity of the

gonococcus to many antibiotics and dyes (34,35).

PI is present in all gonococci regardless of colony phenotype (36), and the subunit's apparent molecular weight (M_r) varies between strains, ranging from 32,000 to 38,000 as determined from sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli technique (37). The subunit M_r of PI is the same for all colony phenotype variants within a single strain, and the M_r does not change with SDS solubilization at 37°C vs. 100°C.

PI is a predominant surface antigen currently being used for serotyping strains of N. gonorrhoeae (38,39). Sandstrom and colleagues have shown with a panel of monoclonal antibodies that there are probably two major "groups" of PI molecules. They have proposed the exsistance of two structural genes and the designations of protein 1a and 1b based on this serological evidence (40). It is not known if these two genes are co-expressed. Swanson (41), using a two-dimensional thin layer chromatography, ^{125}I-peptide mapping procedure, showed that there were two groups of structural homology for PI. One group correlated with high M_r and the other correlated with low M_r. He further demonstrated that for each gonococcal strain, the PI contained unique peptides which were exposed at the membrane surface.

Blake et al. (42) have studied the in situ sensitivity of PI to cleavage by alpha chymotrypsin and trypsin. They described two fragments which remained associated with the membrane and proposed a configuration for PI. They suggested that PI loops back on itself and that the two terminal portions of the protein are buried in the membrane (Figure 1.1).

Recent studies have shown that PI may be transferred between

gonococcal membranes and human RBC membranes (43). The mechanism is not completely understood, but involves the spontaneous insertion of PI into these eukaryotic membranes. Blake showed that PI maintained an orientation in the gonococcal membrane and was sensitive to protease cleavage. When it was transferred to the RBC membrane, it was no longer sensitive to cleavage. PI could only be cleaved by lysis of the RBC and when the cytoplasmic side of the membrane was exposed. Studies using purified PI and artificial "black lipid" membranes confirm that PI rapidly inserts into lipid membranes and does so in a specific orientation. This finding suggests that PI may be involved in the attachment phenonmenon. The insertion of PI may also trigger endocytosis of the gonococci by the host cell (44,45). This is an intriguing possibility and needs investigation.

Protein II

Another prominent outer membrane protein of the gonococcus is PII. It is characterized by "heat modifiability" of the subunit's M_r with an increase in M_r when the specimen is solubilized in boiling 2% SDS as compared to solubilization at 37°C (46,47). The subunit's M_r ranges from 24,000 to 32,000 by Laemmli SDS-PAGE.

In 1978, Swanson demonstrated that several colony variants were possible in a single strain and frequently these had one or more PIIs (8, 136). Swanson has shown that a colony which has the very dark and highly opaque characteristic usually has multiple PIIs. To date, there has been no description of Op colonies containing organisms which do not have a PII. Lamden and Heckles and other workers have shown that in a single gonococcal strain, multiple variants of PII can be selected by

detecting colonial variants (36,47).

The Tr colony phenotype may or may not have OM-associated PIIs (36). It is not known if strains never expressing PII occur in vivo. In the laboratory it is possible to obtain strains by non-selective subculture which no longer form Op colonies. One PII species, found in organisms from a Tr colony phenotype, has been termed "leukocyte association factor" and is associated with increased gonococcal adherence to and increased ingestion by polymorphonuclear leukocytes (PMNs), (48,49). Two PIIs have been associated with this phenomenon: those of M_r 28,000 and 29,000 (at 100°C). Gonococcal strains may express one or both or neither of these proteins. (48) Rest et al. have shown that there is enhanced killing of gonococci with PIIs by human PMNs compared to those without (50). The M_r of the PIIs in their studies were not stated.

Swanson has suggested that PIIs are associated with increased intergonococcal adherence. He demonstrated by transmission electron microscopy (TEM) of organisms in intact Op colonies that they had large zones of surface contact with neighboring gonococci. He proposed that the adhesions lead to increased colonial aggregation and thus, increased colony friability and opacity (8). As will be seen from the data presented here, this association does not always occur and only some of the PIIs are associated with colony opacity and friability.

The presence of PII in the gonococcal OM has been associated with increased susceptibility of killing of the organism by trypsin treatment (51). This effect has been demonstrated by large zones of growth inhibition around trypsin saturated filter paper discs placed on lawns of Op colonies. Also, in liquid cultures, it has been shown that as

much as 200 ug/ml trypsin has little effect on the growth rate of organisms from Tr colonies whereas organisms from Op colonies were killed. Blake et al. extended these studies to the inhibitory effects of alpha chymotrypsin and showed that these effects were directly related to the cleavage of PII and PI. In Blake's structural model of the arrangement of PI, PII, and PIII in the gonococcal outer membrane (Figure 1.1), PII is shown as the most surface exposed molecule with one major cleavage site for trypsin and chymotrypsin (42).

James et al. (52) showed that organisms forming Op colonies were more sensitive to the bactericidal action of normal human serum than isogenic organisms from Tr colonies. It is possible that different complement activation pathways may be involved in serum killing of these two phenotypes, but the studies have not been done. It should be noted that PII is not exclusively involved in serum sensitivity; PI and LPS are both very important antigens in serum bactericidal reactions (52,53,54).

In studies with chicken embryos, Salit and Gotschlich (55) demonstrated that gonococci from Op colonies were less virulent than organisms from Tr colonies. The LD_{50}s were 3 to 10 fold higher for organisms from Op colonies than those from Tr colonies.

Swanson (56) reported TLC peptide mapping results of several ^{125}I-labelled, purified PIIs from different strains and demonstrated a high degree of apparent structural homology. However, each PII peptide map contained several unique fragments. Heckles compared the peptide maps of PIIs extrinsically labelled in whole organisms to those of purified PIIs and found that the maps were very different (128). A few of the peptides which he found in the fully labelled samples corresponded to

those in the extrinsically labelled sample. He suggested that these peptides were surface located.

PIIs may be expressed in the presence or absence of pili. Within a strain, the PIIs in piliated and nonpiliated organisms appear to be the same by several criteria, e.g., molecular weight, peptide map and Western blot reaction with PII specific antiserum (Draper, Brooks, unpublished observations).

Finally, PII is very antigenic both in purified form and in situ. PII is part of the membrane complex which is responsible for serotypic specificity in the serotyping scheme proposed by Johnston (27). It is also a prominent antigen detected by convalescent sera from men and women with gonococcal disease (Brooks, unpublished observations).

Protein III

This gonococcal outer membrane protein was first described in 1980 (57). The protein has an M_r of 30,000 in the absence of beta-2-mercaptoethanol (2-ME) and 31,000 in the presence of 5-8% 2-ME. The observation suggests an internal disulfide linkage. The M_r is the same in all strains and in all intrastrain colony phenotypes (58,59).

PIII in situ, is very resistant to exogenous proteolytic cleavage (42) and does not extrinsically label well with lactoperoxidase catalyzed ^{125}I-iodination (60), suggesting less surface exposure and accessability than PI or the PIIs. Surface crosslinking studies with di-thiobis propionimidate (43,57) showed that PIII is closely associated with PI and may exist in the membrane as a complex of one PIII molecule and three PIs. The exact arrangement of this heterooligomer is not known, but PIII may function in some way to maintain the structural

integrity of the porin diffusion channel.

Peptide mapping studies of Judd (58) showed that there is only one structural homology group for PIII, with no intrastrain unique peptides. Interest in PIII has gained ground as immunologic studies have demonstrated that PIII is antigenically active, serologically crossreacts in most strains, is part of the serotype antigen and co-precipitates with PI in immunoprecipitation experiments (57, 60). PIII appears to be a structurally conserved outer membrane protein.

Pili

The pilin molecule is the most studied of the gonococcal outer membrane proteins and its role in pathogenesis is the most securely established. Pili promote infection by facilitating the initial attachment of the gonococcus to the mucosal surface. Many studies have demonstrated that piliated organisms are more infective in vivo and in vitro (4,5,61,62). Careful and elegant studies were performed by Pearce and Buchanan, and Schoolnik et al. to characterize the pilin subunit (63,64). These investigators studied the characteristics of purified, whole pili attachment to human cells and discovered that the cells of the genital tract have increased numbers of binding sites compared to other cells. Immunologic studies have shown that anti-pilin antibodies, found in urogenital secretions, block the attachment of piliated gonococci to epithelial cells in vitro (1, 65, 66). Pili also have been shown to enhance virulence by promoting resistance of gonococci to phagocytosis by human PMNs and mouse peritoneal macrophages (67,68).

Gonococcal pili display great antigenic diversity and have been characterized and serogrouped by both human and rabbit antisera (69, 70). Human immune sera detect less than 50% cross-reactivity between

pilin molecules while rabbit antisera show less than 10% cross-reactivity. However, the human serologic evidence does suggest that there are common antigenic determinants which could be used as vaccine components. Schoolnik and others have used this premise to pursue a rigorous physicochemical characterization of the pilin molecule (64).

Each pilus filament is assembled from approximately 10,000 pilin subunit molecules of approximately 17.5-21 Kdalton (strain variable). The pilus filament is about 60 angstroms in diameter and can vary in length from 1 to 4 um (6, 71). The pilin isolated from isogenic colonial variants may differ in M_r and also may differ in isoelectric point and bouyant density (72,73,74). Pili, with different attachment properties, have been named alpha and beta and correlated with the transparent and opaque colony phenotypes, respectively (74). Swanson has reported recently that pili from P^+ (piliated) and P^{++} (heavily piliated) phenotypes may vary in molecular weight and antigenic properties, (75). Whether these variations observed by Swanson are similar to those already described by Lambden et al. (72) is not known, but evidence suggests that there may be at least two different pilin molecules within a strain and these pili may be expressed singly or simultaneously and in the presence or absence of PIIs.

Amino acid analysis and tryptic peptide maps of pilin isolated from various strains show that pilins are similar, but not identical (64). Approximately 40-45% of the amino acid residues are non-polar (76) and there are two moles of hexose and two moles of phosphate per mole of pilin. The N-terminal amino acid sequence through residue 59 is identical for pilin isolated from PII^- and PII^+ variants from two gonococcal strains by Schoolnik (64), and contains an unusual N-terminal

amino acid, N-methylphenylalanine. Of the first 20 amino acids, 18 are hydrophobic, and their sequence is very similar to the sequence of pilin from Moraxella sp. and Pseudomonas aeruginosa, and entirely different from the N-terminal sequence for pilin from E. coli (76, 77, 78). The peptide mapping of two gonococcal pilin preparations with 5% shared antigenicity and different M_r showed that at least 30% of the peptides were different and 63% were identical (64). Schoolnik has used these data to suggest that there are regions of the pilin molecule which are unique and possibly surface located and regions which are responsible for the antigenic and functional diversity. Schoolnik has pursued the identification of the functional domains of the pilin molecule by studying fragments prepared by cyanogen bromide cleavage at methionine residues. He has named the three fragments: CNBr-1 (N terminal #1-7 residues), CNBr-2 (#8-84), and CNBr-3 (#85-160). He has shown that purified pili from strain MS11 can hemagglutinate human red blood cells (RBCs) and, by competition assays with fragments, has shown that this binding function can be assigned to the CNBr-2 fragment. He has further found that 87% of the amino acid sequence of the binding domain is conserved amoung pilin molecules. The CNBr-3 fragment shows the greatest structural variability and may be responsible for antigenic diversity of the molecule. He suggested that the pilus filament is assembled from pilin subunits in such a way that the binding regions are exposed along the longitudinal axis and thus, a polyvalent ligand is created. The observed variation in binding of purified pili to human cells could be due to the modification of the binding domain (CNBr-2) by the juxtaposition of the unique, CNBr-3 region of the molecule.

These findings have stirred great excitement and the prospect of a

pilin vaccine. Unfortunately in the rabbit model, the CNBr-2 binding region is not immunodominant when whole gonococcal pili are used to immunize rabbits. This type of antiserum blocks RBC agglutination by homologous pili only. If CNBr-2 fragments are used to immunize rabbits, then the resulting antiserum binds to heterologous as well as homologous whole pili and anti-CNBr-2 IgG Fab fragments block RBC agglutination (64). When monoclonal antibodies are prepared against CNBr-2 fragments, they only block binding of homologous whole pili (Schoolnik personal communication). Therefore, a peptide vaccine for gonorrhea looks promising but more work is needed to characterize the cross-reactivity and the protective power of its antibodies.

The CNBr-3 fragment contains the immunodominant, type specific antigenic determinants of gonococcal pili and it is the portion of the molecule with the greatest amino acid sequence variability. Antisera raised against this portion of the molecule show low cross-reactivity with other CNBr-3 fragments and do not prevent binding of homologous or heterologous whole pili to RBCs.

Lipopolysaccharide (LPS)

LPS (endotoxin) is one of the predominant constituents of the gonococcal outer membrane. It plays major roles in virulence and in the host's immune response to infection.

The structure of LPS in N. gonorrhoeae is similar to that of other gram-negative bacteria. However, there are some differences. In general, the smooth LPS molecule (S-type) is composed of a lipid A moiety, a core oligosaccharide and long polysaccharide side chains (O antigens). There is little evidence that gonococci have true O antigen

polysaccharide. Some extraction experiments have yielded only low molecular weight polysaccharides (79, 80). LPS molecules are linked by phosphodiester bonds to form polymers which have M_rs in the 4,000 to 7,000 range (in contrast to E. coli LPS which has M_r in the millions). The gonococcal lipid A moiety is composed of phosphate and a D-glucosamine oligosaccharide acylated with a variety of long chain fatty acids. The most common acids are beta-hydroxy myristic, lauric, and stearic acid (18, 81). This fatty acid composition also differs from that of the Enterobacteriaceae.

The core oligosaccharide is composed of phosphate, L-glycero-D-manno-heptaose and 2-keto-3-deoxyoctonate (KDO), linked to a series of methylated glucosamine, methylated galactose sugars, and pyranosyl residues (80).

The O-polysaccharides of N. gonorrhoeae, found on S-type LPS, are predominantly composed of 2 amino-2-deoxyglucose, D-glucose, and D-galactose. However, there are other sugars, such as D-galactosamine, L-rhamnose, D-xylose, fucose, and D-mannose which also may occur with strain variation. As much as 20-40% of the O-polysaccharide may uniquely vary from one strain to another (80). Perhaps this variability explains, in part, the difficulty of developing a successful serotyping scheme based on LPS.

As in other gram-negative bacteria, the LPS is found predominantly in the outer leaflet of the outer membrane, and is readily released from the outer membrane as "free endotoxin" contained in outer membrane blebs (82, 83).

Rough, or R-type LPS containing only lipid A and core oligosaccharide, may be produced by N. gonorrhoeae (177). It is not

clear if it is only produced *in vitro* or if it may also occur *in vivo*. R-type and S-type LPS do not always segregate with colony phenotype. R-type is commonly found in T3 and T4 colonies; however, T1 and T2 colonies may have S-type or R-type LPS (80).

Recent work on LPS synthesis suggests that the growth rate of the organism can influence whether or not S- or R-type LPS is produced. Morse et al. (84), using chemostat cultures grown under glucose limitation in a defined medium, found that there was a decrease in carbohydrate content of LPS, an increase in cell surface hydrophobicity, and an increase in susceptibility to bactericidal action of normal human serum (NHS) when the organisms were grown at slower chemostat dilution rates. Organisms grown at faster dilution rates had altered glucose metabolism, increased resistance to NHS, and decreased surface hydrophobicity. Structural analysis of LPS using monoclonal antibodies showed that the LPS from serum-sensitive organisms lacked the O side-chain moiety. This study is important because it shows that the structure of LPS can shift within a single strain when culture conditions change.

Studies to determine the site of bactericidal action of human serum have shown that LPS is the antigen recognized by IgM antibodies. The binding of IgM results in the activation of complement and gonococcal cell death (85, 86, 87). A common antigen found in the core oligosaccharide is a D-galactosamine-O-D-galactopyranosyl-(1--4)-D-glucopyranosyl determinant, (88); the ability of IgM antibodies to bind to this determinant depends on the absence of serotype antigen (O side chains), (89).

LPS is a toxic substance (endotoxin) and can cause endotoxic shock

responses in humans and animals. Much of the host response is due to the lipid A component. Interestingly, gonococcal LPS is exquisitely toxic for human Fallopian tube mucosa. Nanogram quantities of LPS are capable of causing alterations in ciliary motility of epithelial cells and LPS may be the toxin which preferentially kills these cells during infection (90, 91, 92, 93).

Adherence of bacteria to host tissue and its role in pathogenesis

What distinguishes a pathogen from a commensal organism? There are several characteristics (94) and two of them are selective adherence and tissue tropism. These properties have been demonstrated for several mucosal pathogens. For example, when mixed suspensions of Streptococcus pyogenes and E. coli are applied to rat tongue and bladder, the streptococci selectively adsorb to tongue dorsum and the E. coli attaches in larger numbers to bladder mucosa (95). The correlation between increased ability to adhere and ability to cause human genital tract infection has been made for several bacteria by Mardh and Westrom (96). They demonstrated that freshly isolated gonococci, Group B streptococci, and Gardanerella vaginalis attached in higher numbers to vaginal epithelial cells than did strains of E. coli, Enterobacter aerogenes, Bacteroides melaninogenicus, Fusobacteria sp., and Lactobacillus sp. They concluded that the relative degree of adherence correlated well with relative virulence. In the intestinal tract, the ability to adhere also has been correlated with virulence for Vibrio cholera, Shigella flexneri, and enteropathogenic E. coli. These are only a few examples of the binding specificity of pathogens. An attempt to cite them all would take many pages. Several excellent reviews and

monographs are available for more information (94, 97, 98, 99).

We are presented with a large body of evidence which indicates that attachment, or the ability to adhere is an important aspect of virulence. The initiation of disease by Neisseria gonorrhoeae also involves attachment.

Attachment mechanism of N. gonorrhoeae

Man is the only known natural host of N. gonorrhoeae. This phenomenon suggests that there are unique gonococcal recognition sites which only the human mucosal surfaces provide. The preferential attachment of the gonococcus to human genital tissue has been well documented by scanning and transmission electron microscopy studies (SEM) (44, 62). Quantitative binding analyses performed by Pearce and Buchanan indicate that the recognition sites are restricted primarily to the genital tract (63).

In the past decade much time has been invested in studying the attachment mechanisms of the gonococcus. Numerous studies have been performed which demonstrate that pili function as adhesins promoting attachment (63, 66, 71, 75). The classic studies of Kellogg, et al. (4, 5) showed that piliated forms of gonococci were capable of causing disease in male volunteers while nonpiliated, isogenic strains were not. In vitro studies using tissue culture cells, buccal epithelial cells, sperm, Fallopian tube, cervix, or RBCs (61, 62, 63, 64, 65, 100, 101) have supported this finding by demonstrating that piliated gonococci can attach in 10-100 fold greater numbers than nonpiliated gonococci. However, it is important to recognize in all these studies that nonpiliated gonococci also attach to human cells in low numbers (e.g., less than 3% of inoculum). This finding suggests that there are

attachment and recognition factors other than pili on the surface of the gonococcus. One such factor is the surface-located protein, "leukocyte association factor;" it correlates with increased adherence of gonococci to PMNs and "intracellular diplococci" seen on Gram stained smears of gonorrhea exudates (48, 49).

Genetics of *N. gonorrhoeae*

The gonococcus is not genetically well defined. Little is known about the structural genes for the outer membrane proteins and their regulation. There are several reasons for this dearth of information. Firstly, there are no known phages which infect *N. gonorrhoeae*. This deficit has prevented the application of phages as transducing vectors and mapping tools, as it has been done sucessfully in *E. coli*. And secondly, not until 1977 was it shown that a conjugal plasmid existed and promoted DNA exchange (102). Unfortunately, the genetic exchange is limited to plasmid DNA (103) and only to those plasmids carrying antibiotic resistance genes such as penicillinase. Chromosomal DNA is not mobilized during conjugation (104). Thus, no simple tool is presently available for such essential tasks as genetic mapping and construction of isogenic mutants varying in a single locus.

Gonococci do exchange genetic information and this is done by a very efficient transformation mechanism. Piliated organisms are more competent than nonpiliated variants at transformation (105), however, the exact role of pili in the process is not well understood. Gonococci do not have sex pili as in *E. coli*. The DNA uptake is facilitated by an undefined recognition mechanism for gonococcal DNA. The mechanism may

involve recognition of a frequent nucleotide sequence carried on the ubiquitous 2.7 Mdalton cryptic plasmid and on chromosomal DNA by a corresponding gonococcal surface receptor (103, 106). The gonococcal receptor is unidentified. Transformation has been used to map a tightly clustered group of linked chromosomal antibiotic resistance genes (107) and to study the degree of linkage of nutritional mutations (108).

Several groups of investigators are now using E. coli as a host and cloning genes and constructing recombinant plasmids for investigation of gonococcal gene expression and, more importantly, for the genetics of pathogenic mechanisms. For example, Koomey and Falkow have cloned the gene for the gonococcal IgA protease and expressed it in E.coli. They have selected mutant genes and constructed strains of the gonococcus which fail to express this enzyme (109). The next few years should see a dramatic increase in our knowledge of this aspect of gonococcal biology.

Genetics of outer membrane proteins

Little is known about the synthesis and regulation of outer membrane proteins at the molecular level. The bulk of our knowledge about the OM is centered around descriptions of virulence phenomena and functional characteristics. The best information comes from genetic studies of resistance to the bactericidal action of normal human serum and antibiotics.

Mutations at several loci which influence the composition of the cell envelope and outer membrane have been described. Functionally, the level of penicillin resistance is affected. Relatively high levels of resistance can be achieved by the additive effects of several mutations.

Each mutation by itself causes only a small, incremental change in resistance. The markers have been called penA, penB and mtr (110). PenA affects a penicillin-binding protein and increases cross linking of the peptidoglycan; penB is closely linked to a locus affecting PI expression and is identified by a shift of PI to a high molecular weight species. A mtr mutation results in a nonspecific increase in resistance to several antibiotics, dyes, and detergents. It is accompanied by an increased expression in a 52 Kdalton OM protein and changes in the degree of cross-linking of the peptidoglycan (111).

There are also several mutations which cause a phenotypic increase in antibiotic susceptibility. These are env1 and env2. Env2 is the most interesting as it causes a nonspecific drug hypersusceptibility. Its expression can suppress the effects of penA, penB, and mtr and it is associated with a dramatic increase in membrane rigidity, decrease in peptidoglycan cross-linking, a decrease in the M_r of PI, and a decreased synthesis of a 52 Kdalton OM protein (111).

Recently, Cannon has described the locus nmp-1 (new membrane protein-1) which may be the structural gene for PI (112). She demonstrated that penB locus was not the same, but was closely linked to nmp-1. She characterized a mutant nmp-1 allele which caused an increase in the M_r of PI from 35 to 36 Kdaltons in SDS-PAGE gels. How the mutation results in a change in molecular weight is unclear. The nmp-1 gene may be a regulatory gene or it may be involved in the export of PI to the OM or its processing.

A correlation has been found between virulence and resistance to the bactericidal activity of normal human serum for several gram-negative bacteria including the gonococcus. Invasive strains of

gonococci which cause disseminated gonococcal infection (DGI) have been shown to have several combinations of unique characteristics. They are very penicillin sensitive, serum resistant, and have unusual nutritional requirements (auxotrophy) (113, 114, 115). Several investigators have shown that these are independent, phenotypic properties determined by separate loci. Eisenstein et al. showed that penicillin sensitivity was independent from serum resistance, but highly correlated with virulence (116); Mayer reported that the mutations responsible for auxotrophy of DGI strains were unlinked to serum resistance (Mayer 1977). In contradiction, Spratt et al. (118) have described a genetic locus (sac-2) that affects the level of serum resistance and which is linked to arginine biosynthesis. Hildebrant et al. (54) have reported that the transformation from serum sensitive to serum resistant was accompanied by a simultaneous structural and antigenic change in PI. Subsequently Cannon et al. (119) have described the sac-1 locus which affects the level of serum resistance and is closely linked to the nmp-1 locus for PI. This locus maps at a great distance from the sac-1 locus.

As can be seen from the above discussion, numerous loci have been described, but as yet no structural or regulatory genes have been precisely characterized.

Genetics of colonial phenotype variation

The genetic basis of variation in colony phenotype is unknown. It is, however, a fascinating problem and only a few facts are known about the mechanism.

Within a single strain of N. gonorrhoeae there are numerous combinations of types of pili, opacity-associated PIIs; non-opacity

associated PIIs and possibly types of LPS. The rates at which these various surface components "switch on and off" do not suggest mutational events. Specifically, the rates are very high i.e., on the order of 10^{-3}/cfu/generation. Mayer conducted an extensive study on the rates of colony phenotype variation from piliated to nonpiliated and back to piliated states (14). He has also looked at the rates of change from Op (O^+) to Tr (O^-) and O^- to O^+ colonial phenotypes. He tested the influence of 194 compounds and found none which significantly promoted or inhibited transition rates. The compounds tested included amino acids, antibiotics, cations, anions, detergents, carbohydrates, protease inhibitors, chelators, heavy metals, dyes. Also tested were temperature, pH, O_2 concentration, and the presence of DNAase or DNA from Op or Tr colony phenotypes. Below is an excerpt from Mayer's paper where he determined transition rates for opacity and piliation in naturally occurring, agar grown colonies of strain F62.

Table 1.2

	Opacity		Piliation	
	Direction	Rate	Direction	Rate[a]
$F62P^+O^+$	$O^+ \rightarrow O^-$	3.8×10^{-3}	$P^+ \rightarrow P^-$	1.5×10^{-3}
$F62P^+O^-$	$O^- \rightarrow O^+$	2.1×10^{-3}	$P^+ \rightarrow P^-$	3.0×10^{-3}

[a] cfu/generation
(Table reprinted with permission of the publisher.)

From these data Mayer suggested that there was no association between the transition which affects piliation and that which affects colony opacity. Lambden and Heckles (36) also tested a few medium components and could show no change in PII composition when medium components were altered. To date no specific medium or environmental condition has been proved to directly effect a phenotypic shift although

there is abundant, indirect evidence that shifts do occur due to changes in environmental conditions.

Swanson has reported frequencies for opacity/PII shifts in single colonies of strain F62. His results are very similar to those reported by Mayer (3 colonies/1000 progeny). Swanson also observed that the addition or loss of a PII species was a single step phenomenon. That is, as colony variants were formed, the variant organism did not lose or gain multiple PIIs. PIIs were gained or lost one at a time. His results are excerpted below (ref).

Table 1.3 Occurrence of opacity variants among gonococci in single colonies of Strain F62.

Parental colony[a]	Variant colony	Prevalence %
O^-	O^+ (PIIα^+)	0.39 ± 0.13
O^-	O^{++} (PIIβ^+)	0.31 ± 0.15
O^+ (PIIα^+)	O^{++} (PIIα^+, PIIβ^+)	0.37 ± 0.26
O^+ (PIIα^+)	O^-	3.1 ± 1.9
O^{++} (PIIβ^+)	O^-	0.92 ± 0.52
O^{++} (PIIα^+, PIIβ^+)	O^+ (PIIα^+)	0.93 ± 0.45

[a] O^- to O^{++} represent colony phenotypes from transparent or $PII^-(O^-)$ to opaque or PII^+ (O^{++}) and α,β represent outer membrane protein PII content and characteristics. (Table reprinted with permission of the publisher.)

Mayer has speculated that the transitions may be governed or mediated by a mechanism similar to the *Salmonella flagellar* (H antigen) phase variation (120). Norlander et al. (121) had previously suggested that the genetic basis for variation in piliation (P- to P+) was transformation. They suggested that autolytic liberation of DNA and subsequent transformation played a large role in colony variation. They

showed that rates of colony variation for organisms from broth cultures were sensitive to the presence of DNAase in the medium. However, two independent investigators have not been able to reproduce these results (14, 103).

It has been suggested that plasmids may play a role in control of colony variation. This idea is only now under investigation and preliminary reports are contradictory. Mayer tested three plasmid-free strains in his transition rate study (14). He found that one strain had a 10-fold decrease in transition rates (approx. 10^{-4}) when compared to 12 strains with plasmids. However, the two other strains showed no statistical difference in rates when compared to normal, plasmid bearing strains. Sparling et al. (103) have stated that there is no evidence that the ubiquitous 2.7 Mdalton cryptic plasmid is involved in piliation, iron utilization or resistance to serum. Although, they have observed that strains lacking the cryptic plasmid were highly Op in colony morphology. It is interesting to note that the structure of the cryptic plasmid is highly conserved and contains numerous inverted repeats (122, 123). Thus, its structure is suggestive of a regulatory role.

Studies by Meyer and So (124) have provided the first real break through in studies of phenotypic control mechanisms. Their research has concentrated on the genes involved in pilus expression. They have shown that the switch from the piliated to the nonpiliated state involves chromosomal rearrangement and that the switch from Op to Tr may also involve the pilus gene. The switch from nonpiliated to piliated appears to require the rearrangement of a "silent copy" of the pilus gene into an active site for expression (M. So, personal communication).

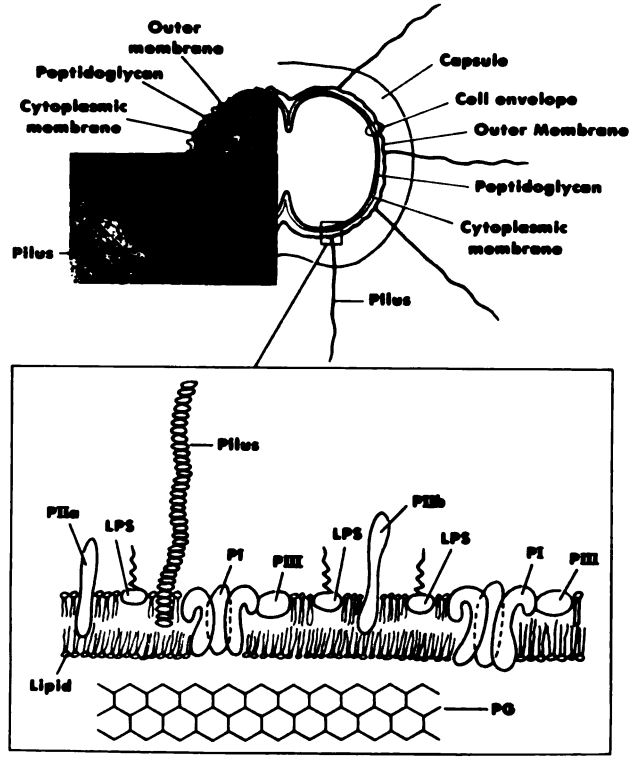
Whatever control mechanism governs the phenotypic variation, its elucidation is crucial to understanding the pathogenic mechanisms of this organism. The ability of the organism to vary the structural components of its outer membrane at high frequency assures that a population of gonococci found at a patient's site of infection will be mixed. Thus, if any of the varible components are associated with enhanced survival in the face of changing environment (host defense), then those organisms will be selected and survive. Such a capability provides a remarkable virulence mechanism.

Table 1.1 Gonococcal Colony Morphology and Phenotype Terminology

Kellogg Classification	1978 Classification	1983 Classification
Colony Type	Colony Type	Colony Type
T_1	P^+Tr	P^+O^-
	P^+Op	P^+O^+
		P^+O^{++}
		P^+O^{+++}
		P^+O^{++++}
T_2	$P^{++}Tr$	$P^{++}O^-$
	$P^{++}Op$	$P^{++}O^+$
		$P^{++}O^{++}$
		$P^{++}O^{+++}$
		$P^{++}O^{++++}$
T_3	P^-Op	P^-O^+
		P^-O^{++}
		P^-O^{+++}
		P^-O^{++++}
T_4	P^-Tr	P^-O^-

Table 1.1 The various gonococcal colony phenotype classification schemes and their terminology are described. P^+ and P^{++} represent piliated and heavily piliated colony colony phenotypes. P^- represents nonpiliated phenotypes. In 1978, the terms opaque (Op) and transparent (Tr) were introduced. The extent of colonial opacity can be quantitated. O^+ to O^{++++} represent degree of colonial opacity from slightly opaque (O^+) to very dark, golden opacity O^{++++}). O^- represents the transparent phenotype. Organisms from Kellogg colony types T_1 and T_2 are infectious.

Figure 1.1 The structural features of the cell envelope of N.gonorrhoeae seen by transmission electron microscopy, or specimens negatively stained with uranyl acetate, and a schematic of the molecular organization of the major components of the outer membrane. Photograph is reprinted with permission of Geo. F. Brooks.



Chapter 2

Structural Analysis of Protein II of *N. gonorrhoeae*

Abstract

PII, an outer membrane protein of *N. gonorrhoeae*, may be variably present and may have several molecular forms within a single strain. A technique was developed to identify organisms within a culture containing PII variants by changes in the colony morphology. Several PII variants in strain F62 and FA1090 were identified, purified and compared structurally. A purification procedure was developed using 1 M NaCl and 1% octyl glucoside. PIIs were analyzed for apparant molecular weight (M_r), amino acid content, isoelectric point and compared by peptide mapping with staphylococcal V8 protease. The PIIs varied in M_r from 30 to 32 Kdaltons at 100°C. Amino acid analyses of purified PIIs showed that they were similar proteins and contained 8-16% basic amino acids and 23-30% hydophobic amino acids. The isoelectric points of PIIs were very basic (greater than 8.5), and were similar for all variants. Peptide mapping indicated that some portions of the molecule were structurally conserved and others were variable. Comparisons of peptide maps from different strains showed that all PIIs were structurally related; however, each PII contained unique regions. It is speculated that variation in structure may have significant effects on the function of PII and the pathogenic mechanisms of the gonococcus.

INTRODUCTION

Protein II (PII) is a major constituent of the gonococcal outer membrane (29). PII has been associated with several biologic properties and it has been suggested that PII is a major virulence factor (43). The putative antigenic variation of PII enhances the marked antigenic diversity of the organism (125). PII may be involved in attachment to tissue culture cells (61, 126), and is associated with interaction of gonococci with human polymorphonuclear leukocytes (48). PII may be important in the antibody-complement mediated bactericidal reaction. The evidence is indirect and the results are variable. (52, 127).

The protein has several unique characteristics which are believed to contribute to this functional diversity. There may be multiple forms of PII expressed by a single strain of N. gonorrhoeae (8, 36) and these vary in apparent molecular weight (M_r) from 24,000 to 32,000 (30). The proteins demonstrate changes in M_r upon heating to 100°C in 2% SDS, and these shifts in M_r are associated with altered binding of specific antibody. (Swanson, Brooks, Draper, unpublished observations) Each PII appears to be antigenically distinct from other PIIs (125). The different PIIs are associated with changes in colony morphology (9). Shifts in the expression of PII "types" occur at high frequency in vitro (14) and in vivo (15).

The molecular basis of the functional diversity of this protein is unknown. It is also unknown to what degree PII structure varies within a strain. Peptide mapping studies have suggested high degrees of

structural relatedness and some unique peptides in PIIs compared from different strains (56, 128).

The following studies were undertaken to more fully understand the structural basis of PII diversity. Two N. gonorrhoeae strains, exhibiting multiple colony phenotypes, were studied systematically for PII variability. The PIIs were purified and analyzed for amino acid content, isoelectric point, and structurally compared to each other by peptide mapping. The results indicated that there were variable as well as constant regions of the molecule in intra-strain variants as well as between strains. PIIs showed similar amino acid contents, close isoelectric points, and were among the most basic of the gonococcal outer membrane proteins.

MATERIALS and METHODS

Growth, maintenance and selection of N. gonorrhoeae colony phenotypes

Neisseria gonorrhoeae were grown on modified gonococcal colony phenotyping agar (PPT) composed of the following per liter: 7.5 gm Thiotone peptone (BBL, Cockeysville, MD), 7.5 gm Proteose peptone (Difco, Detroit, MI) 5 gm NaCl, 4 gm K_2HPO_4, 1 gm KH_2PO_4, 1 gm soluble starch (BBL), and 12 gm Noble agar (Difco). The organisms were passaged daily by single colony transfer with aid of a dissecting microscope (Bausch and Lomb, Rochester, NY). Colony phenotype characteristics were determined by the appearance of colonies observed with diffuse and reflected light sources (9, 136).

The N. gonorrhoeae were laboratory adapted strains F62 and FA1090. Strain F62 was obtained from Dr. John Swanson, Rocky Mountain Labs,

Hamilton, Montana. Colony phenotype variants for strain FA1090 were provided by Dr. Janne Cannon, University of North Carolina, Chapel Hill.

N. gonorrhoeae were stored at -70°C in 5%(w/v) bovine serum albumin (BSA) with 5%(w/v) monosodium glutamate. Phenotypes were stable for at least 1 year in these conditions. Working cultures of N. gonorrhoeae were checked for PII content by SDS-PAGE bi-weekly or with each experiment as needed.

Gonococcal outer membrane isolation procedure

Outer membranes (OM) of N. gonorrhoeae were isolated according to Heckles (46) with a few modifications. Twenty to 35 gm (wet weight) of N. gonorrhoeae, grown on agar for 20 hours, were scraped into 100 ml of 0.2 M lithium acetate (LiAc/HAc) buffer, pH 6.0, with 5 mM ethylenediamine tetraacetic acid (EDTA). The organisms were shaken for 3-4 hours at 45°C in a New Brunswick shaking incubator (New Brunswick Sci. Co., Edison, NJ). The suspension was then placed in a Lourdes tissue homogenizer (Vernitron Medical Products, Carlstadt, NJ), blended for 10 minutes, and centrifuged at 12,000 x g for 10-15 minutes at 4°C. The supernatant was ultracentrifuged at 100,000 xg for 3 hours in a Beckman L2-65B centrifuge with SW25.1 rotor (Beckman Instruments, Palo Alto, CA). The yellow, glassy pellet was resuspended in 0.2 M NaAc/HAc buffer, pH 6.0, and stored at -70°C. From 20 gm of starting material, approximately 8 mg of OM protein could be prepared (Figure 2.1).

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (37). A 30 gm:0.8 gm per 100 ml solution of acrylamide:bis-acrylamide (Sigma) was used to prepare 12.5% separating gels overlayed with 5% stacking gels. All gels were

prepared the day of use and electrophoresed at 40 mamps constant current. Gels were fixed overnight in 25%(v/v) isopropyl alcohol with 10%(v/v) acetic acid (HAc) and stained with 0.25%(w/v) Coomassie Brilliant Blue dissolved in fix solution. Gels were destained with fix solution until background staining was negligible and then air dried between two wetted sheets of dialysis membrane (BioRad Laboratories, Richmond, CA).

Preparation of *N. gonorrhoeae* for SDS-PAGE

Gonococci were grown on PPT medium for 18-22 hours, removed with a cotton swab, and suspended in 5 ml of Dulbecco's PBS. The suspension was adjusted to an optical density of 200 Klett units (Klett Summerson colorimeter, Klett Mgf. Co., NY) and two-2 ml aliquots prepared. The organisms were pelleted by centrifugation at 2000 x g for 10 minutes in an IEC tabletop centrifuge (Damon/IEC Divison, Needham Hts., MA). The pellets were resuspended in 100 ul of H_2O and an equal volume of 2X SDS-PAGE sample buffer. The sample buffer was composed of 2%(w/v) SDS (British Drug House, Poole, England), 0.02%(w/v) beta 2-mercaptoethanol (2-ME), 0.025%(w/v) Bromophenol Blue (Sigma Chemicals, Saint Louis, MO), and 20%(v/v) glycerol in 100 mM Tris/HCl buffer, pH 6.8. For each organism, one sample was solubilized at 37°C for 2 hours and another sample solubilized at 100°C for 10 minutes in a boiling water bath. The samples could be stored at -70°C for reuse and were not reboiled.

OM vesicles were prepared for SDS-PAGE by mixing 100 ul of outer membrane suspension (containing 2 mg protein/ml) with 100 ul of 2X sample buffer. Aliquots were solubilized at 37°C or at 100°C as above.

Protein Assay

Protein was measured by the Peterson assay (129). This assay is a modification of the Lowry procedure performed in the presence of SDS which improves the solubilization of intrinsic membrane proteins. Bovine serum albumin was used as a standard. Freshly prepared standards were included in each protein assay.

Iodination of molecular weight standards

Low molecular weight protein standards, obtained from BioRad, were iodinated by the chloramine T reaction. Two ul of the standards were mixed with 2 ul of water and 20 ul of PBS. Fifteen ul of freshly prepared 1% (w/v) chloramine T (Sigma) and 0.5 mCi of (^{125}I)-Iodine (^{125}I) (100 mCi/ml) (Amersham Corp., Arlington Hts., IL) were added to the mixture and allowed to react for 2 minutes at room temperature. The reaction was stopped by the addition of 15 ul of 25%(w/v) sodium metabisulfite (Sigma) and the mixture chromatographed on Sephadex G-25 equilibrated with PBS. The void volume, containing the standards, was collected and stored at -70°C. A portion of the labelled standards was diluted with SDS-PAGE sample buffer to yield 1 x 10^5 cpm per 10 ul sample.

Extrinsic iodination of *N. gonorrhoeae*

N. gonorrhoeae were suspended in Dulbecco's PBS to an optical density of 200 Klett units. Two ml of the suspension were centrifuged at 2000 x g for 10 minutes and the pellet resuspended in 100 ul PBS. Five ul of lactoperoxidase (160 U/ml) (Calbiochem, La Jolla, CA) and 250 uCi ^{125}I were added, and the reaction initiated by addition of 5 ul of 0.001% (v/v) H_2O_2. After 10 minutes incubation, an additional 5 ul of 0.001%(v/v) H_2O_2 was added. The reaction was stopped at 20 minutes by

the addition of 0.5 ml of 5mM 2-ME in PBS. The labeled organisms were pelleted by centrifugation and washed twice with 1 ml of Dulbecco's PBS. The final pellet was suspended in 100 ul H_2O or appropriate buffer.

Iodination of gonococcal outer membrane vesicles

One hundred to 200 ug protein of outer membranes were ^{125}I labeled by the lactoperoxidase catalyzed reaction in the presence of H_2O_2. To 100 ul of outer membranes in 0.2 M sodium acetate (NaAc) buffer, pH 6.0, 20 ul of lactoperoxidase enzyme (160 U/ml) and 0.5 mCi of I^{125} (100 mCi/ml) were added and mixed. To initiate and maintain the reaction, 10 ul of 0.001% (v/v) H_2O_2 solution were added at 0 and 10 minutes. The reaction was stopped at 20 minutes with 0.5 ml of 5 mM 2-ME and the mixture chromatographed on a Sephadex G-25 column (10x1cm) equilibrated with 0.2 M NaAc buffer, pH 6.0. The void volume was retained and radioactivity per ug protein was determined. ^{125}I-labelled membranes were usually used within 24 hours and could be stored up to one month at -70°C in NaAc buffer.

Densitometry

The dried SDS-PAGE gels were scanned on a Helena Quick Scan RD model densitometer (Helena Laboratories, Beaumont, TX) with a red filter and regular optics. Estimates of densities were the average of at least 3 scans.

Two dimensional gel electrophoresis

To determine isoelectric points of gonococcal surface proteins, the technique of O'Farrell (130) as modified by Ames et al. (131) was applied to outer membrane vesicles. Vesicles were solubilized at 100°C in 2% SDS, buffered with 75 mM Tris/ HCl, pH 6.8. This solution was mixed with Nonidet P-40 (NP-40) (Sigma) lysis buffer to exchange deter-

gents (131). The final SDS:NP-40 ratio was 1:8. Samples containing 25-50 ug of protein were loaded on tube gels containing 2% ampholines (LKB, Rockville, MA), pH3-11, and electrophoresed in a disc gel apparatus (Buchler, Fort Lee, NJ) at 300 volts for 17 hours and at 800 volts for the last hour for a total of 6000 volt-hours. Control gels containing no protein were immediately cut into 1 cm segments, eluted, and the pH determined. Sample gels were equilibrated, and loaded onto 12.5% Laemmli slab gels with 5% stacking gels. The second dimension gels were prepared in a BioRad Protean electrophoresis unit and electrophoresed at 45 mamps. Gels were fixed overnight, stained with CBB and dried. First dimension gels could be stored at -70°C for one week.

Non-equilibrium pH gradient gel electrophoresis

The technique of O'Farrell et al. (132) was applied to OM vesicles. Twenty-five to 50 ug protein samples were prepared as above and loaded onto gels containing 2% ampholines (pH 3-11). Tube gels were electrophoresed for 1600 volt-hours. Before the second dimension was run, gels were equilibrated and the second dimension slab gel was electrophoresed at 45 mamps. Gels were fixed and dried as above. First dimension tube gels could be stored at -70°C for one week.

^{125}I-peptide mapping of protein PII variants

Outer membranes were labelled with ^{125}I by the lactoperoxidase reaction and prepared for SDS-PAGE. Samples for SDS-PAGE were diluted to contain $5x10^5$ cpm per 10 ul and unlabelled OMs were added to bring protein concentrations to 10 ug in the 10 ul sample. After electrophoresis, the gels were briefly stained with 0.025% CBB in 10% HAc with 25% isopropyl alcohol until the bands just appeared. The PII bands were excised, equilibrated with 0.125 M Tris/HCl buffer, pH 6.8, and stored

at -70°C for use the next day.

Peptide mapping was performed with staphlococcal V8 protease (Miles Laboratories, Elkhart, ID) according to Cleveland et al. (133), utilizing the Laemmli gel system. Separating gels of 15% acrylamide with 5% stacking gels were used to separate peptides. For each PII variant from F62 and FA1090, a single gel slice containing 1 x 10^5 cpm and approximately 1-2 ug of PII was loaded into a gel lane and overlayed with 100 nanograms of V8 protease in 20%(v/v) glycerol-Tris buffer, pH 6.8. The samples were electrophoresed at 40 mamps and the gels fixed in 10% HAc with 25% isopropyl alcohol overnight. The next day, the gels were dried between 2 sheets of wetted BioRad dialysis membrane.

For autoradiography, the dried gels were exposed to Kodak X-OMAT AR X-ray film (13x18 cm) overnight with a Dupont Cronex extra lite intensifying screen (Dupont, Wilmington, Delaware) at -70°C. The X-ray film was developed in a Kodak automatic processor.

Protein II purification

Fifteen grams (wet weight) of organisms were suspended in 80 ml of 25% (w/v) ultrapure sucrose (Schwarz/Mann, Orangeburg, NY) in 0.01M Tris, pH 8.0. The suspension was gently stirred at room temperature and at 20 minute intervals the following solutions were added:

1. 10 ml lysozyme (Sigma) (6.4 mg/ml) in 0.25 M Tris, pH 8.0
2. 10 ml sodium EDTA at 20 mg/ml in H_2O
3. 0.2 ml of 4 M $MgCl_2$
4. a few crystals of bovine pancrease type 1-A ribonuclease (Sigma)
5. a few crystals of bovine pancreas type III deoxyribonuclease (Sigma)

The lysate was spun at 13,000 x g for 30 minutes and the pellet resuspended in 50 ml 2% (v/v) Brij 96 (Sigma) in 0.01 M Tris, pH 8.0,

with 5 uM phenylmethylsulfonyl flouride (PMSF) (Sigma) and incubated with gentle stirring at 37°C for 1 hour. The extract was centrifuged at 40,000 x g for 1 hour. A 1.0 gm (wet weight) aliquot of the pellet was suspended in 5 ml of 1% (w/v) octyl glucoside (Sigma) in 25 mM Tris, pH 8.0, with 5 mM EDTA and 5 uM PMSF. The suspension was incubated at room temperature for 1 hour and centrifuged at 100,000 x g in a Ti 75 rotor in a Beckman L5-50B ultracentrifuge for 60 minutes at 4°C. The waxy pellet was resuspended in 5 ml of 1 M NaCl in the Tris/EDTA/PMSF buffer, homogenized by sonication for 10 minutes in a beaker in a Bransonic 12 sonic water bath (Bransonic Cleaning Equipment Co., Shelton, CT) and then incubated as above. The suspension was ultracentrifuged again, and the pellet was resuspended in 5 ml of 1% octyl glucoside with 1 M NaCl in complete Tris buffer and extracted for another hour at room temperature. The extract was ultracentrifuged as above and the PII-rich supernatant was retained. Purification steps were monitored by SDS-PAGE and protein determined. From the 1 gm aliquot of the Brij pellet approximately 0.5 to 1.0 mg of protein II could be prepared. The yield was strain variable.

Amino acid analyses

For amino acid analysis, the PII-enriched fractions were further purified by preparative SDS-PAGE according to Cannon (112). The octyl glucoside PII fractions were dialyzed against 5 uM PMSF at 4°C to remove detergent and salt. The precipitate was harvested by centrifugation at 100,000 x g for 1 hour. The pellet was resuspended in 1 ml of SDS-PAGE sample buffer, boiled for 10 minutes and loaded on a 1.5 mm preparative gel. After electrophoresis, the edges of the gel were removed and stained to locate proteins; the body of the gel was frozen at -70°C

until needed. The stained edges were aligned with the unstained gel and the area corresponding to PII was excised, and macerated in a loose fitting, hand-held tissue grinder (Bellco, Vineland, NJ) in a small volume of 0.1% SDS. The protein was eluted with 0.1% SDS and further dialyzed against 0.1% SDS to remove traces of glycine and PMSF. The dialysate was lyophilized in a Labconco Freeze Dryer 3 (Labconco, Kansas City, MO) in 100 ug protein aliquots.

Purified PIIs were hydrolyzed in 6N HCl for 24 hours in the presence of phenol and sent to UC Davis Health Sciences Research Laboratory for amino acid analysis. Analyses were performed on a Durram D-500 analyzer.

RESULTS

Colony phenotypes and PII variants

Strain F62 is a laboratory strain which had been stabilized (greater than 99.9% homogeneity) into non-piliated (P-) forms of opaque (Op) and transparent (Tr) colony types. The different colony phenotypes were derived from these cultures and the technique of selecting PII variants was developed by trial and error. The initial selection was attempted from a culture of mixed phenotypes. Colonies with obvious differences in opacity, granularity and color were picked and stabilized to 99.9% homogeneity of colony morphology and then tested for PII variation by SDS-PAGE. The PII which was associated with the most opaque colony type (P-O^{4+}) was called PIIa. It was a naturally occurring variant in cultures of the transparent phenotype. Subsequent clones with single PII variants were selected from cultures of the isogenic transparent phenotype by detection of subtle differences in

colonial opacity. One PII-containing variant was found serendipitously in cultures of P-Tr colonies. These colonies were difficult to distinguish from P-Tr colonies containing PII$^-$ organisms. The PII variants from strain F62 were named in order of discovery: PIIa, b, c, d. PII expression usually correlated with varying degrees of colonial opacity from faint, bluish opacity to strong, golden opalescence with high granularity. The colonies also varied in consistency from smooth and butterous to very friable (Table 2.1).

The phenotypic characteristics of the colony variants selected for study from strain FA1090 are seen in Table 2.2, and include five PIIs and one PII$^-$ colonial variant. One phenotype had two PIIs, and two colony variants had transparent appearing colonies even though the organisms contained PIIs (PIIb, PIIc,e).

Outer membrane vesicles were prepared from each colony variant of both strains by lithium acetate extraction and then solubilized at 37^{o}C and 100^{o}C for Laemmli SDS-PAGE. A photograph of the gel is seen in Figure 2.2. The M_r for the 37^o and 100^{o}C forms for each PII were calculated from at least 3 separate experiments and are seen in Table 2.3. The PIIs ranged in M_r from 26.4 to 29.2 Kdaltons at 37^{o}C solubilization and from 30.0 to 32.0 Kdaltons at 100^{o}C solubilization. The difference in weight between the 37^{o}C and 100^{o}C form of a PII ranged from 2,330 (PIIb F62) to 4,750 daltons (PIId FA1090). The difference in M_r between PII variants at a given temperature, e.g. PIIa 37^{o}C - PIIb37^{o}C ranged from 250 to 1370 daltons. There did not seem to be a constant difference of weight at either temperature. Also, the rank order of increasing M_r at 37^{o}C was not the same as rank order at 100^{o}C. These observations show that there is not the same mass increase at 37^o

and 100° when two PIIs are compared. One interpretation of this result is that there may be conformational changes in each PII variant with differential binding of SDS which account for the differences in M_r at 37° and 100°C, and that the amplitude of this change varies from one PII variant to another.

SDS-PAGE gels showed that PII was contained in outer membrane vesicles. To confirm the surface location of PIIs in the OM vesicles, extrinsic iodination was performed on the vesicles and compared to labeled whole gonococci. The results are seen in Figure 2.3. The patterns were very similar. The predominant proteins labeled in whole cells and OM vesicles were PI, PII, and a 58 Kdalton protein. PIII, also an OM protein, did not label well by the lactoperoxidase technique. This observation has been reported by others. PIII is believed to be predominantly buried in the membrane and less accessible to labelling. (59, 134).

Densitometry scans were performed on CBB stained gels of OM vesicles. PII accounted for 4.8 to 20% of total OM protein. Densitometry scans of autoradiograms of OM's showed that PII accounted for approximately 40-50% of the ^{125}I-label (data not shown).

Isoelectric focusing

The methods of two dimensional gel electrophoresis (2D-GE) of O'Farrell and two dimensional non-equilibrium pH gradient electrophoresis (NEpHGE) of O'Farrell et al. were applied to the samples of PII^+ OM vesicles. The purpose was to determine the pIs of the PII variants and the relationship of the pIs to those of other outer membrane proteins.

Initial studies were performed with NEpHGE techniques. The PIIs

were identified as some of the most basic proteins in OM vesicles. All migrated at the front of those proteins approaching the basic end of the gel and were eluted off the gel at greater than 2000 volt-hrs. PIIs were identified on gels by M_r and relative positions compared with those of purified PIIs. An example of a NEpHGE gel is seen in figure 2.4. Both 37°C and 100°C forms of PII migrated to the basic end of the gel suggesting that they are the same protein (Figure 2.4).

Gels containing pH 3-11 ampholines were constructed and an example is seen in Figure 2.5. The pI of PI was about 8.5. PIIs could not be identified on these gels. The PIIs probably failed to enter the gels or were lost as the pH gradient at the basic end destabilized during electrophoresis. The construction of IEF gels with basic range ampholines (pH 8-11) was attempted and failed. Gels consistently failed to polymerize. Thus, from NEpHGE studies and the relationship of PIIs to the location of PI, it was assumed that the pI of PII was greater than pH 8.5. These basic isoelectric points indicate that PIIs will carry positive charges at physiologic pHs.

Peptide mapping

Peptide mapping was performed to investigate the degree of structural relatedness between PII variants within a strain and between strains. Specifically of interest was the extent of conserved and variable regions of the PII molecules.

PIIs were sensitive to staphlococcal V8 protease and were cleaved at 100 nanograms of protease per 2 ug OM protein. The patterns from four experiments for each PII were fairly consistent. The digestion was partial and a fraction of native protein was retained. This phenomenom is probably due in part to the technique of proteolysis in acrylamide

gel slices and is also a characteristic of V8 proteolysis (P. Bavoil, personal communication). The peptide maps are seen in Figure 2.6. The PIIs varied in sensitivity to V8 protease. F62-PIIb was the most sensitive and F62-PIIa was the most resistant to cleavage. All PIIs contained a major cleavage site which generated a fragment at about 18-22 Kdaltons. A second, minor, conserved cleavage site was detected as a band running directly under the native protein (26-28 Kdalton fragment). This fragment can be seen clearly in the lane containing FA1090 PIIe. The other fragments appeared to have unique apparent molecular weights. At least one PII from each strain contained a site which generated a fragment of approximately 24 Kdalton. e.g., PIIb-F62, PIIa-FA1090. Overall, the maps of surface peptides were similar between all PIIs. However, there were distinct differences. These results indicated that there are regions of the molecule associated with the cell surface which are conserved and areas which are unique. Peptide mapping of 37°C and 100°C forms of a single PII yielded the same peptide pattern (data not shown).

Purification and amino acid analyses

A purification procedure was developed to obtain functional PII. PII could be enriched by sequential, differential treatment of whole gonococci with 2% Brij 96, 1% octyl glucoside and salt. The flow chart of the purification procedure is seen in Table 2.5. The purification steps for PIIa from strain F62 are shown in Figure 2.7.

One percent octyl glucoside could extract several OM proteins preferentially, including PI, PIII, 14K, 15K, 21K, and 62Kdaltons. The 14 Kdalton protein was purified by salt treatment. PII was preferentially extracted by treatment of cells with 1% octyl glucoside and 1 M

NaCl. In strain FA1090 some PIIs could be extracted by 1% octyl glucoside alone (data not shown). An another protein (M_r 26,800) was extracted with PIIa from strain F62. This protein reacted with PIIa specific antiserum in the Western blot reaction (Figure 2.8), but it is not clear what relationship it has to PIIa.

PIIs could be extracted in almost pure form by repeating the extraction steps with octyl glucoside alone and salt alone before the final combined extraction. This procedure decreased the contamination of the final step of purification by PI and the proteins of 14 and 15 Kdalton. It was interesting to note that PII lost its heat modifiability during the octyl glucoside extraction procedure. While associated with other gonococcal components PII continued to be modifiable upon heating.

There were some problems with the degradation of PI and PII during the purification procedure. Endogenous protease(s) may have been activated during the salt treatment (42, 135). The 21K and 14 Kdalton proteins were similar in size to proteolytic fragments previously reported for PI, (42). This cleavage could be partially eliminated by the addition of 5uM PMSF to all extraction steps.

Studies of solubility demonstrated that a high concentration of salt (greater than 0.6M NaCl) was required to maintain PII in solution in detergent concentrations of 1-2%(w/v) (data not shown). A soluble protein was defined as one remaining in the supernatant of a solution after centrifugation at 100,000 x g for 1 hour.

Amino acid analyses showed that purified PIIs had similar amino acid content. The results of the analyses are shown in Table 2.4. The proportion of hydrophobic amino acids was 24-30% and was similar between PII variants within a strain and between PIIs in different strains. The

proportion of basic amino acids was 8-16%.

DISCUSSION

In this study the structural variation of PII outer membrane proteins of Neisseria gonorrhoeae was investigated. Specifically studied were the variation of PII proteins within a strain and between gonococcal strains, and the extent the surface regions of the molecule were different. A technique for identifying organisms with PII variants and a PII purification procedure were developed to accomplish these studies.

Previous phenotyping studies have suggested that colonies of organisms containing heat-modifiable proteins (PII^+) were usually Op while those of PII^- organisms were generally Tr (47, 136). Recent studies of gonococcal strains P9 and FA1090 have shown that at least one PII may be associated with the Tr colony phenotype (9, 36, 137). In the studies presented here, it was also shown that organisms with PIIs may be associated with Tr colony phenotypes, and in fact there are several PIIs within a single strain which can be found in Tr colonies. A recent report by Swanson suggested that strain F62 did not produce a PII found in Tr colonies (9). Serendipitously, a PII was identified in gonococci from Tr colonies of this strain. Several additional gonococcal strains were studied (including recent clinical isolates) and it was observed that most strains have Tr colonies whose organisms express at least one PII variant (data not shown). This observation is important because it

means that Tr colonies must be carefully screened before it can be assumed that the organisms are PII$^-$. This finding will impact on all previous studies of Op/Tr differences in function and virulence and may render some invalid.

The best method for selecting PII variants was to screen growth in cultures of the "true" transparent colony type because in these cultures there are always a few Op colonies which occur. These opaque colonies are easily seen and variants are usually readily distinguished from one another in these cultures. Efforts failed at selecting organisms with PII variants within cultures of a PII$^+$ organisms. Although subtle differences in color and granularity are common in gonococcal cultures, colonial opacity variation within the stable PII$^+$ population is usually associated with the production of a second PII variant occurring simultaneously with the first. Early studies by Swanson showed that as the opacity/darkness of a colony increased, multiple PIIs would occur (8). It was found that difference in opacity could also be associated with increased or decreased production of a single protein II as determined by SDS-PAGE of whole cell lysates (data not shown).

Swanson suggested that the mechanism associated with the gain or loss of PII expression was a single step event (9). Thus, switching from the PII$^-$ state (true Tr colony) to the PII a,b,c, or d state, etc. is a single event and subsequent addition of PIIs occurs one at a time. The phenomenon of switching from PII$^-$ state to multiple PII expression has not been described. In the initial selection of PII variants, two were found (PIIc and PIId in strain F62) in cultures of different PII$^+$ variants. However, each of these cultures had a high, initial population of Tr organisms (aprox.30-50%) when the variants were found.

It is possible that these PII variants arose from the PII^- organisms in the culture rather than the PII^+ organisms. Most PII variants could be stabilized in culture after several, single colony passages and were associated with a characteristic colonial phenotype and opacity (Table 2.1). The PII variants which were associated with transparent colony phenotypes were difficult to maintain and therefore, a biweekly check of whole cell lysates by SDS-PAGE was performed.

All PIIs demonstrated heat-modifiability. The mechanism of this phenomenon is unknown although it has been reported for several proteins found in OMs of gram-negative organisms (138). Several theories have been proposed (9, 139, 140). The low molecular weight form may represent a more folded, compact configuration of the molecule which migrates faster on SDS-PAGE gels due to the smaller dimension of its complex with SDS. It has also been suggested that the choice of detergents, gels, and buffer systems for electrophoresis can influence the mobility of proteins (140). The elegant studies of Reithmeier and Bragg (139) on E. coli heat modifiable proteins have demonstrated using sedimentation coefficients, uv spectroscopy, and cirular dichroism techniques that there are conformation differences associated with heating. Swanson has shown that Triton X-100 or Zwittergent 3-14 extraction of PII results in a loss of heat-modifiability (60) and the protein remains in the low molecular weight configuration. We have also observed this with octyl glucoside extraction (Figure 2.7). Curiously, a second SDS-electrophoresis of 37°C forms results in a shift to the 100°C form, without boiling, as if additional treatment with a strong anionic detergent could displace the octyl glucoside, and further unfold the molecule.

Work done by Schweizer et al. (141) on OmpA, the heat modifiable outer membrane protein of E. coli, has shown that when OmpA is purified away from LPS, it looses its modifiability. Heat modifiability may be restored to the protein by reassociation with LPS. This mechanism for the heat modification of a protein's molecular weight may be similar for PII of N. gonorrhoeae. LPS could play a role in generating the 37°C conformation of the molecule during membrane biogenesis. It was observed that purification with octyl glucoside prevented heat modifiability during electrophoresis. When PII was purified, the mild non-ionic detergent octyl glucoside could have replace LPS and held the protein in the 37°C configuration. When detergent was removed with ethanol or replaced with SDS, the PII molecule could have fully denatured with heat treatment.

Heckles compared cyanogen bromide cleavage products of the 100° form of PII and PIIa from strain P9, and showed that two fragments were generated for each PII (142). One pair of fragments gave identical M_rs and the other pair differed. The fragments which differed, varied by the same mass increment as the native PIIs. Thus, in these studies one end of the molecule appeared to contain the additional amino acids.

The M_rs of PII variants were compared at 37° and 100°C for differences in mass. There was no constant, incremental increase in molecular weight between PII variants at 37°C nor 100°C. The differences at 100°C were generally smaller (there was one exception) than differences at 37°. It was difficult to say exactly what the difference in mass was between these PII variants. The determination must await studies with different techniques.

The surface location of PII was confirmed in whole organisms and OMs by extrinsic iodination. It was possible that some PII variants could have been precursor forms of a single PII; however, all PIIs were detected in OM preparations and could be surface labeled. Curiously, one PII in each strain could be extracted into OM vesicles in huge amounts with lithium acetate. (Figure 2.2) e.g., PIIb-F62 and PIIb-FA1090. Proportionally, equivalent quantities of OM vesicles were prepared from each strain. Some PIIs, however, seemed to preferentially seggregate with LiAc-OM vesicles. Other PIIs were relatively difficult to extract into OM vesicles, e.g., PIIa F62, PIId FA1090. An explanation for this phenomenon may be in the mechanism of OM bleb formation and the influence of lithium ions on the distribution of PII.

The isoelectric focusing studies showed that PIIs have similar pIs. All PIIs tested had basic isoelectric points and pIs greater than protein I (greater than pH 8.5). PIIs were identified as some of the most basic of outer membrane proteins. Jones previously reported both acidic and basic pIs for several "opacity associated spots" in strain F62 (143). Our studies were not able to confirm the acidic isoelectric points for PIIs. Perhaps the discrepancy lies in technique. In our NEpHGE study, PIIs were found to migrate off the gel at voltages greater than 1600-2000 volt-hours. Jones et al, used voltages up to 5400 volt-hours during the NEpHGE procedure and perhaps misidentified the PIIs. In studies using the O'Farrell prefocused 2D-GE techniques, PIIs often did not enter the basic end of the pH gradient.

The presence of abundant, positively charged proteins on the surface of the gonococcus could have interesting ramifications on the interaction of the gonococcus with the host. The physiologic pH of the

sites of gonococcal infection may be about pH 4.5 (urethra, vaginal vault) and pH 7.38 (blood, Fallopian tube, tissue). At these pHs, the PIIs would be positively charged. However, the net surface charge for the gonococcus has been reported as negative, for both PII^+ and PII^- organisms within a strain (144). Thus, the charged moiety of PII may be buried in the membrane and hidden from the external milieu or it may be effectively neutralized by interaction with a more abundant, negatively charged surface component such as LPS.

Peptide mapping was performed to evaluate the structural relatedness of PII variants. The surface-exposed portions of PIIs were labeled with ^{125}I. Digestion with V8 protease gave similar peptide patterns with two conserved cleavage sites. There were also several peptides of unique M_r generated for each PII. The results indicated that there was at least two regions of the molecule which were conserved and several regions which were unique. Previous TLC peptide mapping studies by Swanson and Heckles (56, 142) of purified PIIs with trypsin and alpha-chymotrypsin have suggested large regions of structural homology (80-90%) in PIIs purified from different strains. In the studies by Heckles, he suggested that the unique peptides were surface located. The studies reported here are important because they demonstrate that the PIIs contain both variable and constant regions and confirm the findings of previous studies.

The amino acid analyses showed that a substantial proportion of each PII was composed of hydrophobic and basic amino acids. The hydrophobic amino acids may play a role in lipid-protein interactions or some may be located on the outer regions of the molecule. Measurements of gonococcal hydrophobicity by hydrophobic-interaction chromatography

show that whole, nonpiliated gonococcal cells are only slightly hydrophobic and that possession of the opacity associated proteins in the OM increases relative hydrophilicity (144). However, this observation may not be true for all PII variants within a strain. Contact angle studies of water drops placed on lawns of Op or Tr colony variants show that hydrophilicity is strain and colony phenotype variable. In strain F62, Op colonies were more hydrophobic than Tr colonies. In strain MS-11, the converse was true. (D. Brown and J.F. James, unpublished observations). Thus, there may be one PII which is relatively more hydrophobic than others. Its expression could lead to increased colony friability, granularity, and clumping of organisms. In addition, there exists in each strain a PII which is associated with the transparent colony phenotype, a smooth butterous colony, easily suspended in buffer. Presumably growth in these colonies are more hydrophilic than growth in isogenic opaque colonies. However, there is no direct evidence that PIIs are involved in gonococcal hydrophobicity.

Previous techniques for extraction of gonococcal outer membrane proteins were developed for protein I and when applied to protein II have usually had several problems (46, 145). PII is not as easy to extract as PI; it is more basic; it requires high ionic strength plus 1-10% detergent to successfully keep it in solution; when separated from detergent, PII readily precipitates in physiologic solution and buffers. These studies have used acid and ethanol precipitation to remove detergent from PII. For functional studies (see Chapter 3) of purified PII, it was necessary to develope a purification procedure which extracted the protein with as little loss of conformation as possible and from which the detergent was easy to remove. Studies of purification

and analysis of lactose permease of E. coli have successfully used octyl glucoside to extract this protein and functionally reconstitute it into liposomes (146). It was found that octyl glucoside could be used to extract several gonococcal OM proteins (PI,II,III,62K) and that extraction of PII usually required the presence of 1M NaCl. Curiously, some PIIs in strain FA1090 could be extracted with 1% octyl glucoside alone. This observation suggests that the interaction of PII with other membrane components can vary. It suggests that OMs (containing certain PII variants) are somehow organized differently and from these OMs, membrane proteins will extract differently. It is unknown whether this phenomenon is due directly to PII, or is a membrane property which covaries with PII.

The second protein band extracted with PII (M_r 26,800) (Figure 2.7) may represent an extraction artifact, a PII with residual heat-modifiability, or a precursor form of PII. The protein appears to react with immune, PIIa specific serum. The M_r of the purified form was not heat modifiable. The protein could be a glycosylated form of PIIa although reports of glycoproteins in bacteria are extremely rare. That region of the gel was negative for carbohydrates, by tests with Stains All (147), PAS stain, and modified silver stain (148). The protein is not a second PII species because it is not expressed in OM vesicles. The protein could represent a precursor form of PII. This explantion is attractive for the gonococcal protein, although it is unlikely. The phenomenon was observed only with PIIa of strain F62. Elucidation of the role of this gonococcal protein awaits further analyses and biosynthesis studies.

In conclusion, PII variants within a strain are closely related molecules as are PII variants between gonococcal strains. They are among the most basic of the outer membrane proteins. Each molecule appears to have unique, surface-exposed regions as well as conserved regions. The subtle variations in structure may have significant effects on PII function and gonococcal pathogenic mechanisms.

Figure 2.1 The SDS-PAGE protein patterns of the steps in the preparation of the gonococcal outer membranes by lithium acetate extraction. Lane 1,2 starting organism; lane 3,4 pellet from lithium extraction; lane 5,6 supernatant from lithium extraction; lane 7,8 outer membrane vesicles pelleted by ultracentrifugation; lane 9,10 supernatant from ultracentrifugation. Lane 11 molecular weight standards including 92, 68, 45, 31, 21, 14.4 Kdaltons. Lane 7 and 8 contain outer membrane vesicles. Samples from each step were solubilized at 37°C and 100°C respectively. The organism used in this example was strain F62 PIIa.

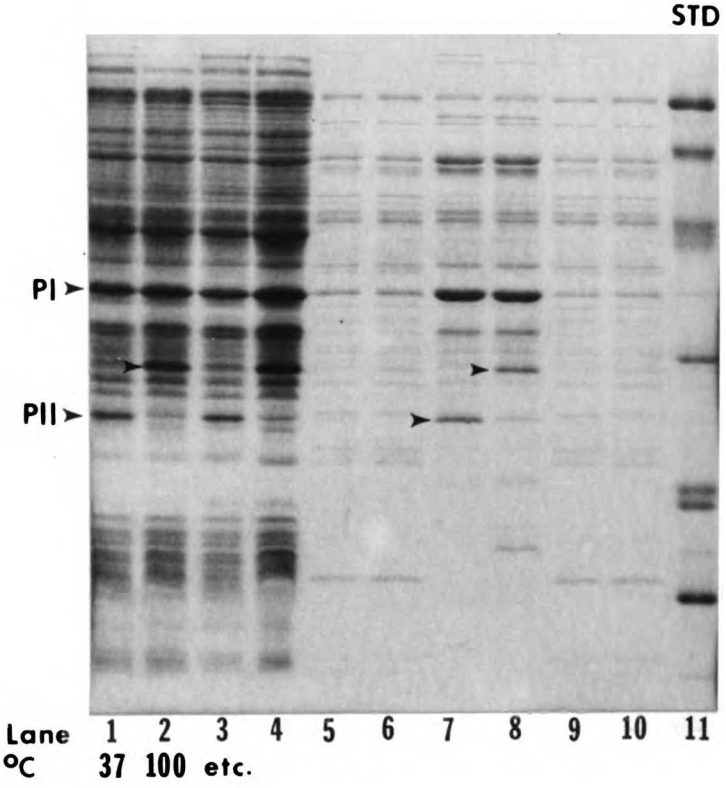


Table 2.1.

Colony Phenotype	PII Composition	Opalesence	Color	Consistence	PII of parent
P-Tr	PII-	none	buff	smooth, butterous	PIIa *
P-Op^{4+}	PIIa	intense, gold granularity	dark brown	very friable	PII$^-$ *
P-Tr	PIIb	faint bluish, no granularity	tan	smooth, butterous	PII$^-$
P-Op^{3+}	PIIc	white, granular	tan	moderately friable	PIIb
P-Op^{2+}	PIId	yellow, orange slight granularity	tan	smooth, butterous	PII$^-$

*These colony phenotypes were provided by Dr. John Swanson; the original strain was isolated by Dr. Kellogg in 1963 and was used to describe his phenotyping scheme (4).

Table 2.1 The characteristics and parentage of the colonial phenotype variants from N. gonorrhoeae strain F62 and their protein II composition. All colony phenotypes contained nonpiliated organisms.

Table 2.2

Colony Phenotype	PII Composition	Opalesence	Color	Consistency
P-Tr	PII$^-$	none	buff	smooth, butterous
P-Op^{3+}	PIIa	golden, very granular	dark brown	very friable
P-Tr	PIIb	bluish, slight granularity	tan	smooth, butterous
P-Tr	PIIc,e	none	buff	smooth, butterous
P-Op^{2+}	PIId	white, granular	tan	slightly friable

Table 2.2 The colonial characterisitcs, phenotypes and the protein II composition of colony variants of N. gonorrhoeae strain FA1090. All organisms were nonpiliated.

Figure 2.2 The SDS-PAGE gel patterns of outer membrane vesicles prepared from N. gonorrhoeae colony phenotype variants in strain F62 and FA1090. The samples were prepared at 37°C and 100°C. The protein II variants are indicated in each sample with an (>). The major outer membrane protein (PI) is indicated with a (>). PIII (>) is the faint band just below PI. A 31 Kdalton molecular weight standard is seen in the far lane.

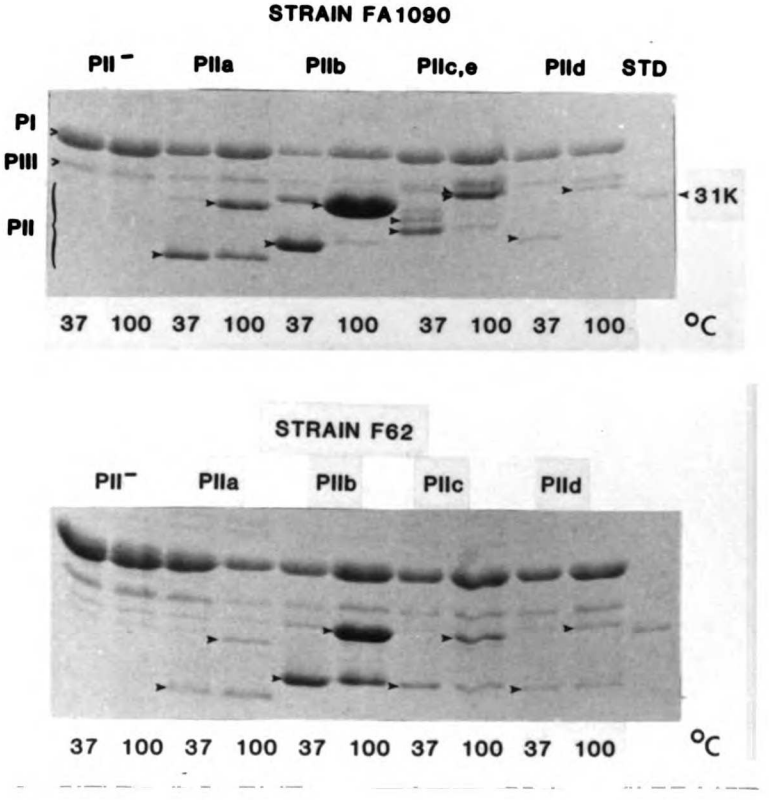


Table 2.3

F62	37°C	100°C
PIIa	26.37 ±.35*	30.15 ±.69
PIIb	28.67 ±.42	30.10 ±.75
PIIc	28.10 ±.34	30.75 ±.59
PIId	27.75 ±.31	31.52 ±.83
FA1090		
PIIa	26.5 ±.27	30.0 ±.48
PIIb	27.5 ±.51	31.2 ±.60
PIIc	28.4 ±.33	31.6 ±.63
PIId	27.3 ±.47	32.0 ±.65
PIIe	29.2 ±.39	32.0 ±.35

*In Kdaltons ± standard error; calculated by R_F from SDS-PAGE

Table 2.3 The apparent molecular weights of the protein II variants of N. gonorrhoeae strain F62 and FA1090. The molecular weights were determined by SDS-PAGE of samples which were solubilized at 37°C and 100°C.

Figure 2.3 The autoradiograms of ^{125}I-labeled outer membrane vesicles (panel A) and selected whole cells of N. gonorrhoeae strain F62 (panel B). Panel A: lane 1,2,3,4 contained outer membranes from FA1090 PII a,b,c,d positive organisms respectively; lane 5,6,7,8 contained outermembrane from F62 PII a,b,ce,d positive organisms respectively. The PIIs (➤)label in both outer membranes and whole organisms confirming their surface location. PIIa in strain F62 and PIId in strain FA1090 did not label well. PI and a 58 kdalton protein labelled in outer membrane preparations. Panel B: the predominant proteins labelled were PI and PII. PII (➤) demonstrates heat modifiability of its apparant molecular weight.(see text for details.) Lane 1,2 F62 PIIa containing organisms; lane 3,4 PIIb containing orgainisms; lane 5,6 PII^- organisms.

Figure 2.3, panel A. ^{125}I-labelled gonococcal outer membrane vesicles from organisms containing PII variants.

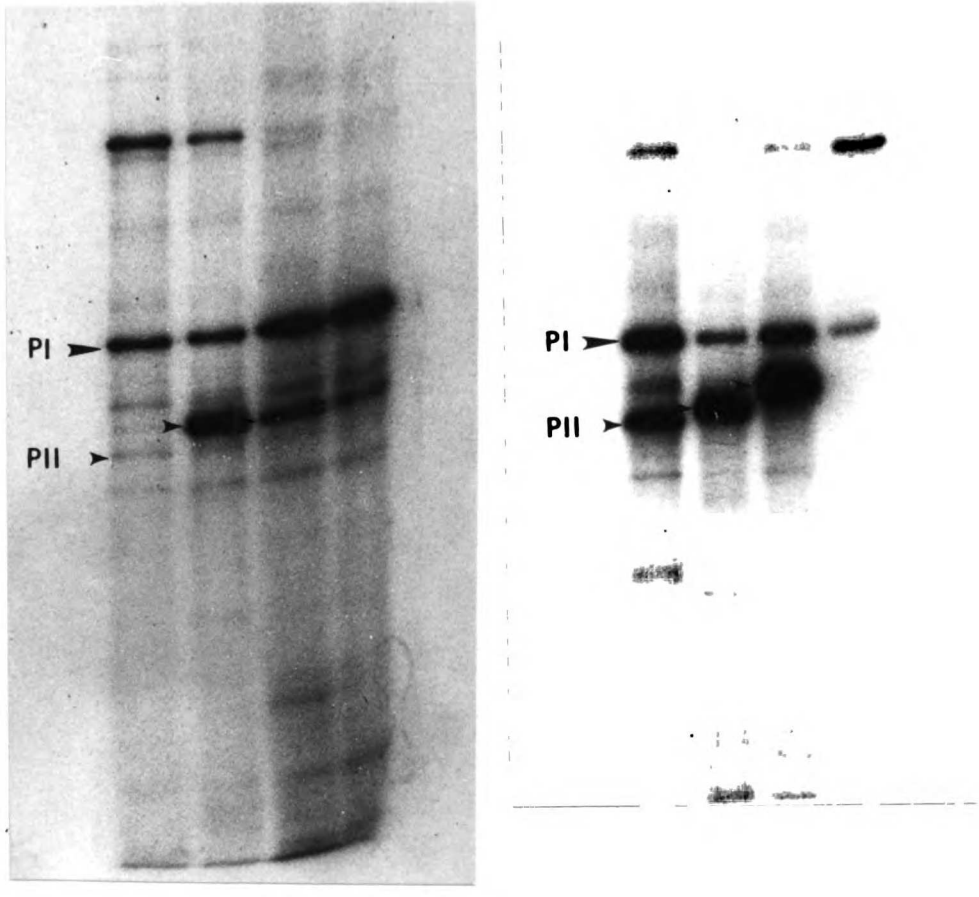


Figure 2.3, panel B. Examples of ^{125}I-labelled whole gonococci from different colony phenotypes of the same strain.

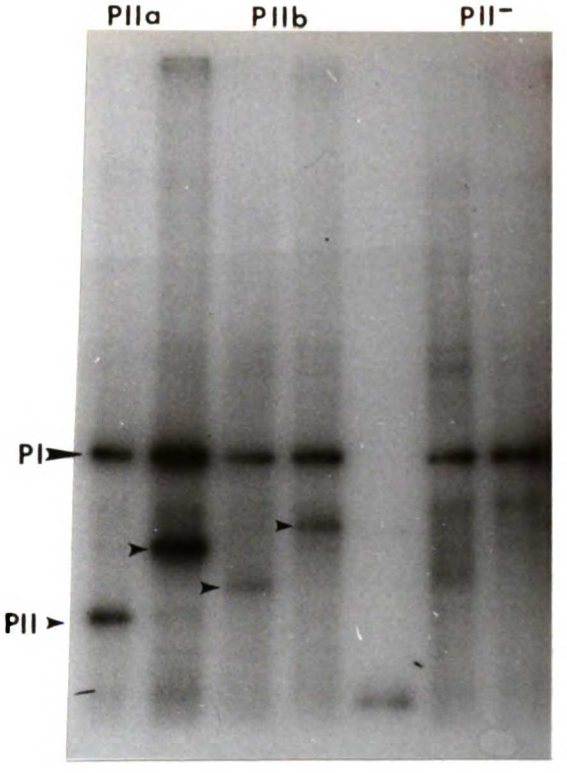


Figure 2.4 Panel A: non-equilibrium pH gradient gel electrophoresis (NEpHGE) of outer membranes from strain F62 containing PIIb. The PII migrates at the front of the proteins approaching the basic end of the gel. Panel B: NEpHGE of purified PIIa from strain F62. The sample contains both the 37° and 100°C forms of the protein and these migrate to the same pI position.

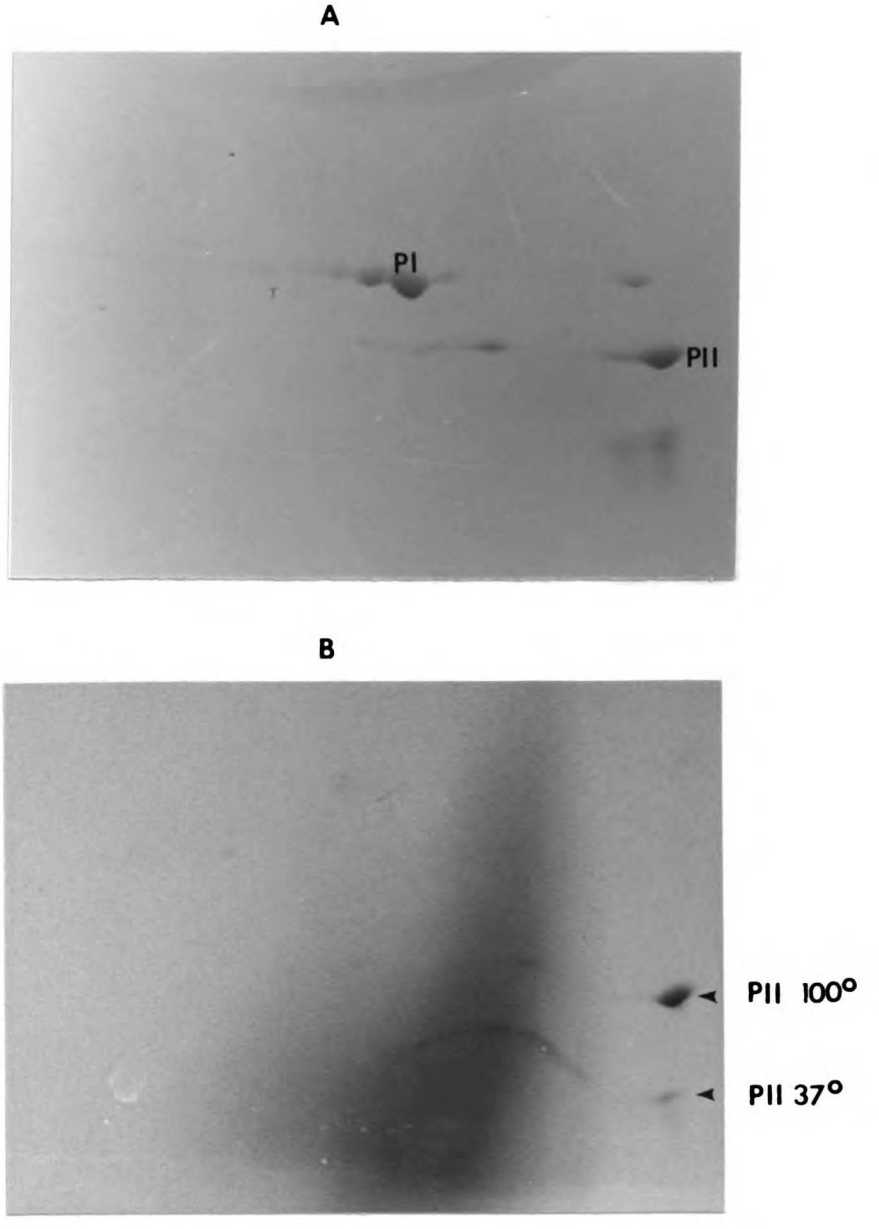


Figure 2.5 Two dimemsional gel electrophoresis of proteins in outer membrane vesicles from organisms containing PIIa. PII has a more basic pI than PI and usually is not seen on this type of gel.

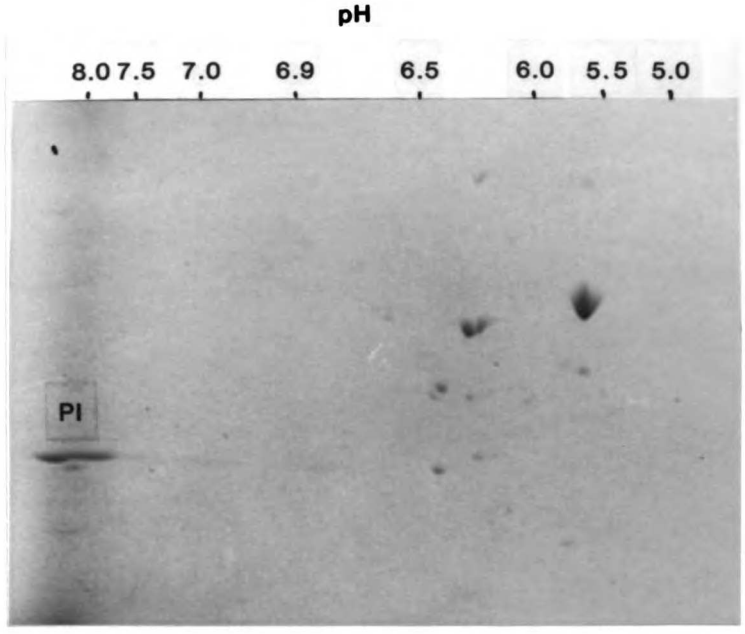


Figure 2.6 Staphlococcal V8 protease peptide mapping of ^{125}I-labeled protein IIs from N. gonorhoeae strains F62 and FA1090. Protein IIa, b, c, and d from strain F62 are seen in lanes 1 through 4; protein II a, b, d, c, and e from strain FA1090 are seen in lanes 5 through 9. The molecular weight markers are indicated by arrows (➤) at 31, 21 and 14 K daltons. There is a conserved major cleavage site at 19 to 21 Kdaltons. The 24 K dalton fragment of F62-PIIb is very faint and can be detected with longer exposures times of the autoradiograph.

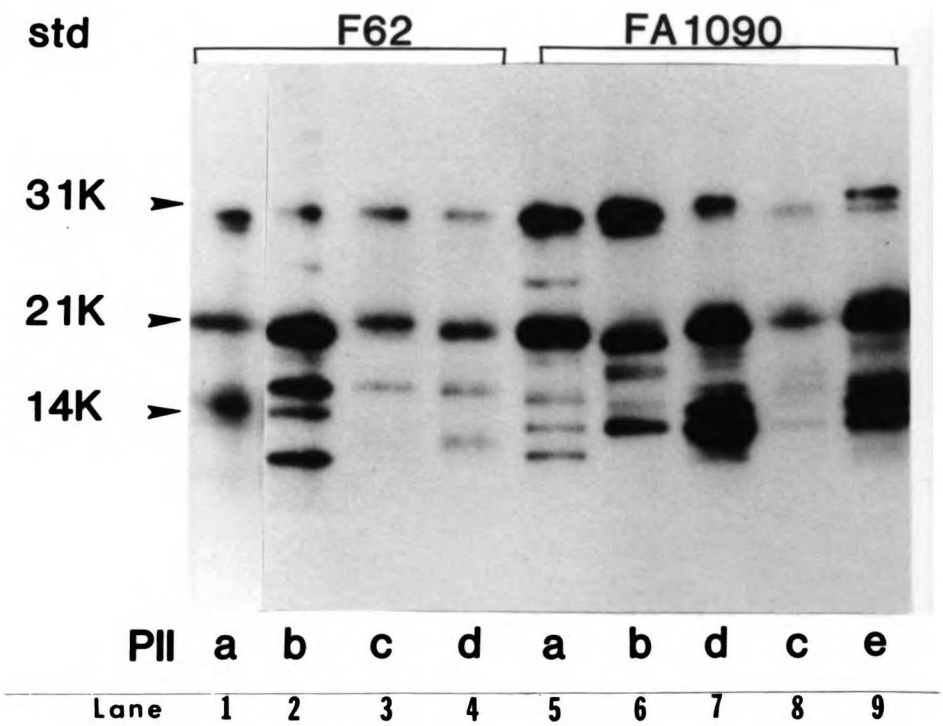


Table 2.4

	Strain F62				Strain FA1090			
	PIIa	b	c	d	PIIa	b	c	d
asx[a]	24	18	15	28	24	28	28	29
thr	8	7	7	11	10	6	9	11
ser	18	10	25	33	23	11	22	20
glx	26	17	21	32	37	28	32	32
pro	9	2	6	9	8	9	9	8
gly*	--	--	--	--	--	--	--	--
ala	22	19	19	17	20	25	28	28
val	16	17	9	14	14	17	16	25
ileu	10	6	5	8	6	9	8	8
leu	14	10	8	13	12	13	12	15
tyr	8	8	5	6	9	10	10	12
phe	7	5	4	5	7	7	7	9
his*	--	--	--	--	--	--	--	--
lys	11	10	7	8	9	13	12	15
arg	12	12	4	7	9	12	7	14

a cys, met, trp not determined
* levels not valid due to extraction, purification procedure.

%hydrophobic	29	32	23	24	25	30	26	30
%basic	12	16	8	8	10	13	13	13

Table 2.4 The amino acid analyses of N. gonorrhoeae outer membrane protein II variants from strain F62 and FA 1090. The hydrophobic amino acids included alanine, valine, leucine, isoleucine, tryosine and phenylalanine. The basic amino acids included lysine, arginine, and

Table 2.5 The flow diagram of the steps involved in the purification of PII from N. gonorrhoeae with the detergents Brij 96, octyl glucoside, and salt. See text for details.

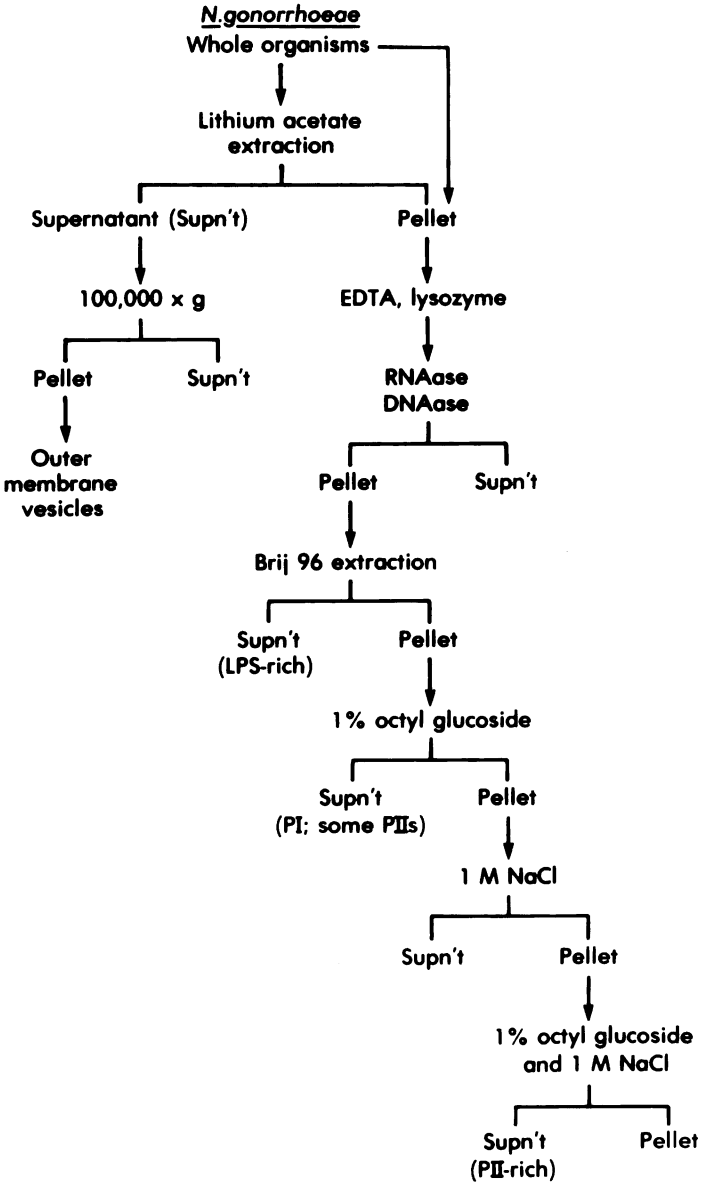


Figure 2.7 The effect of octyl glucoside and 1 M NaCl on the sequential extraction of protein IIa from N. gonorrhoeae strain F62. Samples for each fraction were solubilized at 37° and 100°C. The SDS-PAGE protein patterns of the starting material (lane 1,2); pellet (lane 3,4) and supernatant (lane 5,6) from 1% octyl glucoside extraction; pellet (lane 7,8) and supernatant (lane 9,10) from 1 M NaCl extraction; pellet (lane 11,12) and supernantant (lane 13,14) from 1% octyl glucoside and 1 M NaCl extraction. Molecular weight standards are 92, 66, 45, 31, 21, and 14.4 Kdaltons (lane 15). The major protein (26,000 daltons) extracted in lanes 13,14 reacts with rabbit PIIa specific antiserum in the Western blot assay. The minor band at 26,800 daltons, extracted with the PII also reacts with PIIa specific antiserum.(<)

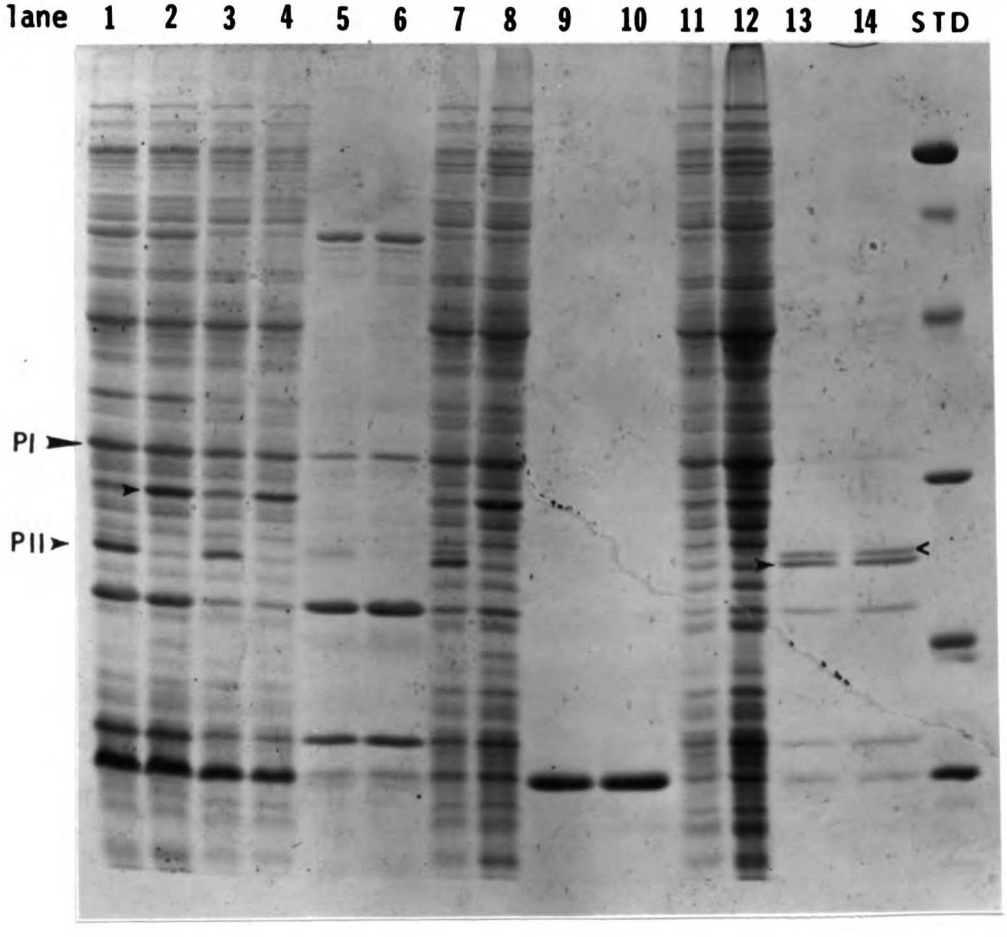
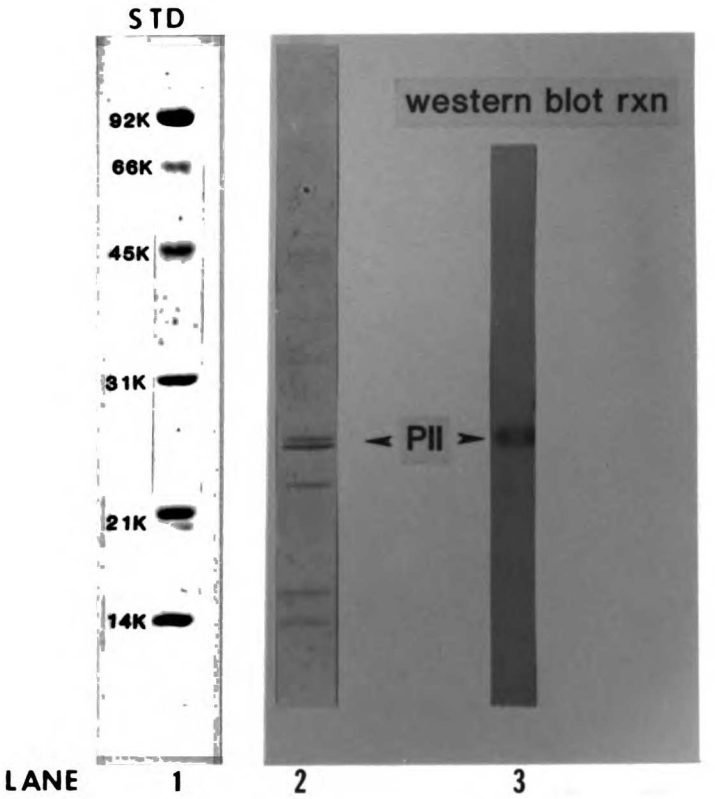


Figure 2.8 The Western blot reactions of proteins purified by sequential extraction of N. gonorrhoeae with 1% octyl glucoside and 1 M NaCl and reacted with rabbit PII specific antiserum. Lane 1 molecular weight standards, lane 2 extracted proteins, lane 3 blot reaction. PII is indicated with and arrow. The protein just above PII also reacts with the serum.



Chapter 3

Functional Analysis of PII

Abstract

The binding of N. gonorrhoeae to human dells in the absence of pili accurrs by an unknown mechanism. The role of gonococcal outer membrane protein, PII, was investigated using organisms containing PII variants. A binding assay using PII^+ and PII^- OMs was used to characterize the mechanism of binding to HeLa 229 tissue cluture cells. The binding of ^{125}I-labelled OMs was pH dependent, reached equilibrium at 60 to 90 minutes, and was partially reversible. Binding of PII^- OMs occurred and the binding of PII^+ OMs was associated with a 2-10 fold increase. Binding was not always saturable and Scatchard analysis indicated a complex binding mechanism with variable rates of attachment and receptor density. Attachment of PII^+ OMs in the presence of competing 100 fold excess PII^- OMs indicated that 80% of the binding was due to the PII^- mechanisms. Binding of PII^+ OMs in the presence of IgG or Fab fragments from immune, PII specific rabbit antiserum demonstrated only 20% inhibition of binding. Pretreatment of HeLa cells with various enzymes, metal ions, charged molecules, hydrophobic, or hydrophilic peptides indicated that the mechanism may involve a protein, a hydophobic component and a negative charge. Nitrocellulose blots of HeLa proteins incubated with ^{125}I-labelled OM vesicles showed a 15,000 dalton protein

to which vesicles strongly bound. The results of these studies suggested that the binding mechanism is a multifactorial process involving specific and nonspecific recognition; PII enhances binding but may not be directly involved in the mechanism.

Introduction

The event initiating bacterial infection is believed to be attachment of the organism to the host's mucosal surface (94). In gonococcal infection, the attachment process is probably multifactorial. Initial adherence is mediated by pili (63, 64, 71) and involves a specific, carbohydrate recognition mechanism (64). A secondary attachment mechanism which binds the gonococcal membrane to the host cell membrane presumably involves other surface component(s) (Figure 3.1.). The secondary mechanism is not well understood and may involve specific recognition (ligand -receptor interaction) as well as nonspecific processes, such as ionic or electrostatic forces. This chapter considers the possiblity that PII may be involved in the secondary attachment process (61, 126, 127).

Previous attachment studies with whole gonococci have shown that there is a difference in attachment between organisms from P++Op and P++Tr and P-Op and P-Tr colony phenotypes to human cell lines. (61, 150). Gonococci from Op colonies have PIIs while gonococci from Tr colonies may or may not have PIIs. Organisms containing a PII usually bind in higher numbers than those without PII. Recently, Heckles et al. (127) have suggested that PII promotes "attachment specificity" to certain human cells and tissues. They suggested that the different PIIs expressed by a single strain had varying affinities for the different

cell types and thus, influenced the site of adherence. eg., urethra, cervix, Fallopian tube, joint, etc. Studies by King and Swanson have shown that gonococci which contain certain PIIs attach more to human polymorphonuclear leukocytes (PMNs) (48, 49). Direct evidence, however, for the role of PII in the secondary mechanism is lacking.

Adherence studies have shown that attachment of whole gonococci may be measured with radiolabelled organisms and human cells in tissue culture. (61, 150). Amoung the problems encountered with such assays were high day to day variation in binding and low sensitivity. Within a single day, experimental results were highly reproducible because the substrate (cells) and organisms were from single cultures. However, on different days, several intervening bacterial subcultures and cell passages were enough to introduce substantial variation. A new assay was needed to allow more sophisticated analysis.

The purposes of the following studies were as follows: i) to develop an assay system which had low day-to-day variability and high sensitivity; ii) to utilize the improved assay to better define the role of PIIs in the attachment process; iii) to characterize and obtain a more quantitative analysis of the secondary binding system(s) and its parameters; and iv) to begin characterization of the cell-surface receptor(s) involved in the secondary attachment process.

From these studies it was found that PII is involved in attachment, but not in the classic sense of a "lock and key" mechanism. The data presented here are consistent with the possibility that there is another component to the secondary attachment mechanism which PII influences and PII is not a ligand.

MATERIALS and METHODS

N. gonorrhoeae: growth and maintenance.

Laboratory adapted strains of F62 and FA1090 were grown and maintained on clear gonococcal colony phenotyping agar. Each organism exhibited several colony phenotypes which could be correlated with variation in PII content (Chapter 2).

Isolation of gonococcal outer membranes.

Outer membranes (OM) of N. gonorrhoeae were isolated according to Heckles (46) with slight modifications. Twenty gm (wet weight) of N. gonorrhoeae grown on PPT agar medium for 20 hours, were scraped into 100 ml of 0.2 M LiAc/HAc buffer, pH 6.0, with 5 mM EDTA. The organisms were shaken for 3-4 hours at 45°C in a New Brunswick shaker incubator. The suspension was placed in an Lourdes tissue homogenizer chamber and blended for 10 minutes. The homogenate was centrifuged at 12,000 x g for 10-15 minutes at 4°C and the supernatant carefully removed to prevent contamination with the cell pellet. The supernatant fluid was ultracentrifuged at 100,000 x g for 3 hours in a SW25.1 rotor (Beckman, Palo Alto, CA). The yellow, glassy pellet was resuspended in 0.2 M NaAc/HAc buffer, pH 6.0, and stored at -70°C.

Iodination of gonococcal outer membrane vesicles.

One hundred to 200 ug of OM protein was ^{125}I-radiolabeled by a lactoperoxidase catalyzed reaction in the presence of H_2O_2. To a 100 ul volume of OMs in 0.2 M NaAc buffer, pH 6.0, 20 ul of lactoperoxidase enzyme (160 U/ml) (Calbiochem, La Jolla, CA) and 0.5 mCi of I^{125} (100 mCi/ml) were added and mixed. To initiate and maintain the reaction, 10 ul of .001% (v/v) H_2O_2 solution was added at time 0 and 10 minutes. The

reaction was stopped with 0.5 ml of 5 mM beta-mercaptoethanol (2-ME) at 20 minutes. The mixture was immediately chromatographed on a Sephadex G-25 column (10 x 1cm) equilibrated with acetate buffer. The void volume was retained and radioactivity and protein content were determined. ^{125}I-labelled membranes were usually used within 24 hours and could be stored for one month at -70°C.

BINDING STUDIES

Preparation of HeLa 229 cells

HeLa 229 cells were a gift from Dr. Ernest Jawetz, Dept Microbiology, University of California, San Francisco. They were routinely cultured in MEM with 10% calf serum, glutamine and antibiotics as previously described (61). For binding studies, a 150 cm^2 flask (Falcon Labware, Oxnard, CA) containing 48 hour old, confluent monolayers of HeLa 229 cells were rinsed with pancreatin-versene-trypsin (PVT) solution containing 0.06% (w/v) hog pancreas trypsin 1:250 (ICN, Cleveland, OH); 0.01% (w/v) Pancreatin 5X (ICN); 0.05%(w/v) ethylenediamine tetraacetic acid (EDTA or versene) (ICN), in 0.8%(w/v) NaCl, 0.04%(w/v) KCl and 1 mM Na_2HPO_4, pH 7.6, with 0.001%(v/v) phenol red. The cells were lifted with fresh PVT and suspended in 10 ml of Eagle's MEM (Microbiological Assoc., Walkersville, MD) medium without antibiotics, and with 10% heated calf serum. A sample was removed and the concentration of cells/ml determined by using a Neubauer chamber (Spencer Co, Buffalo, NY). HeLa cells were diluted to 2-5 x 10^5 cells/ml in serum-supplemented MEM without antibiotics, and 1.0 ml aliquoted into each well of a Falcon 24 well tissue culture plate (VWR Scientific Co) containing a sterile, 15 mm circular glass coverslip

(Bellco Glass, Vineland, NJ). The cells were incubated overnight at 37°C with 5% CO_2 in air for 20 hours and used the next day.

Inoculum preparation.

Dilutions of ^{125}I-radiolabelled OMs were made in buffered M199 (Microbiological Assoc.), and the OM concentrations usually ranged from 0.5 ug/ml to 10 ug/ml. Buffers varied with experimental design, but were usually 100 mM in concentration. One ml of inoculum was used per coverslip and duplicate coverslips were tested. All OM preparations were mildly sonicated in a beaker in a Bransonic 12 water bath for 10 minutes before use. The water bath temperature was monitored and remained constant at 22-24°C. Trypan blue exclusion studies of HeLa 229 cells in M199 medium showed greater than 99% viability after 60 minutes incubation. Long term viability (more than two hours) was greater at pH 7.4 (greater than 96%) than at pH 6.0 (greater than 90%).

Saturation assay

Confluent monolayers of HeLa 229 were prerinsed with 1 ml of M199 (Microbiol. Assoc.) buffered with 100 mM potassium phosphate buffer, pH 7.38, or 100 mM NaAc buffer, pH 6.0, immediately before use. The pre-rinse buffer was not allowed to sit on the cells for longer than 5-10 minutes. One ml of inoculum was added and the trays of coverslips incubated at 37°C with CO_2 for 60 minutes without shaking. All concentrations were tested in duplicate and sixty minutes was determined as the optimum incubation time from equilibrium studies.

The inoculum was removed by aspiration and 1 ml of buffered M199 was gently added to wash the cell surface. The wash was removed immediately and the coverslips placed in a glass tube (VWR, 13 x 100 mm) and counted in a Packard gamma counter (Packard Instruments, Downers

Grove, IL.) for one minute.

Equilibrium assay

Coverslips containing confluent HeLa 229 monolayers were prerinsed with appropriate buffer and inoculated with 1 ml of buffered M199 warmed to 37°C, containing 5 ug radiolabelled OM protein. Coverslips were incubated with OMs for varying times at 37°C in 5% CO_2 in air. Time points were tested in duplicate. The inocula were removed at intervals from 0 to 240 minutes. At each incubation time the inoculum was aspirated and the coverslips gently washed with 1 ml of M199. ^{125}I bound to coverslips was determined in a Packard gamma counter for one minute.

Reversibility assay

Coverslips with HeLa 229 monolayers were prerinsed with buffered M199 and inoculated with 1 ml of M199 containing ^{125}I-labelled OMs at a concentration of 5 ug protein/ml. Inocula were incubated with cells for 30 minutes at 37°C with 5% CO_2 in air, then removed by aspiration. The coverslips were washed once with 1 ml of M199, the wash removed, and a second 1 ml of M199 added. The second wash was removed at 0 time, or up to 120 minutes later. Time points were tested in duplicate. The amount of bound counts remaining on the coverslip was determined.

Influence of pH

Inocula, containing 8 ug/ml of ^{125}I-labelled OM vesicles, were prepared in medium M199 buffered at either pH 5.5, 6.0, 6.5, 7.0, 7.38, or 8.0. For pH 5.5 and 6.0, 100 mM NaAc buffer was used; and at the other pH's, 100 mM phosphate buffers were used.

Coverslips, containing confluent HeLa 229 monolayers, were prerinsed with the appropriate buffer and 1 ml of inoculum was added.

Trays were incubated for 60 minutes, coverslips were rinsed with 1 ml M199 and counted.

Protein assay

Protein was measured by the Lowry technique as modified by Peterson (129). The assay is performed in the presence of SDS to improve the solubilization of intrinsic membrane proteins.

Antisera production

Young adult, female New Zealand white rabbits (2.5 Kg) were used for production of antisera. Rabbits were housed in animal care facilities of UC Medical Center and allowed to acclimatize for 2 weeks before immunization. All animals were bled for base titers from the peripheral ear vein by a vacuum pump connected to a rabbit ear jar (Bellco). Rabbits were immunized subcutaneously in 2 locations with an equal mixture of purified PIIa from strain F62 (200 ug protein) and complete Freund's adjuvant (GIBCO). Four weeks later the animals were bled and reimmunized with immunogen mixed with incomplete Freund's (ICFAg). Titers and reactivity were determined by Western blot reaction. Three to 4 weeks later, the rabbits were bled, titers were determined, and the animals reimmunized with ICFAg if necessary. Total antigen per rabbit was 400-600 ug protein.

Absorption of rabbit antisera

Each immune rabbit serum was absorbed with whole N. gonorrhoeae by mixing 100 ul of serum with 2 x 10^9 bacteria of appropriate colony phenotype and incubating at 4°C for 1 hour. Each serum was exhaustively absorbed by repeating the treatment 3 to 4 times with fresh organisms.

Sera were also absorbed with viable HeLa 229 cells by mixing 100 ul of serum with 1 x 10^8 cells and incubating at 4°C for 1 hour.

Rabbit IgG purification

Rabbit IgG was purified from whole, immune rabbit serum by affinity chromatography using protein A coupled to Sepharose CL-4B (PA-Sepharose) (Sigma, St. Louis, MO). One gram of PA-Sepharose was swollen in PBS and a 4 ml column poured in a 10 ml syringe fitted with 18 gauge needle. The column was equilibrated with PBS, the effluent connected to a BioRad UV monitor and strip chart recorder (BioRad, Richmond, CA)) and monitored at 280 um. Three to 5 ml of whole rabbit serum was processed at a time; after loading, the column was washed with 10 ml PBS or until the UV monitor returned to zero OD units and then 10 ml of 0.58%(v/v) HAc in saline was added to elute bound IgG. The IgG rich fractions were combined, dialyzed against PBS overnight, concentrated in dialysis bags to one half of their original volume with polyethylene glycol (PEG) 20,000 and then redialyzed overnight against PBS. IgG was aliquoted in 0.5 ml volumes and stored at -70°C. The IgG concentration was determined by the Peterson protein assay (ref); the purity was tested by immuno-electrophoresis using a Hyland immunoelectrophoresis kit (Hyland Diagostics, Round Lake, IL) and goat anti-rabbit whole serum (Cappel, Cochranville, PA).

Preparation of Fab fragments

Twenty to 30 mg of rabbit IgG was suspended in 0.1 M NaAc buffer, pH 5.5, with 2 mM EDTA and 1 mM cysteine. Papain (Sigma) was added to a final concentration of 1 ug per 100 ug IgG. The digestion mixture was filter sterilized through a pre-wetted 0.22um Millipore filter (Millipore Corp., Bedford, MA) and incubated at 37°C for 15 hours. The digestion was stopped by the addition of p-hydroxymercuribenzoate (Sigma) to a final concentration of 1 mM. The mixture was dialyzed

overnight against PBS at 4°C. The dialysate was chromatographed over a PA-Sepharose column. The void volume, rich in Fab fragments, was collected. Fc fragments and undigested IgG were retained on the column and eluted with 0.58% HAc in saline. The purity of the Fab fraction was determined by SDS-PAGE.

SELECTIVE BINDING ASSAY

This assay was designed to detect the selective binding of solubilized, I^{125}-labeled gonococcal proteins to HeLa 229 cells and was modified from the technique of Krause and Baseman 1982 (151).

Extrinsic labelling of N. gonorrhoeae and extraction of proteins

N. gonorrhoeae strain F62 was grown overnight on PPT agar, the growth removed with a cotton swab, suspended in 4 ml Dulbecco's PBS and adjusted to 200 Klett units optical density. The organisms were pelleted by centrifugation, resuspended in 200 ul of PBS, and iodinated by the lactoperoxidase technique. The ^{125}I-labelled organisms were pelleted by centrifugation, washed twice in PBS, and the final pellet resuspended in 1 ml of SDS-DOC solubilization buffer composed of 35 mM Tris, pH 8.2, with 0.25 M NaCl, 1.6% (w/v) deoxycholate (DOC), 0.1%(w/v) SDS and 1 mM phenylmethylsulfonylflouride (PMSF). The radiolabelled organisms were solubilized overnight at room temperature and the extract centrifuged for 10 minutes in a microfuge (Beckman).

Preparation of HeLa 229 Cells

HeLa 229 cells were grown as monolayers in 150 cm^2 plastic flasks to confluency, washed 4 times with PBS, and were lifted with 0.02%(w/v) EDTA in PBS. The cells were gently vortexed and pelleted by low speed centrifugation. The pellet, containing about 1.5 x 10^8 cells, was suspended in 20 ml PBS and 25% glutaraldehyde (specially pure, Sigma)

was added dropwise to a final concentration of 2.5%(v/v) glutaraldehyde. The cells were lightly fixed in this solution for 1 hour at 4°C with gentle stirring. The HeLa cells were washed extensively with PBS to remove all traces of fixative, and then incubated with 0.2% BSA in PBS for 1 hour at room temperature. The cells were then washed twice with M199 buffered with 0.2 M NaAc, pH 6.0, and 5 mM EDTA, and suspended to 1 x 10^6 cells/ml. The assay was also performed at pH 7.4 by suspending fixed HeLas in M199 prepared with 0.2 M phosphate buffer.

Binding Assay

One ml of glutaraldehyde fixed HeLa cells in M199 acetate buffer were mixed with 100 ul of ^{125}I gonococcal extract, containing about 1-2 x 10^7 cpm, and incubated for 30 minutes at 37°C with occasional mixing. The cells were pelleted by centrifugation in Beckman microfuge for 30 seconds and washed extensively with SDS-DOC solubilization buffer. The cell pellet was suspended in SDS-PAGE solubilization buffer and boiled for 10 minutes. A portion of the extract containing 5 x 10^5 cpm was loaded on a Laemmli PAGE gel and electrophoresed. Autoradiograms were made by exposing dried gels to Kodak AR X-OMAT AR X-ray film with Dupont Cronex intensifying screen.

RECEPTOR CHARACTERIZATION STUDIES

HeLa 229 cells on coverslips were pretreated with a variety of substances including carbohydrates, peptides, ions, and enzymes for 60 minutes. Coverslips were washed once with 1 ml of M199 in the appropriate buffer and the rinse removed immediately. One ml of inhibitory substance in M199 was added and the coverslips incubated at 37°C. The fluid was removed, the coverslip rinsed with 1 ml of buffer, and a

inoculum of ^{125}I-OMs (5 ug/ml) added. Duplicates were tested for each condition. At the end of the 60 minute incubation with OMs, the coverslips were washed with 1 ml of fresh M199 and counted.

SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (37) and a BioRad Protean apparatus was used. In general, 12.5% acrylamide separating gels were overlayed with 5% stacking gels and all gels were made the day of use. All samples were solubilized in sample buffer as previously described (see Chapter 2).

Staining

The SDS-PAGE gels (0.75mm) of outer membrane vesicles were stained with silver according to Tsai and Frasch (148). Gels were air dried between 2 sheets of moistened, BioRad dialysis membrane. Other gels were stained with 0.25%(w/v) Coomassie brilliant blue R-250 (Sigma) in 25%(v/v) isopropanol with 10%(v/v) HAc in water.

Extrinsic labeling of HeLa 229 cells with ^{125}Iodine

HeLa 229 tissue culture cells were grown to confluence (48-72 hrs) in 150 cm^2 flasks, the medium removed and the monolayers gently rinsed with Hank's balanced salt solution (HBSS) (Microbiological Assoc.) three times. The cells were dissociated with 0.05%(w/v) Na EDTA buffered with 1 mM Na_2HPO_4 with 0.15 M NaCl and 5 mM KCl, pH 7.6. The cells were washed with PBS, centrifuged at 500 rpm in an IEC clinical, tabletop centrifuge for 10 minutes and the pellet resuspended in PBS at a concentration of approximately 2 x 10^7 cells/ml. A 200 ul aliquot of cells was removed for iodination. Twenty ul of lactoperoxidase (100 U/ml) (Calbiochem, La Jolla, CA) and 1 mCi of ^{125}I (100 mCi/ml)

(Amersham Corp.) were mixed. Ten ul of a freshly prepared solution of 0.001% (v/v) H_2O_2 were added at 0 and 10 minutes and the reaction stopped at 20' with 0.5 ml of 5 mM 2ME (Sigma). The cells were immediately centrifuged and washed 3 times with PBS. The final pellet was suspended in 200 ul H_2O and mixed with 200 ul of 2X SDS-PAGE sample preparation solution. The sample was solubilized in a boiling water bath for 10 minutes.

Iodination of molecular weight standards

BioRad low molecular weight protein standards were iodinated by the chloramine T reaction. Two ul of molecular weight standards were mixed with 2 ul of H_2O and 20 ul of PBS. Fifteen ul of freshly prepared 1% (w/v) chloramine T (Sigma) and 0.5 mCi of ^{125}I (100 mCi/ml) were added to the mixture and allowed to react for 2 minutes. The reaction was stopped by addition of 15 ul of 25% (w/v) Na metabisulfite (Sigma). This mixture was immediately chromatographed on Sephadex G-25 equilibrated with PBS. The void volume was collected and stored at -70°C until used. A portion of the labelled standards was diluted with SDS-PAGE sample buffer to yield 1 x 10^5 cpm per 10 ul sample.

Iodination of protein A

Purified, soluble staphylococcal protein A (Sigma) was iodinated by the chloramine T reaction. Ten ug of protein A (in 10 ul) was added to 20 ul of PBS and 15 ul of 1% (w/v) chloramine T. After the addition of 0.5 mCi (100 mCi/ml) of ^{125}I the reaction was allowed to proceed for 2 minutes at room temperature. The reaction was stopped with 15 ul of 25% (w) Na metabisulfite and the mixture immediately chromatographed on a Sephadex G-25 column (15 x 1 cm) equilibrated in PBS. The void volume was collected and radioactivity determined. ^{125}I-protein A was

prepared every 2 months and stored at -70°C.

Western blot assay

Rabbit antisera were characterized by Western blot assay and antigen antibody reactions were detected with ^{125}I-labelled protein A. The method of Burnett (152) was modified for use as follows:

A sample containing 10-20 ug protein of OM vesicles was prepared for SDS-PAGE and loaded on a 12.5% acrylamide separating gel with a 5% stacking gel and electrophoresed using the Laemmli buffer system. The gel was immediately blotted onto Sartorius nitrocellulose paper (0.45 um)(Sartorius) overnight by electrophoresis in a BioRad Transblot apparatus with a buffer containing 1.4% (w/v) glycine, in 25 mM Tris, pH 8.3, with 20% (v/v) methanol. The transfer was carried out at 30 volts and 0.1 amps for 18 hours. The blot was air dried briefly and soaked in 5% (w/v) Fraction V bovine serum albumin (BSA) (Sigma) in PBS for 30 minutes at 37°C with shaking. The blot was removed and reacted with antiserum (diluted 1:100 in BSA-PBS) for 90 minutes with shaking at room temperature. The blot was then washed with PBS for 10 minutes followed by 2 washes of PBS with 0.05%(v/v) NP-40 detergent (Sigma) for 20 minutes and finally rinsed in PBS for 10 minutes. The blot was then reacted with ^{125}I-protein A diluted to 1 x 10^5 cpm/ml in BSA-PBS for 30 minutes, and washed extensively as above. The blot was air dried, wrapped in Saranwrap and exposed to Kodak X-OMAT AR X-ray film with a Cronex intensifying screen at -70°C, usually overnight.

Transmission electron microscopy (TEM)

Outer membranes from N. gonorrhoeae were sonicated briefly and diluted to 250-500 ug protein/ml in H_2O and a 10 ul aliquot loaded on a Formvar coated, deionized copper grid. The membranes were allowed to

sit on the grid for 60 seconds and excess fluid shaken off. The excess moisture was removed from the grid surface with filter paper (Whatman No. 1) by capillary action and immediately a 10 ul drop of 1% phosphotungstic acid, pH 5.0, applied for 5-10 seconds. The acid was removed by blotting on the edge of filter paper and the grid allowed to air dry. The grids were viewed in a JOEL electron microscope at 20,000 x magnification.

RESULTS

Section 1. Development of assay

Previous work (62, 63, 153) showed that there could be a variation in binding of whole N. gonorrhoeae to genital tissues (endocervix and fallopian tube epithelia) from a single patient. Similar tissues from different patients also exhibited variation in binding and only small quantities of each tissue were available for assay. For quantitative studies a human cell type which could provide an abundant, reproducible substrate was necessary. Thus, human cells in tissue culture were selected and several cell lines were found to be suitable for attachment studies (61). HeLa 229 cells were selected for these studies for several reasons: i) they were easy to grow and maintain in tissue culture; ii) they were human cells initiated from an adenocarcinoma of the female genital tract (154, 155); iii) Some TEM studies have shown that HeLa cells do not ingest gonococci (150); iv) and importantly, a difference in the binding of gonococci from Op and Tr colonies could be detected (61).

In an attempt to reduce day-to-day variability of binding when whole organisms are used, OMs were prepared by the lithium acetate. The yield of outer membranes by this procedure was lower than other available procedures, however, it provided less cytosol and cytoplasmic membrane contamination of the OM preparations (data not shown). Further, the OM vesicles were prepared by blebbing from the surface of the organism with gentle conditions (200 mM salt). Other OM preparation techniques required detergent extraction, 6 M urea treatment, or ethanol

precipitation which could modify the surface and affect the configuration of the membrane constituents and conformation of the membrane proteins (28, 29, 145).

OM vesicles could be iodinated with ^{125}I to provide a high specific activity (0.00001 ug/cpm) allowing a detection sensitivity of 10 nanograms of protein bound per coverslip.

OM vesicles from each organism were analyzed for size distribution by transmission electron microscopy (TEM). A representative micrograph is in Figure 3.2. Most OM vesicles existed as blebs of similar morphology. Sonication appeared to aid in preparation of uniform vesicles. Thus, all OM inoculum were gently sonicated for 10 minutes in a water bath sonicator before use. Occasionally, after sonication large membrane complexes could be seen by TEM, but these were rare (less than 1%) (see Figure 3.2).

OM preparations were checked for the presence of pili which bind to HeLa cells, and would give false levels of OM attachment. The presence of pilin was probed for by the Western blot technique utilizing antiserum prepared against the common antigenic domain of all gonococcal pilin (fragment CNBr-2, Chapter 1) and reacting it with all OM preparations. The antiserum was kindly provided by Dr. Gary Schoolnik, Stanford University, Stanford, CA. The antiserum reacted with pilin positive organisms from both strain F62 and FA1090 (data not shown). No pilin antigen was detected in any OM preparation.

Cell density studies were performed to determine if crowding of HeLa cells on the coverslips could cause a change in attachment levels. The results are seen in Table 3.1. Cell density variation from 1×10^5 cells/ml to 1×10^6 cells/ml showed no significant difference in

attachment. Only the inoculum density of 5 x 10^4 gave very sparsely seeded coverslips and the increase in attachment seen in this case may represent stick to glass of OM vesicles. It has been reported that whole gonococci stick to glass cover slips. Cell densities of 1-5 x 10^5 cells/ml gave uniformly confluent monolayers and this concentration range of HeLa cells was used for all subsequent experiments.

The parameters of binding which are frequently analyzed include saturation, equilibrium, and reversibility. These types of assays are important because the results yield estimates of binding sites, affinity constants, and the number of different binding systems. For each type of assay, the test conditions were developed for use with OMs and optimized for the collection of data.

Saturation

PII^+ and PII^- OM variants of strain F62 were tested in initial studies and all assays were carried out at pH 6.0. Increasing quantities of ^{125}I-labelled vesicles were tested for binding over a 4 $\log_{10}$ concentration range. Low concentrations (0.004-3.0 ug protein/ml) showed plots of binding with continuously changing slopes. At concentrations of 1-5 ug protein there was a faster change in slope than at lower concentrations and at approximately 5 ug/ml the amount of binding appeared to plateau. (Figure 3.3) At concentrations greater than 20 ug protein/ml, HeLa cells rapidly lost viability, possibly because of LPS toxicity. There was a substantial amount of scatter and variability of binding at higher protein concentrations with the PII^+ OMs. Reproducibility within a single experiment was excellent. The concentrations of 0.25-10 ug protein/ml were chosen for future assays. A standard 60 minute incubation time was chosen from equilibrium studies (see below).

Equilibrium

The ability of the binding system to reach a steady state was tested. Assay conditions were studied over a four hour period. Previous conflicting reports of attachment studies of whole organisms by James et al., (61) and Gubish et al. (150) suggested that equilibrium was acheived at 1 hour or at 4 hours, respectively. The results for PII^+ and PII^- OMs are seen in Figure 3.4. At 60 minutes equilibrium was reached for both OMs and this incubation time was used for all saturation studies. Cells on coverslips also were monitored for sloughing over this time period. At pH 6.0, and with up to 60 minutes of incubation, there was no apparent sloughing and crystal violet staining of the coverslips showed monolayers to be confluent. Monolayers remained confluent at pH 7.38 up to four hours.

Reversibility

The OM binding mechanism was studied for reversability. Initial studies were done with 4 ug OM protein/ml. Cells were incubated for varying times with OMs, the inoculum removed and fresh buffer added. The proportion of bound counts which could be eluted was determined. The results are seen in Figure 3.5 and showed that outer membrane attachment tended to become irreversible at longer incubation times. Long loading times (60 minutes) gave scattered points which were difficult to interpret and in some instances appeared to give continued binding even after the inoculum had been removed (data not shown). Thirty minutes incubation time was chosen because it gave detectable levels of binding and a greater reversibility than long incubation times. A study of wash conditions was made to improve reproducibility and circumvent the artifact of continued binding after inoculum removal. Results were

compared with an additional wash step as follows;

Assay

1 prerinse cells with 1 ml buffer
2 inoculate with OM, incubate 30 minutes
3 remove inoculum

Procedure I	Procedure II
4 add 1 ml buffer, incubate	4 rinse with 1 ml buffer,remove
5 remove buffer, count coverslips	5 add 1 ml buffer, incubate
	6 remove buffer,count coverslips

The results of the comparison are seen in Figure 3.6 (panel A). With only one wash, the duplicate coverslips at most time points gave poor reproducibility, more scatter around a fitted curve, and a curious increase in binding.

Data from experiments with two washes (panel B), demonstrated that a portion of the bound OM protein (30%) could be removed rapidly with the two washes and with time, an additional (20%) could be "eluted." It should be noted that not all of the OM protein was reversibly bound. The procedure with two washes provided better reproducibility, less scatter, and was used in all subsequent reversibility assays.

Reproducibility of the binding of OM vesicles prepared from the same organism at different times was tested. The time course extended over a 13 month period and separate batches of OMs were prepared from strain F62 PIIa^{+}. The results are in Table 3.2. Between batches there could be as much as 50% change in binding. However, within a batch, the binding levels were not significantly altered when the membrane vesicles were stored at -70^{o}C for 6 months.

Previous studies have reported a difference in attachment levels of organisms from different colony phenotypes with a change in pH (61,

127). Our studies expanded on those observations to see if there was any influence of pH on attachment of OMs from organisms containing PII variants. The results for variants from strain F62 and FA1090 are seen in Figure 3.7. Binding patterns for PII variant OMs from both strains showed similarities. The lowest attachment was seen at pH 6.5-7.0 with a slight rise at more acidic pH's and dramatic increases at alkaline pH's. There were subtle differences in rank order of binding (from most to least adherent) of OMs at each pH; at some pH points there appeared to be no binding differences. Two pHs were chosen for further study of binding characteristics: pH 6.0 to represent cervical milieu, and pH 7.38 to represent tissue and blood sites.

Section 2. Binding Studies

All OM vesicles containing PII variants from strain F62 and FA1090 were compared in saturation, equilibrium, and reversibility assays. The purpose of these studies was to compare the binding parameters of the OMs and to attempt to correlate the results with PII variation. All assays were performed using duplicate coverslips and most assays were repeated. The concentration of 5 ug/ml OM protein was used as an internal control. Binding levels at this concentration had to agree between the saturation, reversibility, and equilibrium assays for a given OM, or the results were considered invalid.

Saturation

The effect of increasing concentrations of free ligand (OMs) on the level of binding was determined for each OM preparation. The binding of OMs containing different PII variants is seen in Figure 3.8. Some PII^+ OMs consistently bound in higher amounts than PII^-. These included OMs with PIIa and c in strain F62 and PIIa, c, and e from strain FA1090. At

pH 6.0 there were distinct differences in binding. The differences ranged from 2 to 14 fold for F62 and 2 to 7 fold for FA1090. Specifically, the shapes of the binding curves were varied indicating different rates of attachment (see Scathard analysis). At pH 6.0, not all curves were convex; some were curvilinear (F62 PIIa,b,c) and concave (FA1090 PIIc,e,b). The curvelinear plots indicated that saturating concentrations of ligand were not achieved. Such plots of binding data can represent complex binding systems with multiple, independent receptors, or systems with a single ligand and multiple receptors of different affinities. At pH 7.38, there was a decrease in binding of several OM variants (e.g., F62 PII a,c,d; FA1090 PIIc,e,Tr). In general there was a flattening of curves at pH 7.38 compared to pH 6.0. One interpretation of these results could be that at pH 6.0 there were several binding systems including a saturable and a non-saturable mechanism. It is possible that both systems operate at pH 6.0 and that saturable system does not "operate" at pH 7.38. This interpretation then suggests that there is a change in the number of binding sites with shift in pH and that there is a pH sensitive component to the binding mechanism.

Equilibrium

The results are seen in Figure 3.9. The change in pH dramatically altered binding results. At pH 6.0, in all assays, the binding of OM variants reached equilibrium at about 60-90 minutes and was stable up to 4 hours. The data showed that there was a rapid, initial binding of 16-25% of the inocula within the first minute. At pH 7.38, equilibrium was generally not achieved. FA1090 variants showed similar behavior and their maximum binding levels were similar. F62 plots showed non-equili-

brium conditions and dramatic differences between variants. These equilibrium plots were indicative of binding systems which were irreversible and which "ate ligand."

Reversibility

The reversibility of OM binding was studied and the results are seen in Figure 3.10. In general, the binding of OMs was not fully reversible. A portion of bound OMs could be rapidly removed within 1 minute and rates of reversibility (slopes) appeared to be different for each variant. There was a slight effect of pH on reversibility. Binding at pH 7.38 appeared slightly more reversible than binding at pH 6.0 (Table 3.3). In strain F62 and in strain FA1090, there were distinct differences in reversibility between OM variants (F62 PIIa vs. PIIc (pH6.0); FA1090 PIIc,e vs.PIIb at pH 6.0.) and for individual OMs at different pHs (FA1090 PIIc,e; F62 PIIa).

Scatchard analysis

By this technique several binding parameters may be calculated. The number of binding sites may be estimated from the intercept of the X-axis, the affinity constant may be calculated from the slope and the number of binding systems may be estimated by the shape of the curve. e.g. straight lines mean single systems and curved lines usually mean multiple systems. (156, 157, 158).

The saturation data for three OM variants from each strain were transformed and graphed for Scatchard analysis. (Figure 3.10B and 3.10C) Preliminary analysis of the data suggested that i) each OM binds with a unique attachment constant, ii) the total number of binding sites were different for each OM and iii) for F62 PIIa OMs, several binding systems may be involved. A word of caution here; these estimations are based on

data generated from a binding system(s) which is not fully reversible. This fact invalidates the mathematical premise of the Scatchard analysis and thus, calculations of specific values of intercepts and slopes were not done.

Competition assays

The analyses of the attachment assays have suggested several different binding systems. PII^- as well as PII^+ OMs can bind to HeLa cells. If PII is involved directly in binding, then the mechanism by which PII^- membranes bind should be distinct from the PII^+ mediated mechanism. This hypothesis was tested by competition assay. The binding of PII^+ OMs was measured in the presence of unlabelled, excess PII^- membranes (100 fold). The results are in Figure 3.11. PII^- OMs were very effective competitors and reduced the binding of PII^+ OMs by approximately 80% at pH 6.0 and pH 7.4. Results may be due to steric hindrance of binding. However, one interpretation suggests that a large portion of binding is due to the non PII-mediated mechanism of adherence and that PII^+ and PII^- membranes may compete for the same receptor on the surface of the HeLa cell.

Competition assays were performed for both PII^+ and PII^- vesicles to determine if multiple receptors (specific and non-specific) might be involved. In principle, if multiple types of receptors exist for a given ligand and there is at least a 100-1000 fold difference in association constants, then unlabelled ligand will saturate first those systems with limited binding sites and high affinity constants. Thus, the binding under these conditions will be a measure of more numerous, "non-specific," low affinity binding sites. The results are seen in Figure 3.12. Unlabelled OM vesicles competed poorly with labelled

vesicles. Two conclusions may be made from these experiments. Firstly, this observation means that ^{125}I labelling may have interfered with or denature the surface ligand. Secondly, correction of the non-competition attachment data with the competition data yielded straight lines and suggested that there were "nonspecific" (unsaturable) binding sites involved in the attachment.

A good way to demonstrate multiple receptors for a ligand is to do a binding assay with purified ligand. Scatchard analysis of this assay will generate curved lines if there are multiple binding sites. For this type of assay it is assumed that the ligand is monomeric and in solution. Competition assays with unlabelled, purified protein PIIa and $PIIa^+$ OM vesicles were attempted. These experiments were unsuccessful (data not shown). The concentrations of detergent and salt required to keep PIIa in solution readily solubilized the HeLa monolayers. Two purification procedures for PIIa were tried; Zwittergent 3-14 of Blake et al. (145), and the one developed utilizing octyl glucoside (see Chapter 2). Both PII extractions showed similar solubilization of monolayers from residual detergent. In the absence of detergent and salt, PIIa precipitated.

Selective binding assay

Another approach for determination of the direct role of PII in gonococcal binding was tried. This method was developed by Krause and Baseman (151) to detect the selective binding of proteins (from a mixture of detergent solubilized ^{125}I-labelled proteins) to cellular surfaces. This procedure was applied to ^{125}I-labelled gonococcal surface proteins and glutaraldehyde-fixed HeLa cells. The autoradiogram of the different fractions is seen in Figure 3.13. Numerous proteins

were ^{125}I-labelled by the lactoperoxidase technique and only a portion of these proteins could be extracted by DOC-SDS detergent mixture. The proteins remaining attached to HeLa cells after extensive washing were predominantly PI and PII. The predominant protein from solubilized PII$^-$ organisms which selectively bound to HeLas was PI. In the final pellet for PIIa$^+$ organisms the 100^{o}C and the 37^{o}C form of the PII were detected. Perhaps some of this PII remained in the low molecular weight configuration during boiling because of HeLa cell association or the detergents which were used to extract it.

Antibody inhibition assays

Rabbit antibodies were prepared against purified PIIa, for testing of inhibition of binding of PIIa$^+$ OM vesicles to HeLa 229. The antisera were characterized by Western blot assay and tested against whole gonococcal lysates. The reactions showed one major reactive band corresponding to the molecular weight of PIIa. There were no reactive bands in PII$^-$ organisms (Figure 3.14). Preimmune rabbit serum gave no reaction by Western blot assay.

The effect of 10% whole rabbit serum on attachment can be seen in Figure 3.15. Immune antisera inhibited attachment greater than 75%; however, normal rabbit serum from the same animal gave identical inhibitory results. Efforts were made to further characterize the nature of the inhibition in each serum. Immune and preimmune rabbit sera were exhaustively absorbed with HeLa cells and PII$^-$ gonococci in an attempt to remove cross-reactive and interfering antibodies. There was no change in results; all absorbed sera, immune and preimmune, gave 75-80% inhibition of binding of OM vesicles. The IgG fraction was purified from each serum to look at its inhibitory power (Figure 3.15). Purified

IgG (anti-PIIa) had a small effect on attachment at a 1:20 dilution even when pre-absorbed with HeLa cells (panel C).

The proportion of IgG in the attachment mixture was titrated to see if antibody concentration was optimal for maximum effect. The titration for immune IgG is seen in Figure 3.16. The optimal IgG concentration was determined from three separate experiments as approximately 250 ug IgG/ml. Maximum inhibition of binding achieved was variable and ranged from 35-50%. The average was 43%. Preimmune IgG was titrated only twice due to limited supplies and gave a consistent 20% inhibition of attachment in concentrations from 10 to 300 ug IgG/ml (data not shown).

When the effect of preimmune IgG was subtracted from that of immune IgG there was only a 20% inhibition of specific binding in optimal conditions. This effect was not impressive and the significance of the inhibition was borderline. The results of these experiments were difficult to interpret.

The immune IgG was treated with papain and Fab fragments prepared. Inhibition assays were performed with these and the results were inconclusive. High concentrations of Fab fragments (1000 ug/ml) were required to demonstrate any effect on the binding of PII^+ OMs to HeLa 229 cells. Inhibition was only slight (10%). It is commonly known that the avidity of an antibody changes with valency and thus it is possible that the failure of the Fab fragments to inhibit binding is a meaningless result. Also, it must be remembered that the initial IgG did not appear to inhibit well and thus may have had low avidity or poor, initial specificity for the binding site "determinant."

Section 3. Preliminary characterization of receptor(s)

HeLa cells were pretreated with a variety of carbohydrates, peptides, ions, and enzymes. Two OM variants (PII^- and PII^+) were tested from each gonococcal strain. The PII^+ variant was selected by the criterium of enhanced binding when compared to PII^- (PII^+ greater than PII^- at pH 7.4). The results are seen in Table 3.4. In general, the results were complex. The HeLas were treated with a mixture of glyosidases to see if there was a carbohydrate on the cell surface which was involved in recognition. The carbohydrates cleaved by C. lampus mixed glycosidases included mannose, glucose, galactose, fucose, xylose, N-acetylglucosamine, and N-acetylgalactosamine. These sugars did not appear to be involved in recognition or binding. However, negative results in such studies are not helpful because these enzymes may not be active against complex substrates.

Sialidase, which cleaves neuraminic acid residues, appeared to have some effects on binding. It enhanced F62 OM binding and it inhibited FA1090 OM binding. This effect may be a function of the change in surface charge by the removal of a negatively charged carbohydrate moiety rather than the carbohydrate per se. Polyphosphate, a negatively charged tetramer, inhibited OM binding in FA1090 strains by at least 20% (beyond variance). DEAE-dextran, a positively charged polymer, had no effect (FA1090 PII^+) or enhanced binding. These results indicated that by increasing the net positive charge of the HeLa surface, attachment could be enhanced. Dextran by itself had no effect. The lack of effect of dextran on binding supported the idea that it was the charged moiety of the DEAE-dextran that had an effect on binding. Polylysine (positively charged) enhanced binding in FA1090. This result was

intrepreted cautiously because polylysine makes everything "sticky". From tests with various metal ions, it appears that Ca^{++} may be significantly involved in binding. Calcium dramatically enhanced binding in three of four variants. Iron (Fe^{++}) had a moderate enhancing effect on binding of FA1090 variants, and Mg^{++} ion had no effect.

The effects of proteases, including trypsin, decreased binding in most variants. In both strains, the predominant effect was on the binding of PII^{+} OMs. The result indicated that a protein on the surface of the HeLa cells may be involved. Alternatively, residual enzyme may have been present after rinsing of the HeLa cells. Several OM proteins of the gonococcus, including PII are sensitive to proteases and trypsin and the inhibition may be due to their cleavage rather than a HeLa component.

Synthetic polypeptides were tested to see if changing the conditions of attachment to more hydrophobic (poly-valine) or hydrophilic (poly-glutamine) conditions could influence binding. The results for polyvaline indicated that increasing hydrophobicity, increased binding. Increased hydrophilicity in the presence of poly-glutamine, decreased binding. One interpretation of the data suggests a hydrophobic component to the binding mechanism. These observations were only considered as preliminary. For these synthetic polypeptides, there could be multiple effects including a non-specific effect analogous to the effect of polylysine.

The effects of the large, acidic polysaccharide heparin and the small, basic protein protamine sulfate were difficult to explain. Heparin had an inhibitory effect on FA1090 OMs and no significant effect on F62. Protamine sulfate had only a slight enhancing effect on the

binding of FA1090 OMs. Both molecules have charged moieties as well as carbohydrate or protein components. Their charges at physiological pH are positive for protamine, and negative for heparin. If it is their charged moieties which effect the binding, this would support the idea that decreased binding is due to competition with a negatively charged gonococcal ligand.

An attempt was made to characterize the molecular weight of the HeLa cell surface receptor. A nitrocellulose blot technique was used to demonstrate the HeLa molecule to which gonococcal OM vesicles bind. HeLa cell lysates were electrophoresed in SDS-PAGE conditions, transferred to nitrocellulose, and the blots reacted with ^{125}I-labelled OM vesicles. The "binding reactions" were detected by autoradiography (Figure 3.17). Preliminary results showed a HeLa protein of 15,000 M_r which was strongly bound by OM vesicles from both PII^+ and PII^- vesicles. Another band at 13,000 was less strongly detected. A third band was bound by only PII^+ vesicles. This band had an M_r of approximately 33,000 and the binding was not as strong as the binding to the 15 Kdalton band. HeLa membrane proteins were extrinsically labelled with ^{125}I to validate the surface location of the putative receptors. The 15 Kdalton band labelled heavily with iodine and the results for the 33 Kdalton band were inconclusive because the band fell in a heavily labelled area of the autoradiograph (data not shown). The blot reactions were compared to SDS-gel patterns HeLa 229 and appeared to correspond to several prominent bands.

DISCUSSION

Much emphasis has been placed on the attachment process as a pathogenic mechanism in Neisseria gonorrhoeae infections (61,63,64,65). The classic studies of Kellogg and others have demonstrated that virulent organisms must express pili to cause disease, and that pili are directly involved in initiating attachment of the organism to the host mucosa (4,5,66,71). The secondary process(es) which binds gonococcal outer membrane to host cell membrane and which in turn leads to "endocytosis" of the gonococcus, is not well characterized. Protein PII has been implicated in this process and its association with phagocytosis of gonococci by human polymorphonuclear leukocytes has been established (48). Recent studies have extended the role of PII in attachment to other human cells. Sugasawara et al (126) could block 50% of the binding of non-piliated PII^+ gonococci to HeLa cells by a monoclonal antibody directed against a PII antigen. Attachment studies using whole N. gonorrhoeae have shown a high correlation with PII. Organisms containing PII usually attach in higher number than organisms lacking PII (61). These studies have been hampered by high variability and poor reproducibility.

The purpose of the present studies was to precisely define the role of PII in gonococcal binding to human cells and tissues. For this purpose a more reproducible, sensitive binding assay was needed. In these studies an assay was developed which increased the reproducibility, sensitivity, and specificity over attachment assays with whole organisms. The assay used OMs dramatically decreased day-to-day variability, and had good reproducibility. However, there still

existed some variability which could be an inherent characteristic of cells in tissue culture and gonococci in laboratory culture.

The gonococcus is a highly adaptable organism, with antigenic diversity and multiple niches in the human host. It has a variable OM composition which responds to changes in the environment. Thus, it should not be so surprising that there could be subtle day-to-day variation of gonococcal characteristics in culture which in turn cause changes in binding patterns. Tramont and Wilson (159) have clearly demonstrated the dramatic variability of gonococcal adhesion to buccal cells from the same donor and the inherent difficulties of such experiments. Efforts are needed to eliminate the variability contributed by both cells and gonococci to improve the quality of data generated in binding studies.

Many features of the attachment assay were standardized. To minimize HeLa cell variability, HeLas were grown in a specific manner and time sequence over the 4 days prior to the assay. They were plated on coverslips at a certain density and used after a specific growth period. Outer membrane preparations used on multiple days decreased day-to-day variability and increased data reproducibility. Aliquots of OM vesicles could be frozen and stored with little effect on binding. However, the experiment of reproducibility of attachment levels from batch to batch of the same phenotype (Table 3.2) clearly showed that there were shifts and variations in the organism which occurred in culture with time. Shifts occurred even with an experienced bacteriologist, well-trained in the colony phenotype morphology, maintaining cultures and exerting selective pressure during daily subculture.

Several investigators have studied the influence of pH on attachment (61,150,160,161) of whole gonococci to human cells. In these studies, when pHs from 4.5 to 7.2 were tested, maximal attachment of whole organisms occurred at acid pHs and minimal at neutral (7.2). Gubish reported a pH maximum of 6.5 for T_4 gonococci (PII^-) binding to HeLa cells and minima at pH 6.0 and pH 8.0 (150). James et al (ref) reported maximal bindings for Op phenotypes (PII^+) at acidic pHs and maxima for Tr phenotypes (PII^-) at 7.2-7.4. In the OM studies, a dramatic maximum binding was found at alkaline pH (8.0) for all OM vesicles and minimal binding at pH 6.5-7.0. All variant vesicles showed increased binding at acid pHs when compared to pH 6.5-7.0. The discrepancy in reported binding maxima (at alkaline pH's) between whole organisms and outer membrane vesicles may be a result of the OM preparation procedure, or strain variability. Trust et al. (161) reported a similar influence of pH on the binding pattern of PII^- whole organism to buccal epithelial cells. These investigators also made the interesting observation that gonococcal binding to red blood cells was not influenced by pH and remained constant from pH 4.5 to pH 7.5. From these studies it becomes evident that different host cells may have different binding mechanisms and thus, variable responses to pH.

The selective binding assay suggested that both PI and PII were involved in the binding of the vesicles to the HeLa cells. The evidence is not definitive because glutaraldehyde-fixed HeLas cells were used in the assay which may have introduced binding artifacts. However, this evidence supported the role of PII in attachment and the studies by Blake and Gotschlich which showed that PI could be transferred from whole gonococci into human RBC membranes (45). It is possible that this

spontaneous PI insertion phenomenon is an attachment mechanism. A role for PI in the binding process would provide a putative mechanism for the binding of PII- OM vesicles and organisms to the surface of HeLa and other human cells.

Antibody studies did not help define the role of PII in binding. The addition of PII-specific immune serum dramatically inhibited attachment, however, it was not possible to prove that IgG antibody was responsible for the effect. Titration experiments of immune IgG suggested a 50% inhibitory effect could be achieved with greater than or equal 300 ug/ml. About one-half of this effect could be attributed to the presence of any non-immune IgG molecule in the reaction mixture. The substraction of the non-immune (control) values from immune values, left 20% specific inhibition of binding. It was not clear if this was a significant value. One would like to see 80% inhibition in the presence of specific antibody as an indicator of a direct role for PII in gonococcal binding. The levels of 20% inhibition were suggestive of some sort of a role for PII, but not a direct, "lock and key model" role. Fab fragment studies also were inconclusive. The fragments had a slight enhancing effect on binding which could not be readily explained. At very high concentrations of fragments there was a very slight inhibition of binding which could be due to steric effects.

King and Swanson have studied antibody inhibition of gonococcal attachment to human PMNs (48). The "leukocyte association factor" is a high molecular weight PII and immune rabbit serum blocked about 75% of the gonococcal association with the leukocytes. In these studies, normal control serum inhibited attachment 16-40%. King and Swanson did not address this finding. It is possible that PII plays a direct role

in N. gonorrhoeae attachment to PMNs, but this conclusion should not be extrapolated to other cells and tissues. It is possible that there is another component in serum which blocks attachment.

An alternative explanation for results of antibody studies was poor specificity of serum, i.e., the antibody present bound to PII determinants which were not involved in binding. The antigenic determinants of the binding site may not be as immunogenic in the rabbit as other determinants on the PII molecule. This phenomenon has been reported for gonococcal pilin. The peptide domain involved in attachment has poor immunogenicity (64).

There is additional evidence which supports this idea. It has been shown in these studies and in work by others (Swanson) that the low M_r form (37°C) of PII does not bind antibody well in Western blot assay conditions. It is the heat modified form (100°C form) that binds large quantities of antibody. This observation suggests that the 37°C form has somehow masked the 100°C form's immunodeterminants. The observation also indicates that there are portions of the molecule which may have poor immunogenicity.

The analysis of the data from the saturation, equilibrium, and reversibility studies suggested that there was no simple, single binding system which could explain attachment of non-piliated organisms to HeLa cells. Scatchard analyses of data suggested multiple, complex binding systems. The characteristics of binding for each outer membrane preparation appeared different. However, as stated, this Scatchard analysis should be interpreted cautiously. The conditions of reversibility were not met by the gonococcal OM attachment system(s). Thus, the quantitative values calculated from an partially reversible

system should only be considered "observed" values, or estimates measured under specific conditions.

The inherent problem in these studies is the multicomponent nature of both the "ligand" - OM vesicles and the "receptor" - the HeLa cell. The assay at best, quantitates the "observed" binding which may be a sum of several binding systems. This status is complicated by the fact that mathematical models have not been generated yet to describe multifactorial binding of organisms to mucosal surfaces. It may be that there are no "simple" formulas and sophisticated computer techniques will be needed to sort out the multiple systems. Improvements in measuring binding parameters will come only as surface ligands are identified, purified, and characterized in assay.

The use of HeLa cells, a transformed cell-line, may not be an appropriate substrate. Conclusions made here should not be extended to in vivo situations. HeLas may have abnormal surface components which do not correspond to normal tissue. However, the cells do exhibit similar binding patterns to normal human cells in tissue culture and thus, may be suitable for preliminary studies. Efforts were made to "standardize" the cell characteristics through consistency of manipulations, and culture techniques. It was assumed that the cell characteristics could be stabilized and were reproducible. (This is the definition of a "cell line" made by the National Cell Culture Collection.) Comparisons of HeLa data generated over several years for gonococcal attachment, indicated that in general, the cell line was stable. However, some studies also clearly indicated that HeLa, as well as other mammalian cells, can and will respond to subtle changes in cell cycle and nutrients by changes in membrane composition (162,163,164,165,166). It

has been reported that HeLa membrane composition may be altered by fatty acids, hormones and sugars. Thus it is possible that some variation in attachment can be attributed to HeLa variblitity. For future studies, a normal human genital tissue in tissue culture is needed.

The preliminary experiments designed to characterize the nature of the HeLa receptor indicated that ionic interactions could be important. Negative charges on the gonococcus may play a role in the binding of both PII^- and PII^+ outer membranes to HeLa cells. It was not possible to detect a consistent pattern of results which distinguished binding mechanisms of PII^- and PII^+ vesicles. The inconsistencies could mean that there was strain variability in the binding mechanism.

The results of the inhibition experiments with DEAE-dextran and polyphosphate suggest that a molecule with a negative charge, found on both PII^+ and PII^- variants, could be involved in the binding mechanism. There are two such known surface molecules: LPS and polyphosphate. Gonococcal polyphosphate has only recently been described and has been found intracellularly and extracellularly (Vogel 1983). The substance appears to be loosely bound to the surface and may serve as a capsule as well as a high energy source. An argument against an attachment role of polyphosphate is that it is also found on (and in) non-pathogenic Neisseria. Noegel has demonstrated that non-pathogenic Neisseria do not attach to human genital tissue (167). LPS is a possible candidate for an attachment ligand. It is negatively charged and complexes with Ca^{++} ions in the outer membrane. Recent work by Warren et al. (168) suggests that LPS intercalates into the outer lipid leaflet of the human RBC membrane and thereby causes morphologic, shape changes. Zlydaszyk has reported that lipopolysaccharide was efficiently endocytosed by

parenchymal cells of the liver *in vivo* and *in vitro*, and by HeLa cells (169). It was found bound tightly to the nuclear membrane of the cells. Recent studies by Kihlstrom have shown that purified LPS from Salmonella LPS-mutants, can alter the binding of this bacterium to HeLa tissue culture cells (170). Thus, this molecule has inherent characteristics which facilitate membrane association. Trust (161) has pursued this possibility and tested the ability of gonococcal core oligosaccharide, covalently linked to methyl BSA, to inhibit gonococcal attachment to buccal epithelial cells. He could demonstrate no significant inhibitory effects. This result does not necessarily mean that LPS is not involved in attachment. Trust coupled methyl-BSA to the LPS moiety through the free carboxyl groups of the core oligosaccharide and thus, may have destroyed the charged nature of the molecule. Further studies are needed to define the role of LPS in attachment.

In summary, the secondary binding mechanism is a multifactorial process and involves irreversible binding. Selective binding studies show that PI and PII remain tightly associated with the HeLa membrane even in the presence of detergents. The presence of PII promotes OM binding to HeLa cells, but antibody inhibition studies suggest it may not be directly involved as a ligand. The secondary mechanism may involve negative charges on the gonococcus, and may have a hydrophobic component. From these studies it was not possible to determine if a PII specific receptor was involved in the secondary attachment mechanism, although a receptor candidate of 15,000 daltons was identified for the mechanism of attachment of PII^+ and PII^- OMs.

Figure 3.1 The primary and secondary attachment systems of N. gonorrhoeae during infection of the host mucosa. Pili are primary gonococcal attachment effectors and the secondary effectors are unknown.

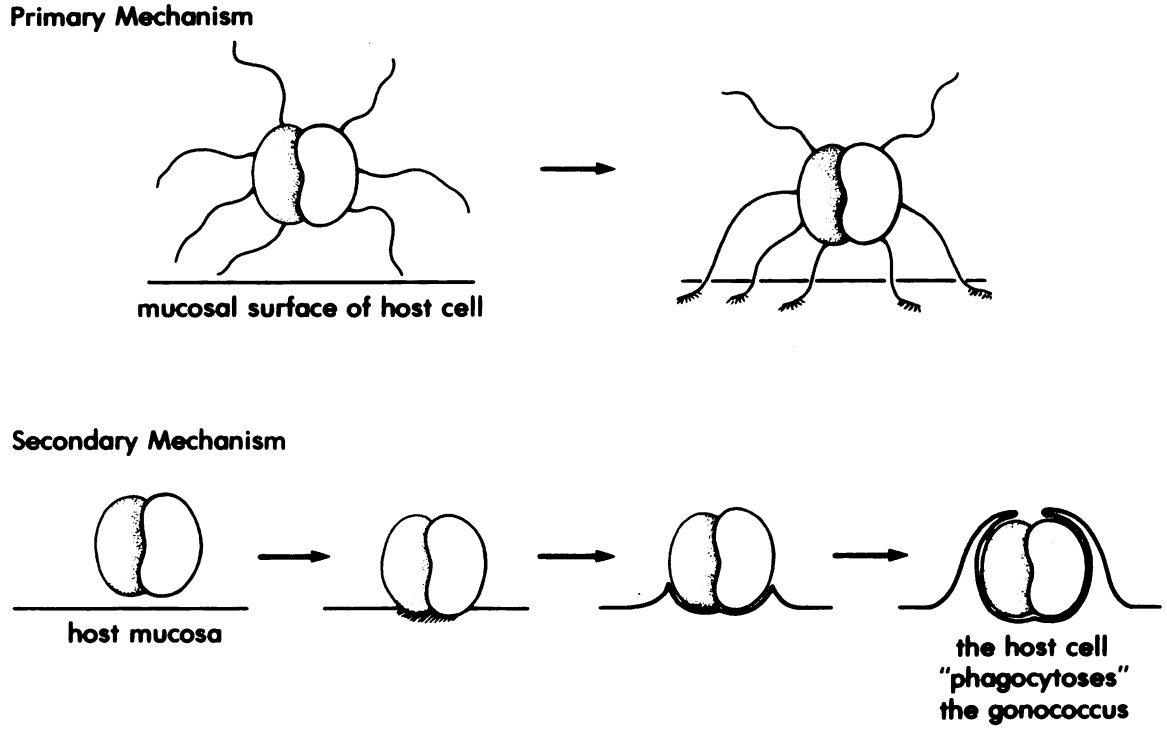


Figure 3.2 Transmission electron micrograph of phosphotungsic acid stained outer membrane vesicles from N. gonorrhoeae. When sonicated, the outer membranes do not vary in size greatly. Occasionally aggregates do occurr (↖). magnification 46,000X.

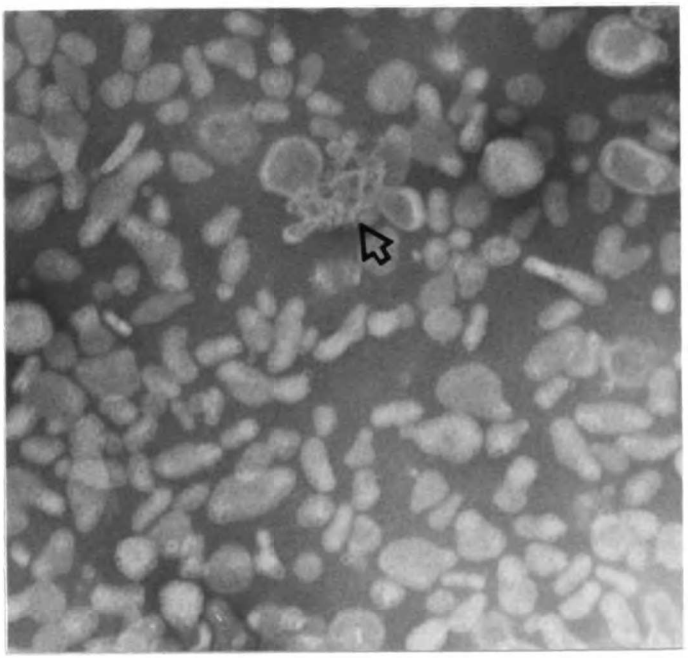


Table 3.1.

	cpm bound/coverslip			
Hela density[a]	1 ug/ml[b]	5 ug/ml	9 ug/ml	Comments
5×10^4	5.02×10^3[c]	3.65×10^4	5.16×10^4	sparse
1×10^5	4.85×10^3	2.10×10^4	4.10×10^4	confluent
5×10^5	4.79×10^3	1.69×10^4	4.00×10^4	confluent
1×10^6	4.89×10^3	2.15×10^4	4.45×10^4	very dense multiple layers

[a] concentration of Helas/ml used to seed coverslips.

[b] concentration of free outer membrane vesicles used as inoculum.

[c] average of two experiments

Table 3.1 The effect of varying cell density on the attachment of ^{125}I-labeled outer membrane vesicles to HeLa 229 cells. Coverslips were seeded with with varying concentrations of HeLa cells and attachment assays performed. There was no significant effect on binding except at low seeding concentrations.

Figure 3.3A The effect of varying the concentration of free outer membrane protein over a 4 $\log_{10}$ range on the levels of attachment gonococcal strain F62 to HeLa 229 cells. The experiment was done to determine the range of concentrations needed to demonstrate saturation of binding sites. Outer membranes containing PII^+ (•) and PII^-(o) were tested. Binding for both types of outer membranes appeared to saturate at about 5-7 ug/ml. This graph represents the data of the upper range of concentrations tested. Points represent the mean +/- standard deviation of at least six separate experiments.

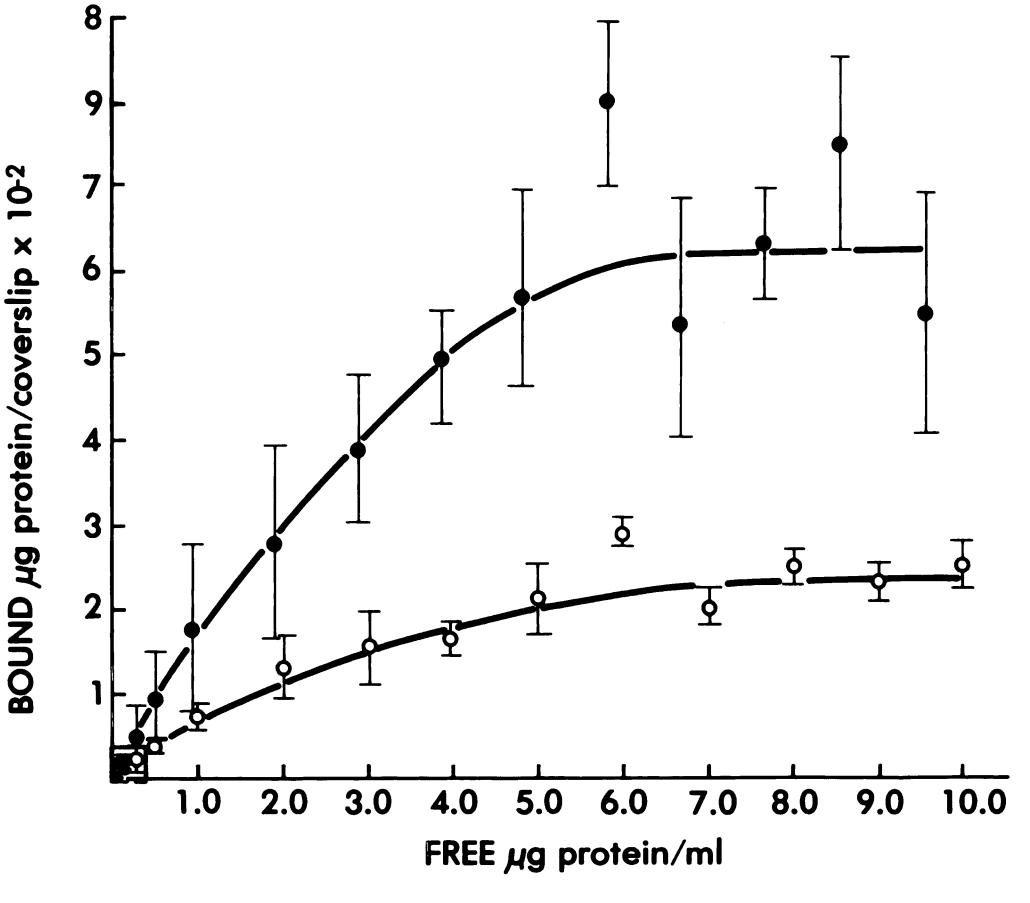


Figure 3.3B An inset of the data in Figure 3.3A for the low concentration range. Points represent the mean +/- standard deviation of at least three separate experiments.

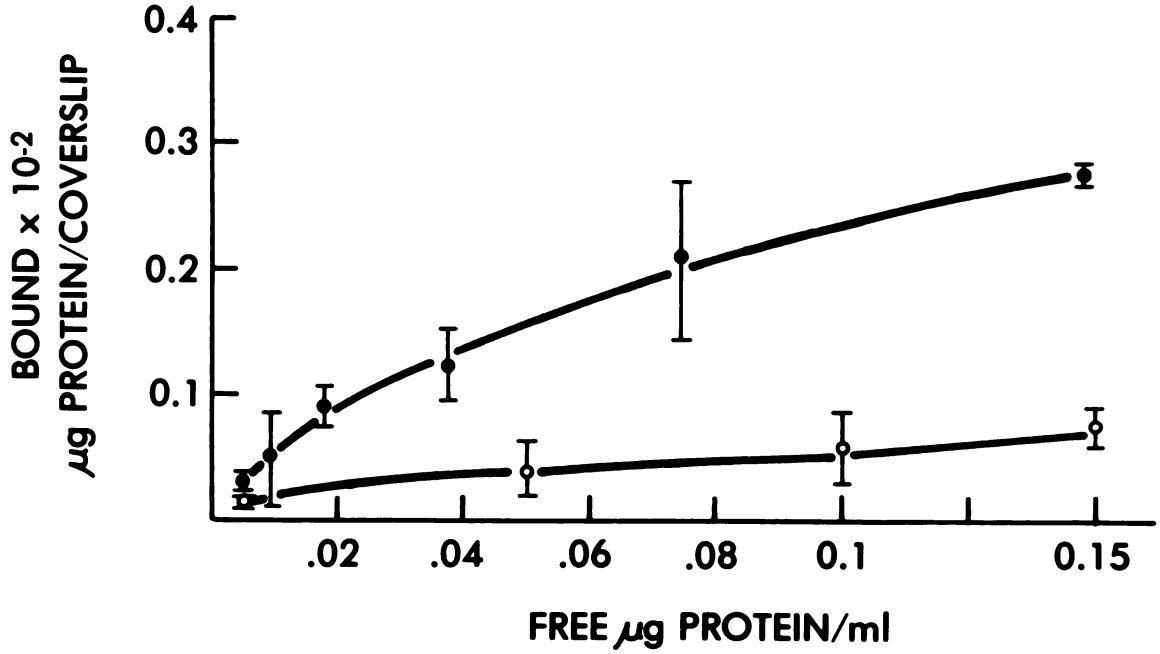


Figure 3.4 The effect of varying the time of incubation on the binding of gonococcal outer membranes to HeLa 229 cells. The binding of PII^+ (●) and PII^- (o) outer membrane appeared to reach equilibrium at 60 to 90 minutes. The points represent the mean +/- standard deviation of at least 3 separate experiments.

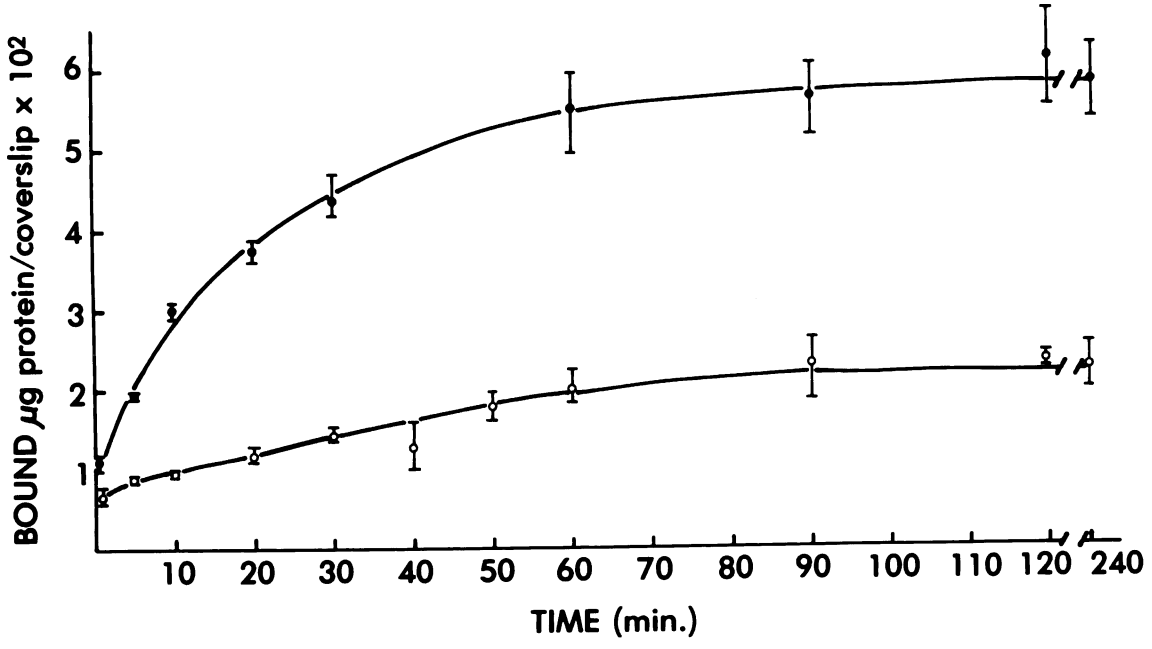


Figure 3.5 The effect of different incubation times on the reversability of binding of gonococcal outer membrane vesicles from strain F62 containing PIIa to HeLa 229 cells. At different incubation times (↓) the free outer membranes were removed and fresh medium added.

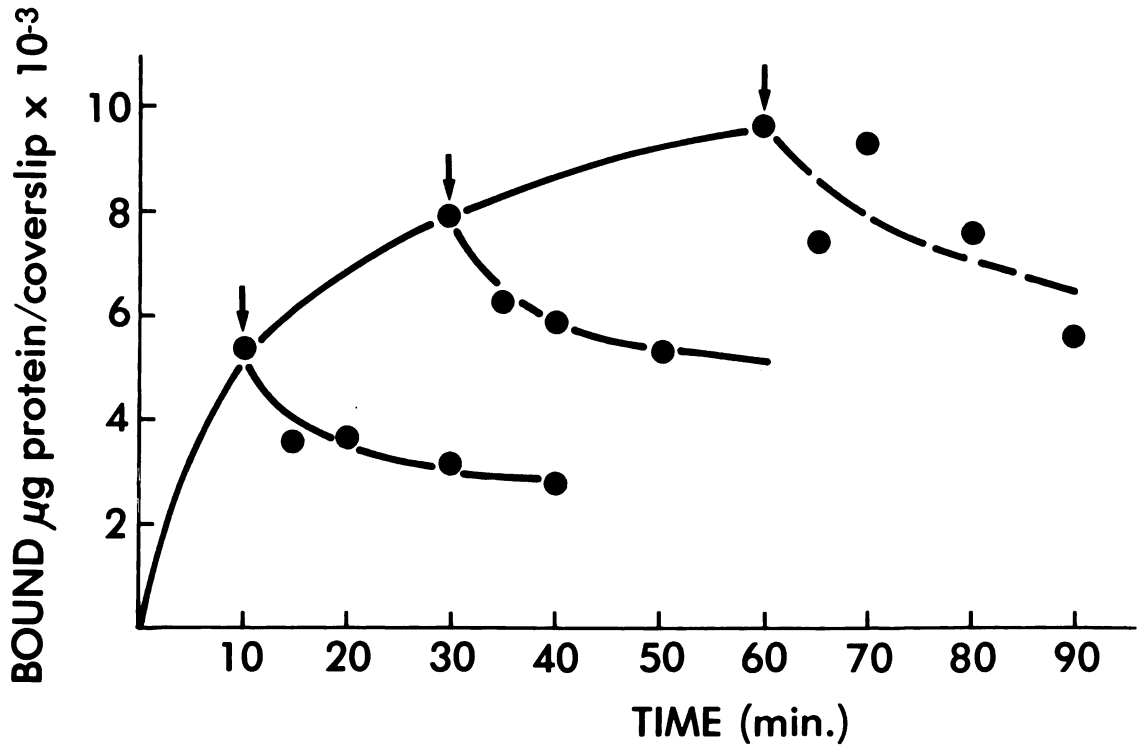


Figure 3.6 The effect of different washing conditions on the detection of reversible binding of gonococcal outer membranes to HeLa 229 cells. Panel A: the effect of one wash step. Panel B: the effect of two wash steps. (see text for details.) The points connected by lines represent the range of binding.

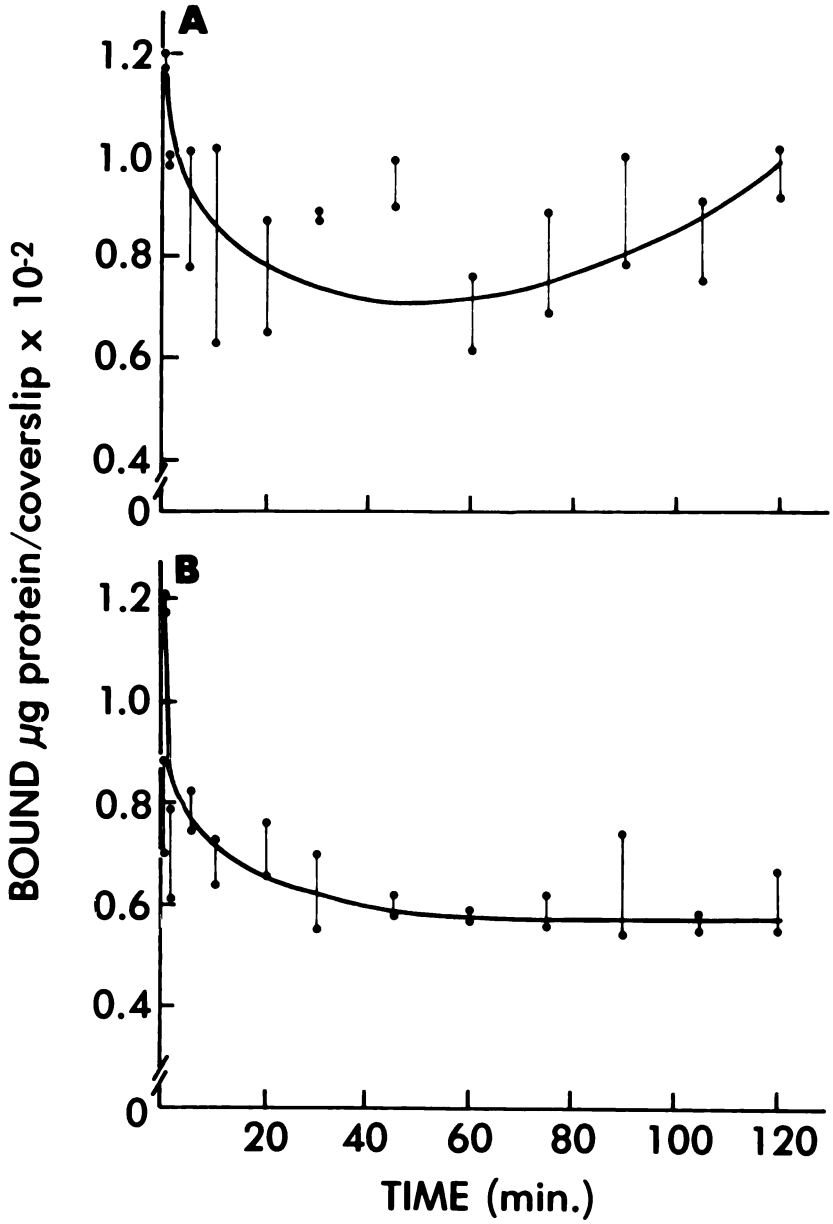


Table 3.2.

Batch No.	date prepared	date of assay	A bound (ug)*	B bound (ug)
10	2.16.82	4.19.82	0.03	0.053
		7.22.82	0.05	0.065
39	4.6.83	5.4.82	0.09	0.12
		5.6.82	0.08	0.13
		9.15.83	0.11	0.12

* ug outer membrane protein bound to coverslip

Table 3.2 The reproducibility of binding to HeLa 229 cells of different preparations of outer membrane vesicles from the same organism. HeLa cells were inoculated with either 5 ug/ml outer membrane protein (A) or with 10 ug/ml (B). Within a batch of outer membranes, there was good reproducibility of binding. Between batches of outer membranes there could be 50% variation in binding.

Figure 3.7 The influence of pH conditions on the binding of gonococcal outer membrane vesicles to HeLa 229 cells. Outer membranes were prepared from various colony phenotypes of strain F62 and FA1090 and contained different protein IIs. The PIIs are indicated in the key and Tr represents PII$^-$ OMs.

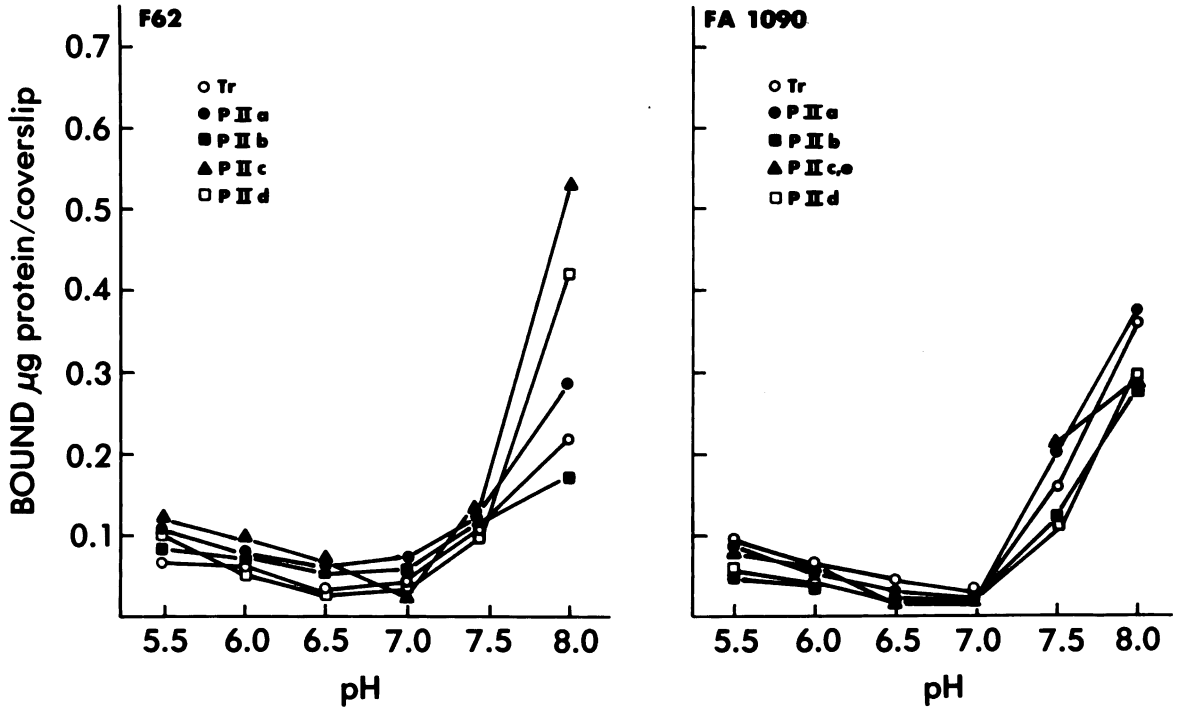


Figure 3.8 A comparison of binding curves of N. gonorrhoeae outer membrane vesicles from strain F62 and FA1090 containing PII variants. The levels of binding were tested for saturation at pH 6.0 and pH 7.3. The PII variants are indicated in the key and Tr represents PII⁻ outer membranes.

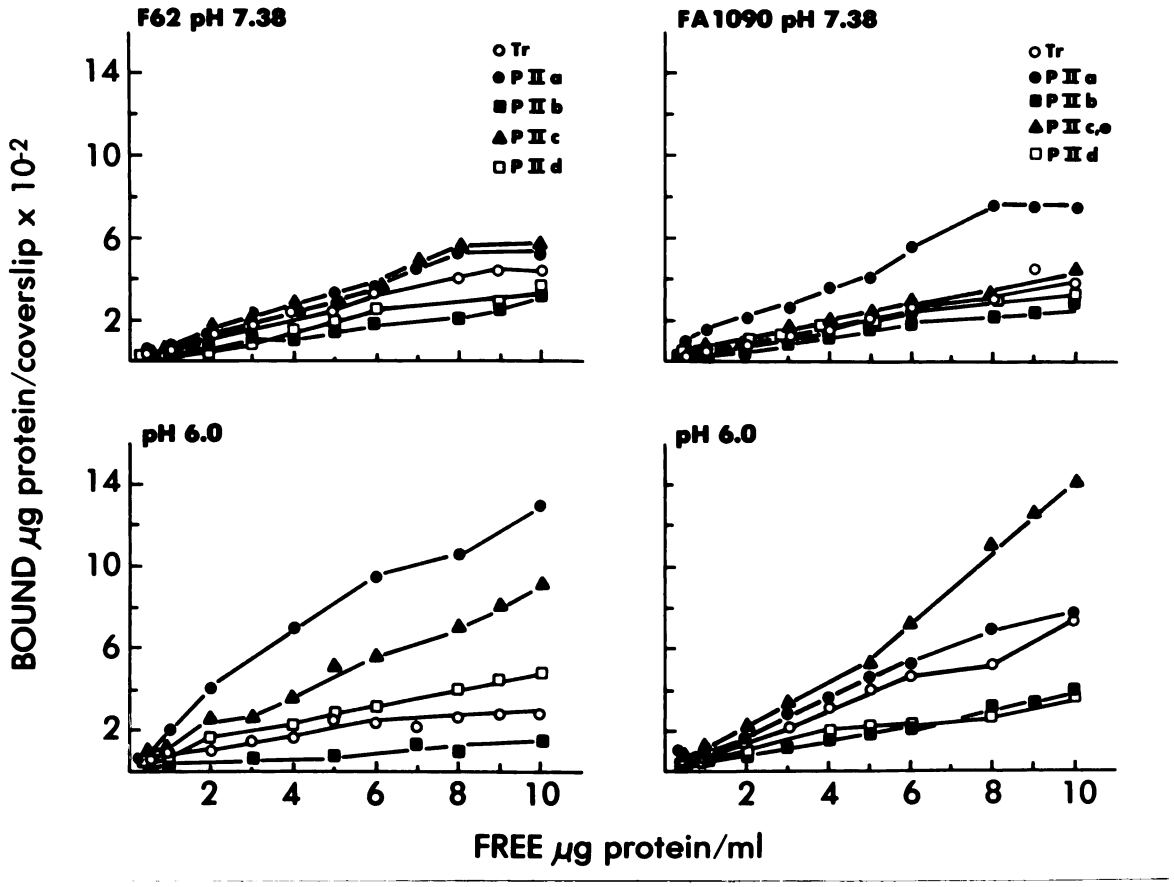


Figure 3.9 A comparison of equilibrium conditions at two pHs for N. gonorrhoeae outer membrane vesicles from strain F62 and FA1090 containing PII variants. Equilibrium of binding was not achieved at pH 7.38 by most outer membranes. The PII variants are indicated in the key and Tr represents PII$^-$ outer membranes.

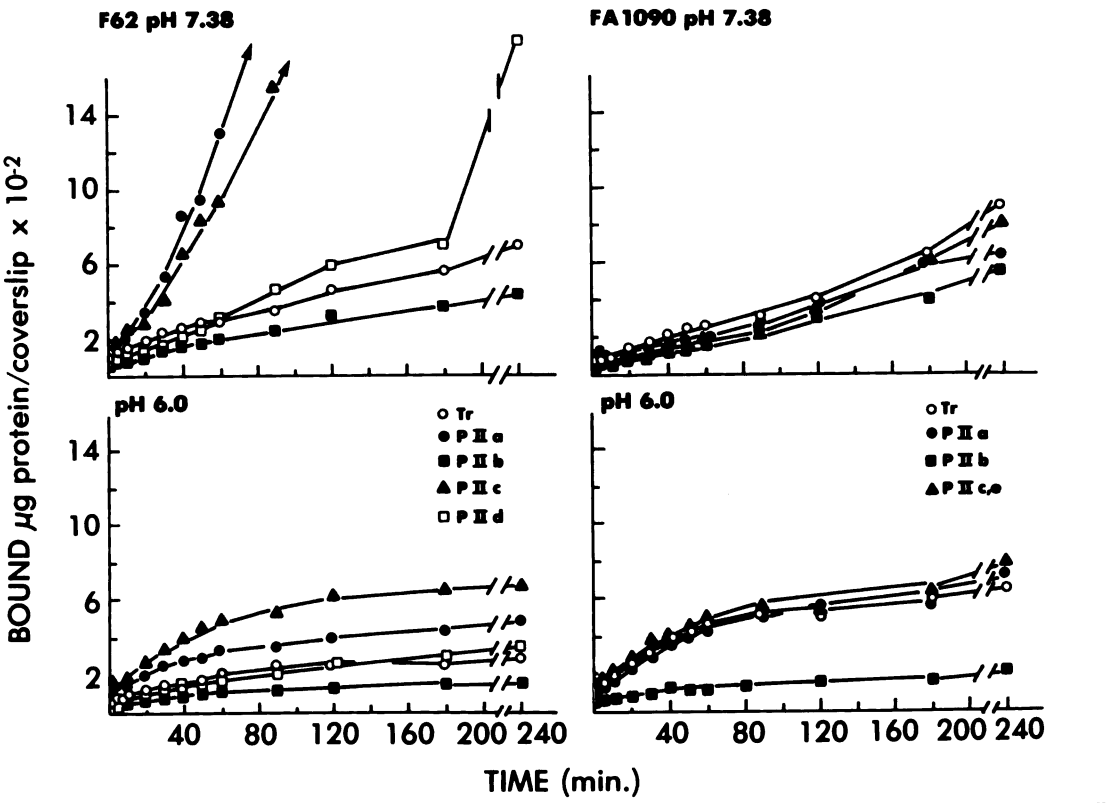


Figure 3.10A A comparison of the reversibility of the binding of gonococcal outer membrane vesicles to HeLa 229 cells. Outer membrane vesicles were prepared from different colony phenotypes from strain F62 and FA1090 containing PII variants . Reversibility of binding was tested at pH 6.0 and 7.38. The PII variants are indicated in the key and Tr represents PII$^-$ outer membranes.

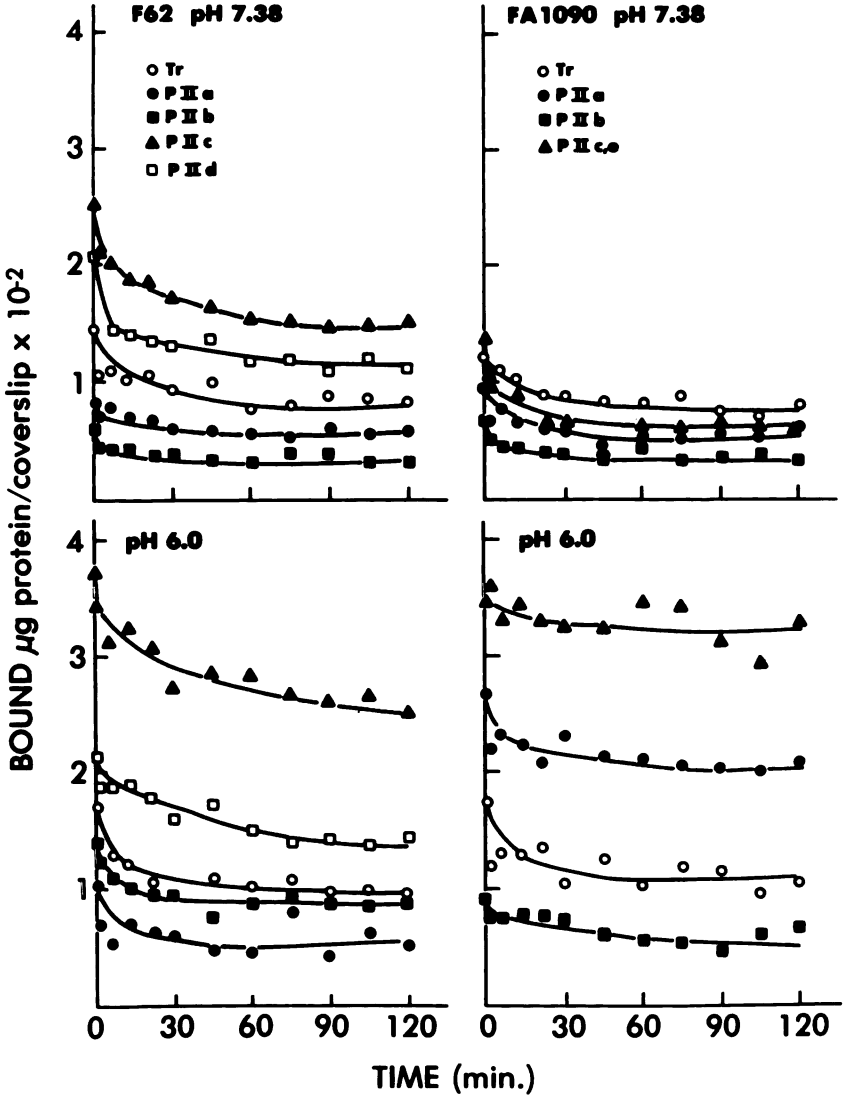


Figure 3.10B and C Scatchard plots of binding data from gonococcal attachment to HeLa 229 cells. The results of the saturation assays for OM variants from strain F62 and FA1090 in Figure 3.8 were transformed and graphed. The OMs were PII$^-$(△) or contained PIIa (●) or PIIb (○). Straight lines represent single binding systems; curved lines represent multiple binding systems. Notice the effect of pH on the number of binding sites (intercept of x-axis). Figure 3.10 C is on the next page.

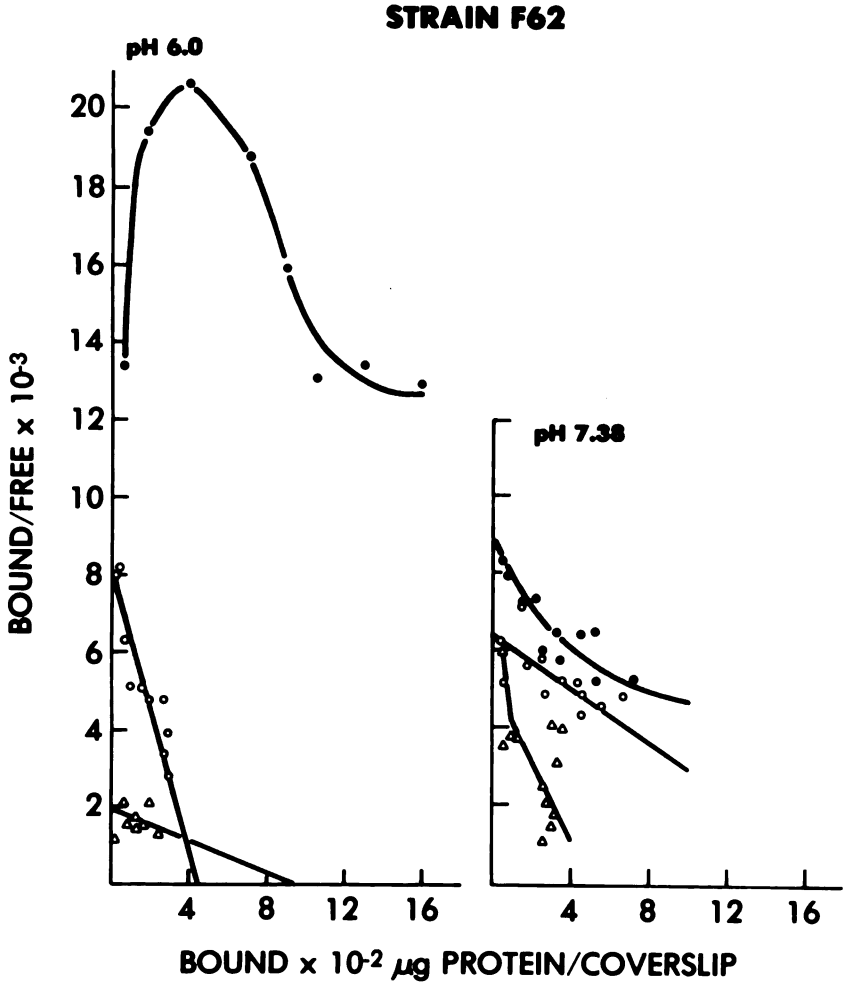


Figure 3.10 C

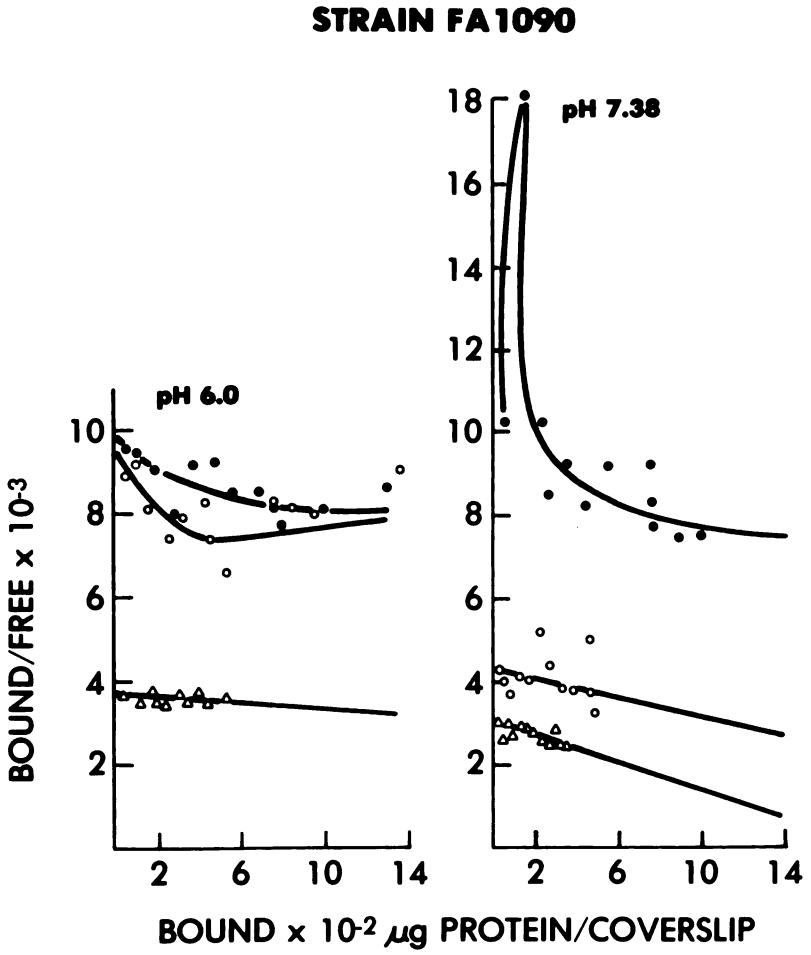

STRAIN FA1090
pH 6.0
pH 7.38
BOUND/FREE x 10-3
BOUND x 10-2 µg PROTEIN/COVERSLIP
2
4
6
8
10
12
14
16
18

Table 3.3.

Strain	PII composition	percent of binding reversed pH 7.38	pH 6.0
F62	PII$^-$	44	41
	PIIa$^+$	18	50
	PIIb$^+$	50	36
	PIIc$^+$	40	32
	PIId$^+$	47	33
FA1090	PII$^-$	38	39
	PIIa$^+$	47	25
	PIIb$^+$	44	44
	PIIc,e$^+$	57	7

Table 3.3 The reversibility of binding of N. gonorrhoeae outer membrane vesicles to HeLa 229 cells at two pHs. The percent of reversible binding was determined after 60 minutes of incubation with fresh buffer and calulated from total ug protein bound minus ug protein remaining on the coverslip after washing.

Figure 3.11 The attachment of PIIa$^+$ outer membranes (●) to HeLa 229 cells and attachment in the presence of unlabeled, competing (100X) PII$^-$ outer membranes (○). Attachment was tested at two pHs.
Panel A: pH 6.0; panel B: pH 7.38.

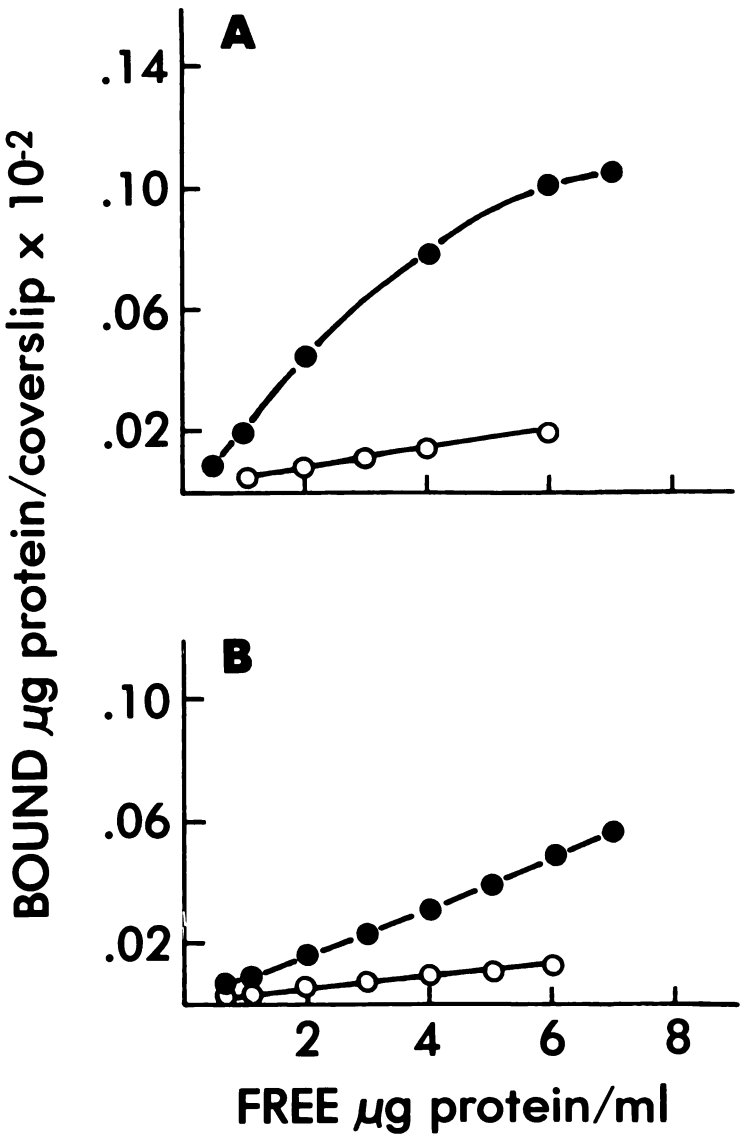


Figure 3.12 The attachment of PII$^+$ (■) and PII$^-$ (o) outer membranes to HeLa 229 cells and attachment in the presence of competing, unlabeled homologous membranes (----) at two pHs. Panel A: PII$^+$. Panel B: PII$^-$.

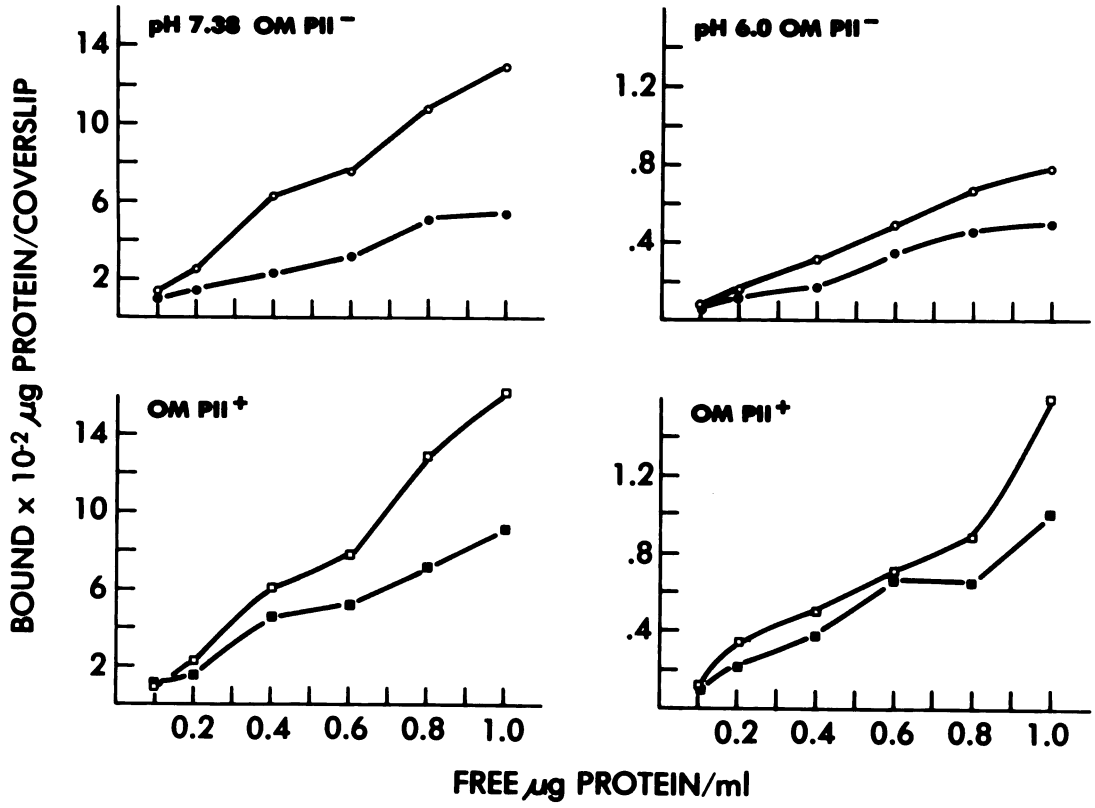


Figure 3.13 Autoradiograms of the study of selective binding of proteins extraced from different colony phenotypes of N. gonorrhoeae strain F62 ^{125}I-labeled gonococci were solubilized in DOC-SDS buffer and mixed with glutaraldehyde-fixed HeLa 229 cells. The gonococcal proteins remaining bound to the surface of the HeLa cells before and after extensive washing were determined. PI is indicated with a (►) and PIIs are indicated with (>).

Panel A: ^{125}I-labeled whole gonococci from PII$^-$ and PII$^+$ colony phenotypes. Lane 1, PII$^-$; lane 2, PIIa; lane 3, PIIb; lane 4, PIIc.

Panel B: DOC-SDS solubilized gonococcal proteins extracted from organisms in panel A: the protein solutions were used as inocula in the binding assay. The content of the lanes is the same as in panel A.

Panel C: Gonococcal proteins adherent to HeLa cells before washing. Lane 1, PIIa; lane 2, PIIb; lane 3, PIIc.

Panel D: Gonococcal proteins adherent to HeLa cells after extensive washing. Lane 1, PIIa; lane 2, PII$^-$; lane 3, PIIb; lane 4,PIIc.

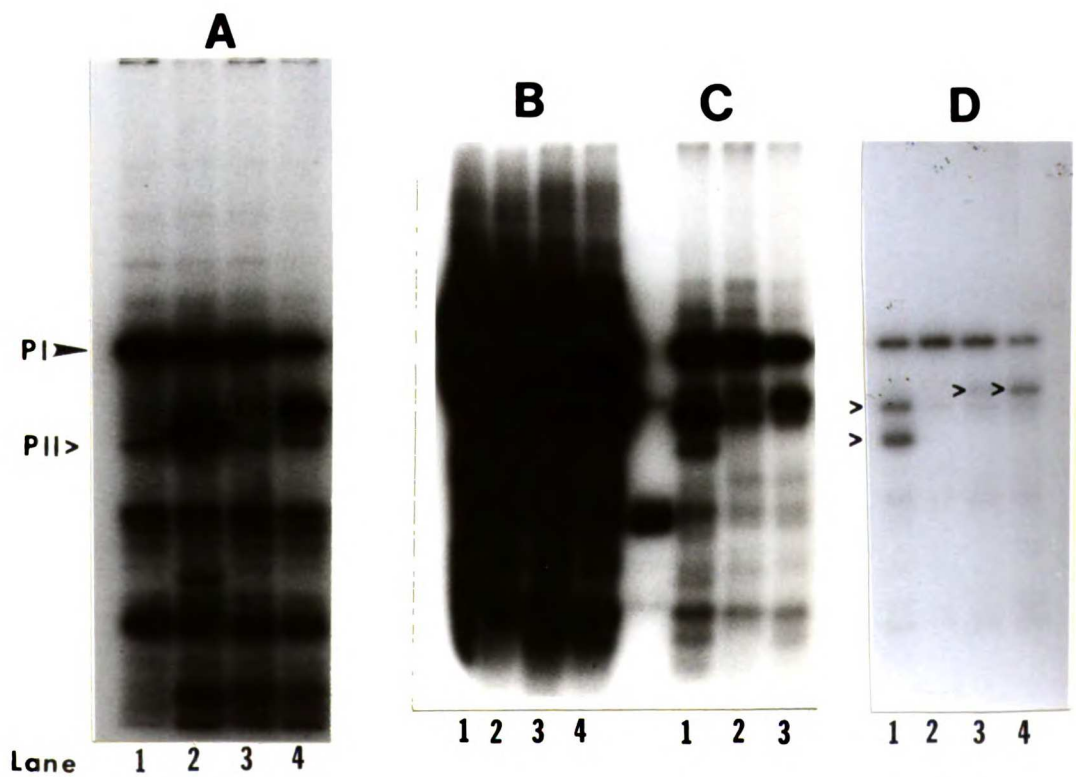


Figure 3.14 Western blot technique was used to characterize the specificity of the rabbit antisera R11 (panel A) and R12 (panel B). Both sera were prepared by immunizing rabbits with purified protein PIIa from N. gonorrhoeae strain F62. Dilutions of 1:100 of each serum were tested against nonpiliated, PII^- (lane 1) and $PIIa^+$ whole organisms (lane 2), outer membrane vesicles of organisms containing PII variants including PIIa (lane 3), PIIb (lane 4), PIIc (lane 5), PIIc (lane 6) PIId (lane 7), and purified PIIa (lane 8). Notice the difference in the immune response of the two rabbits to the same antigen.

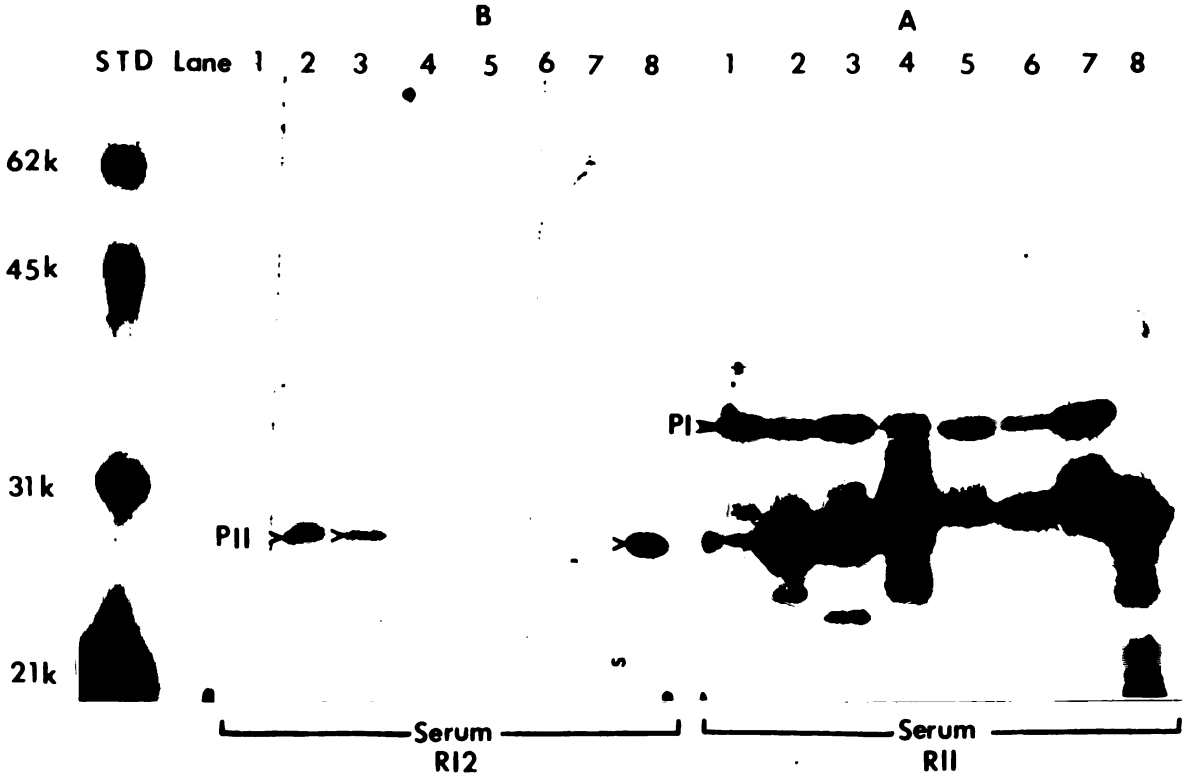


Figure 3.15 The effect of normal and immune rabbit serum (PIIa specific) on the attachment of N. gonorrhoeae outer membranes from PII$^+$ organisms to HeLa 229 cells at pH 7.38. Panel A: attachment in the absence of serum (○) and attachment in the presense of normal preimmune serum (●) and immune serum (△). Panel B: attachment in the absence of serum (○) , and in the presence of immune serum (△), normal serum (○), immune serum preabsorbed with HeLa cells (□), and normal serum preabsorbed with HeLa cells (■) and PII$^-$ organisms(▲).Panel C: The attachment in the absence of IgG (○) and in the presence of immune IgG (△) and immune rabbit IgG preabsorbed with HeLa 229 cells (▲). The concentration of serum used was a 1:20 dilution of whole serum. The concentration of IgG was 50 ug/ml.

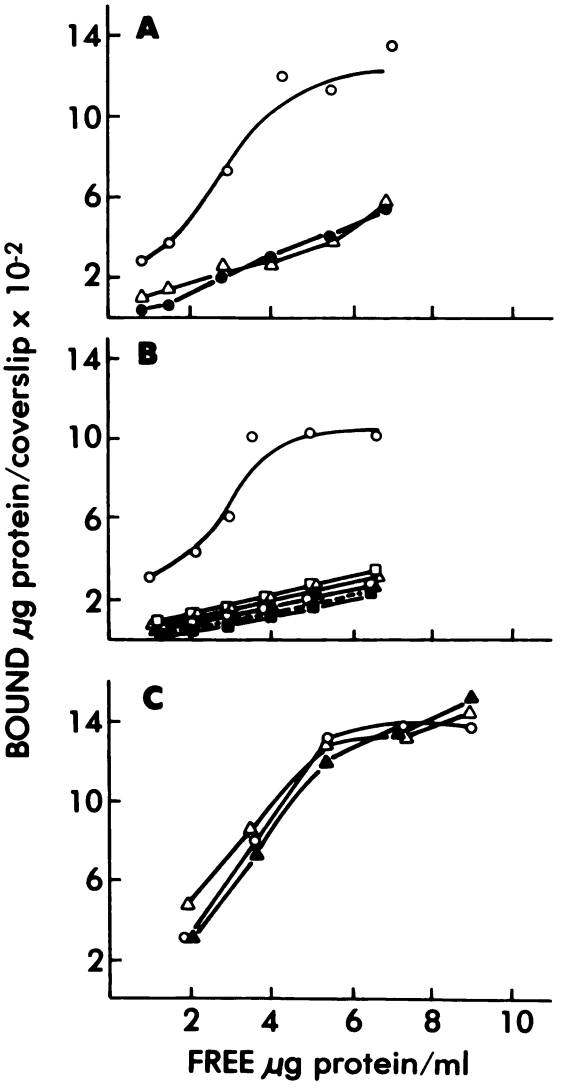
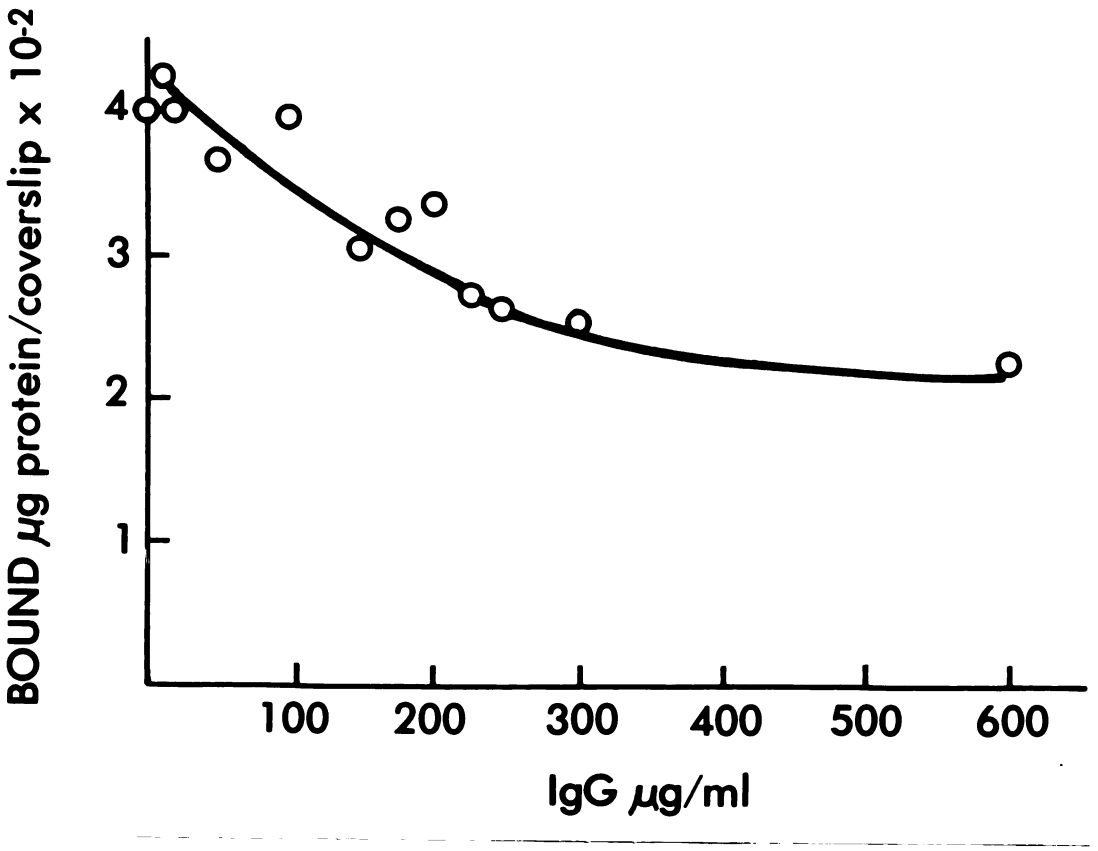


Figure 3.16 The inhibitory effect of increasing concentrations of immune rabbit IgG (PIIa specific) on the attachment of PIIa containing gonococcal outer membranes to HeLa 229 cells.

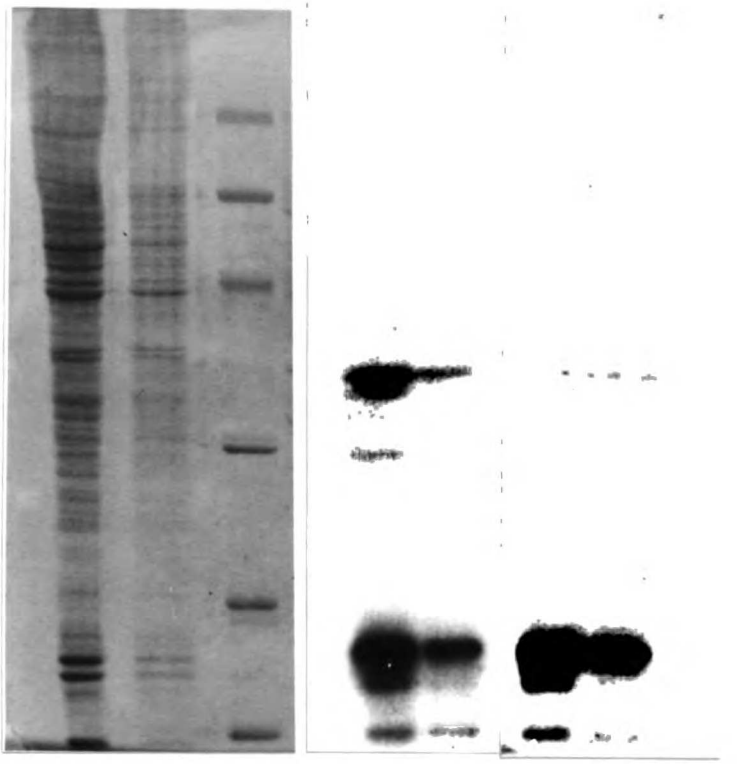


	F62		FA1090	
	PII$^-$	PIIa$^+$	PII$^-$	PIIa$^+$
Control[a]	1.00	1.00	1.00	1.00
Variance	±0.27	±0.12	±0.16	±0.05
1. 10 uM glucose [b]	1.20	0.90	0.75	0.61
2. 1 mg/ml polyvaline	1.60	1.56	1.29	1.17
3. 1 mg/ml polyglutamine	0.90	0.85	0.70	0.65
4. 1 mg/ml polylysine	1.16	0.78	1.47	1.14
5. 500 ug/ml tripoly PO_4	0.98	1.02	0.64	0.70
6. 1 U/ml neuraminidase	1.37	1.16	0.75	0.78
7. 1 mg/ml mixed glycosidases	1.07	0.97	0.87	1.01
8. 5 ug/ml trypsin	0.82	0.58	0.83	0.40
9. 1 mg/ml protamine SO_4	1.10	0.99	1.30	1.07
10. 5 ug/ml protease	0.45	0.28	0.80	0.47
11. 1 mg/ml heparin	1.12	1.13	0.54	0.51
12. 1 mg/ml DEAE-dextran	1.67	1.34	1.31	1.07
13. 1 mg/ml dextran	1.18	0.94	0.89	1.05
14. 0.1 mM $FeCl_3$	1.28	1.00	1.24	1.44
15. 0.1 mM $MgCl_2$	1.26	0.96	0.87	0.96
16. 0.1 mM $CaCl_2$	1.73	1.53	1.13	1.25

a base level attachment, normalized to 1.0 ± variance.
b % of control level/100

Table 3.4 The effect of various compounds on the attachment of outer membrane vesicles from two strains of N. gonorrhoeae to HeLa 229 cells. Outer membranes from PII$^+$ and PII$^-$ clones were prepared from strain F62 and strain FA1090. The results were the averages of two separate experiments.

Figure 3.17 A nitrocellulose blot assay for the HeLa 229 receptor of nonpiliated gonococcal outer membranes from PII^+ (panel A) and PII^- (panel B) organisms. Blots of HeLa cell lysates were reacted with ^{125}I-labeled outer membranes and the proteins binding outer membranes detected by autoradiography. Assay blots were compared to SDS-PAGE gel patterns of HeLa 229 cells. The prominent bands detected in the assay may be surface located in the HeLa cell. Lane 1: HeLa cells prepared by the "shake-off" technique from monolayer cultures. The preparation is rich in mitotic cells. Lane 2: HeLa cells prepared by harvesting monolayer cultures with sodium EDTA. Molecular weight standards included 92, 66, 45, 31, 21, 14.4 Kdaltons.



Chapter 4

Antigenic analysis of PII

Abstract

The antigenic heterogeneity and homology of variants of N. gonorrhoeae outer membrane protein, PII, were investigated by Western blot reactions of peptide fragments generated with staphylococcal V8 protease and PII specific rabbit antiserum. Reactions for PII variants a,b, and c from gonococcal strain F62 and PIIb from strain FA1090 with F62-PIIa specific antiserum detected common antigens in all four PII variants. Comparisons of peptide maps of ^{125}I-labelled PIIs with Western blot reactions indicated that not all peptide fagments were detected by the serum. These studies indicated that there were antigenically unique as well as conserved regions in each PII. Coomassie brilliant blue and silver staining of SDS polyacrylamide gels of whole organisms or OM vesicles was used to characterize proteins which varied in the PII$^+$ and PII$^-$ colony phenotypes. The Western blot technique was also used to charterize antigenic differences. A 58 Kdalton surface protein was found to vary in abundance with PII expression, and 32 and 65 Kdalton proteins were associated with the PII$^-$ phenotype. The conclusions from these studies were that several proteins vary on the surface of the gonococcus changing its composition and antigenic characteristics. Also, the PII molecule contains constant as well as antigenically unique regions. These results suggest that the surface of

the gonococcus is a dynamic mosaic which confers flexibility and adaptability and which in turn, may alter the pathogenic process.

INTRODUCTION

Some gonococcal outer membrane (OM) constituents, including pili, protein I, and LPS are known to have marked or moderate antigenic diversity between strains (64,70,79,171,172,173). Gonococcal PII also appears to be antigenically heterogeneous. Peptide mapping studies of PIIs from different strains have shown that there is a high degree of structural homology (60,125). However, each PII also contains unique peptides. Diaz and Heckles (125) observed that PII specific antisera, for PII variants in strain P9, showed very little cross-reactivity in ELISA reactions. When heterologous whole organisms or OMs were added to the ELISA reaction, there was very little inhibition. The explanation offered by these investigators for this interesting observation was that the membrane surface regions of each PII molecule were unique because of the antigenic heterogeneity. It is, however, not known to what extent the PIIs are antigenically similar or if there are immunodominant or immunorecessive portions of the PII molecule as there are with pili (64).

In this Chapter, the antigenic relationships of PIIs from strain F62 and FA1090 were investigated. A Western blot peptide mapping technique was developed to compare the antigenic similarity of peptides from different PIIs. Several peptides, in PIIs purified from different strains, were found to contain common as well as unique antigens.

The antigenic variation associated with the PII^+ and PII^- phenotypes in N. gonorrhoeae strain F62 also was examined and the

results are reported in this Chapter. The studies were limited by the antisera available, which were primarily reagents developed for other studies. However, a few interesting observations were made and several antigens were found to vary with the PII^+ to PII^- shift. These observations may have future impacts on studies of antigenic diversity and function of gonococcal outer membrane proteins.

MATERIALS and METHODS

N. gonorrhoeae strains

N. gonorrhoeae strains F62 and FA1090 were grown and maintained as described in Chapter 2. The colony phenotypes used included piliated P++Op and P++Tr, and nonpiliated P-Op and P-Tr from strain F62; a nonpiliated P-Tr phenotype producing PIIb was used from strain FA1090. The PII variants in strain F62 included PIIa, b, or c. Outer membranes were prepared only from nonpiliated organisms.

Preparation of outer membranes

Outer membranes of N. gonorrhoeae were isolated according to Heckles (46) with slight modifications. Ten to 35 gm (wet weight) of N. gonorrhoeae, grown on PPT agar for 20 hours, were scraped into 100 ml of 0.2 M LiAc/HAc buffer, pH 6.0, with 5 mm EDTA. The organisms were shaken for 3-4 hours at 45°C in a New Brunswick shaker incubator. The suspension was then placed in a Lourdes tissue homogenizer chamber and blended for 10 minutes. The homogenate was centrifuged at 12,000 x g for 10-15 minutes at 4°C and the supernatant carefully removed. The fluid was ultracentrifuged at 100,000 x g for 3 hours in a Beckman

SW25.1 rotor in a L2-65B ultracentrifuge. The yellow, glassy pellet was resuspended in 0.2 M NaAc/HAc buffer, pH 6.0, and stored at -70°C.

Rabbit immunization and antisera production

Young adult, female, New Zealand white rabbits (2.5 Kgm) were used for production of antisera. Rabbits were housed in the animal care facility of UCSF and allowed to acclimatize 2 weeks before immunization. All animals were bled for base titers from the peripheral ear vein by use of a vacuum pump connected to a rabbit ear jar (Bellco). Rabbits were immunized subcutaneously in 2 locations with an equal mixture of immunogen (200-400 ug protein) and complete Freund's adjuvant (CFA). Four weeks later animals were bled and reimmunized with immunogen in Incomplete Freund's adjuvant (ICFA). Titers of reactive antibodies were determined by Western blot reaction. Three to 4 weeks later, the rabbits were bled, titers and specificity determined, and the animals reimmunized with antigen in ICFA if necessary. The total amount of antigen injected into each rabbit was 300-800 ug protein. Antisera for purified PIIa (F62) and OM vesicles with and without PIIs were prepared with this procedure.

Antisera were prepared against whole organisms of strain F62 of colony phenotypes P++Op and P++Tr. The PII content of these phenotypes was PIIa and PII^- respectively. Antisera against whole N. gonorrhoeae strain F62 phenotypes were prepared by Dr. Jim Douglas (current address: Dept. Microbiology, University of Hawaii, Honolulu) and Dr. Neylan Vedros (Dept. Medical Microbiology, University of California, Berkeley, CA).

Adsorption of rabbit antisera

Immune rabbit sera were absorbed with whole N. gonorrhoeae by

mixing 100 ul of serum with 2 x 10^9 bacteria of appropriate colony phenotype and incubating at 4°C for 1 hour. The sera was exhaustively adsorbed by repeating the treatment 3 to 4 times with fresh organisms.

Western blot assay

Rabbit antisera were characterized by Western blot assay; antigen-antibody reactions were detected with ^{125}I-labeled protein A. The method of Burnett (152) was modified for use as follows:

N. gonorrhoeae OM vesicles from the various PII phenotypes were prepared for SDS-PAGE. A sample containing 10-20 ug protein was loaded on a 12.5% acrylamide separating gel with a 5% stacking gel and electrophoresed at 40 mamps in the Laemmli buffer system. The gel proteins were immediately transferred onto 0.45 u pore nitrocellulose paper (Sartorius) by electrophoresis overnight in a BioRad Transblot apparatus using 1.4% (w/v) glycine, 25 mM Tris, pH 8.3, and 20% (v/v) methanol. The electrophoresis was carried out at 30 volts and 0.1 amps for 18 hours. The blot was air dried briefly and soaked in 5% (w/v) BSA (Sigma) in PBS (BSA-PBS) for 30 minutes at 37°C with shaking. The blot was removed and reacted with antiserum diluted (usually 1:100) in BSA-PBS for 90 minutes at RT with shaking. The blot was then washed with PBS for 10 minutes followed by 2 washes of PBS containing 0.05%(v/v) NP-40 detergent for 20 minutes and finally rinsed in PBS for 10 minutes. The blot was reacted with ^{125}I-protein A diluted to 1 x 10^5 cpm/ml in BSA-PBS. The blot was finally washed extensively as above and air dried for autoradiography. The blot was wrapped in plastic wrap and exposed to Kodak X-OMAT AR X-ray film with a Cronex intensifying screen at -70°C.

Iodination of staphylococcal protein A

Purified, soluble Staphylococcal protein A (Sigma, St. Louis, MO) was iodinated by the chloramine T reaction. 10 ug of protein A (in 10 ul) was added to 20 ul of PBS and 15 ul of 1% (W/V) chloramine T (Eastman Kodak). 0.5 mCi (100 m Ci/ml) of ^{125}I-Iodine (Amersham) was added and the reaction allowed to proceed for 2 minutes at room temperature. The reaction was stopped with 15 ul of 25% (w/v) Na-metabisulfite and the mixture immediately chromatographed on Sephadex G-25 column (15 x 1 cm) equilibrated in PBS. The void volume was collected and radioactivity determined. ^{125}I-protein A was prepared every 2 months and stored at -70^{o}C.

Iodination of molecular weight standards for SDS-PAGE

BioRad SDS-PAGE low molecular weight standards were iodinated by the chloramine T reaction. Two ul of molecular weight standards were mixed with 2 ul of H_2O and 20 ul of PBS. Fifteen ul of freshly prepared 1% (w/v) chloramine T (Eastman Kodak) and 0.5 mCi of ^{125}I (100 mCi/ml) (Amersham Corp.) were added to the mixture and allowed to react for 2 minutes. The reaction was stopped by the addition of 15 ul of 25% (w/v) Na-metabisulfite (Sigma). This mixture was immediately chromatographed on Sephadex G-25 equilibrated with PBS. The void volume was collected and stored at -70^{o}C until used. A portion of the labeled standards was diluted with SDS-PAGE prep solution to yield 1 x 10^5 cpm per 10 ul sample.

Western blot peptide mapping

Selected PIIs were prepared as previously described (see Chapter 2: peptide mapping) except unlabeled OM preparation were used as a source. The Cleveland technique for peptide cleavage was performed with 100 nanograms of V8 protease (133). Immediately after electrophoresis, gels

were transferred to a BioRad Transblot apparatus and the peptides transferred to nitrocellulose paper as described under Western blot assay. The nitrocellulose blots were reacted with rabbit immune antisera and antibody reactions were detected with ^{125}I-labeled staphylococcal protein A (IPA) and autoradiography. Western blots of gonococcal proteins reacted with IPA alone gave no detectable reactions.

Purification of PIIa by Zwittergent 3-14 extraction.

PIIa was extracted from N. gonorrhoeae strain F62 by a modified procedure of Blake (personal communication, Rockefeller University, NY) using differential solubilization of whole gonococci by the zwitterionic detergent, Zwittergent 3-14 (Calbiochem, La Jolla, CA). This PII was used to prepare PII specific antiserum.

Ten grams (wet weight) of whole gonococci, or organisms pelleted from the LiAc extraction of OMs, were suspended in 100 ml of 100 mM Na acetate buffer, pH 4.0, with 0.5 M NaCl and 5% (w/v) Zwittergent 3-14. (Zwit. 3-14). The suspension was sonicated for 10 minutes in a Bransonic 12 sonic water bath (Branson Cleaning Equipment,Inc., Palo Alto, CA) and then stirred at room temperature for 30 minutes. Absolute ethanol was slowly added to a final concentration of 20% and the solution gently stirred with a glass rod. The adherent DNA strands were removed and the solution was centrifuged at low speed (1000 rpm) in a clinical tabletop centrifuge for 10 minutes. Absolute ethanol was added to a final concentration of 75% and the 75% ethanol solution was placed in an ice bath for 2-3 hours or at 4^{o}C overnight. The precipatate was centrifuged at 20,000 x g in a Sorvall SS-34 rotor for 30 minutes. Approximately 2.0 gm of material, rich in OMs, was recovered. The pellet was suspended in 10 ml of 5% (w/v) Zwit. 3-14 in 50 mM Tris HCl,

pH 8.0, with 20 mM EDTA and extracted overnight at room temperature.

The extract was centrifuged at 3,000 xg for 20 minutes and 9.5 ml of the supernatant was loaded on a CM-Sepharose CL-4B column (1.6 x 10 cm) equilibrated with 5 mM Tris/HCl, pH 8.0, with 20 mM EDTA and 0.5% (w/v) Zwit. 3-14 at room temperature. The column was washed once with one column volume of equilibration buffer and the retained PIIa eluted with a step gradient of NaCl. Ten ml of 0.25M, 0.5M and 1.0M NaCl in Tris equilibration buffer were used in the elution of PII from the column. PIIa eluted with 0.5M NaCl. The 3 ml column fractions were monitored at 280 um with a BioRad UV monitor (BioRad, Richmond, CA) connected to a 1 mV strip chart recorder. The purity of PII rich fractions were monitored by SDS-PAGE and Coomassie brilliant blue (CBB) stained gels. The protein content was determined by the Peterson modification of the Lowry assay (129).

RESULTS

Whole cell lysates of N. gonorrhoeae colony variants were compared by SDS-PAGE and CBB staining to test for differences in protein patterns (Figure 4.1). The only obvious difference in protein bands was the presence (PII^+) and absence (PII^-) of PII. PII was identified by the heat modifiability of its M_r, at 37°C compared to 100°C. In strain F62, there were subtle differences detected in quantities of PIIs among the variants. The PIIa phenotype variant appeared to have abundant quanties of PII. Organisms with PIIb appeared to have small quantities of this protein. Outer membrane vesicles were prepared to improve detection of

differences of surface proteins.

The OMs were compared by SDS-PAGE and the gels were stained with silver (Tsai and Frasch 1982) (Figure 4.2A) The presence/absence of a PII was the predominant difference in over 50 proteins appearing in the gel patterns. There were also subtle differences in one or two high molecular weight proteins (50 to 60 Kdaltons, lane 3). The differences were not consistent and did not segregate with the PII^- or PII^+ phenotype. A 58K protein did appear to vary in quantity between PII^+ and PII^- variants with silver staining. This protein was particularly prominent in F62 PIIa OMs. (Figure 4.2A, lane 2). However, this difference could not be confirmed with CBB stained gels of OMs (CBB is a quantitative stain) (Figure 4.2B).

When OM vesicles were extrinsically labelled with ^{125}I, most of the high molecular weight proteins did not label (see Chapter 2, Figure 2.3) and thus, these may not represent surface proteins. The 58K protein did label in most stains and was probably located on the OM surface.

Antigenic differences of PII^- and $PIIa^+$ OMs were examined using Westen blot reactions. Antisera prepared against whole organisms were reacted with OMs from PII^+ and PII^- variants (Figure 4.3A). The anti-P++Tr serum detected several antigenic differences (panel A). A 29 Kdalton protein in PII^+ OMs was detected and was the PIIa variant found in the P-Op phenotypes. There was also an intensity difference in the reaction of the 58 Kdalton protein. There was more 58 Kdalton antigen detected in the PII^+ OMs than in the PII^- OMs. There were antigens on the 32 and 65 Kdalton proteins which were predominantly detected in the PII^- OMs. A low molecular weight band (approximately 12 Kdalton) was more prominent in the PII^- than in the PII^+ OMs. Absorption of the

anti-P++Tr serum with PII$^+$ organisms from P-Op colonies, reduced the intensity of most bands. The 12, 58, and 65 Kdalton proteins remained prominent after absorption.

Reactions of OMs with antiserum against whole organisms (anti P++Op) detected similar antigenic differences as those described above (Figure 4.3A, panel B). The PII gave a very strong reaction in PII$^+$ OMs but the band was missing in the PII$^-$ OM reactions; the 58 Kdalton protein was more prominent in PII$^+$ than in PII$^-$ OMs. When the antiserum was absorbed with organisms from P-Tr phenotypes, there was very little change in reaction patterns. The reaction with the 32 Kdalton protein was decreased in the PII$^-$ OMs.

These results indicated that with the variation in expression of PII, there also covaried a difference in the abundance of other proteins contained in OM vesicles. Whether or not these were surface proteins was unclear. Extrinsic iodination studies suggested that the 58 Kdalton protein could be surface located. In these studies, the 32, and 65 Kdalton proteins did not label and may have been contained inside the vesicles.

Western blot reactions with antiserum against PIIa OMs and reacted with PII$^+$ and PII$^-$ OMs (Figure 4.3B, panel A), showed simple differences between PII$^+$ and PII$^-$ phenotypes detected with this antiserum, the 58 Kdalton antigen was not detected. The major proteins detected were PI, PIII, PII, 22 Kdalton, and LPS. PII was the only protein remaining at the same intensity of reaction after absorption of the serum with PII$^-$ organisms. The PI reactive antibodies were not entirely absorbed. The Western blot reaction with the second antiserum (anti-PIIb OM vesicles), detected a difference in the 58 Kdalton and 44 Kdalton proteins in the

PII^+ OMs compared to the PII^- membranes (Figure 4.3B, panel B). When the serum was absorbed with PII^- organisms, there was a reduction in the reactivity of PI, PIII, and LPS for both types of membranes. PIIa was not detected by this PIIb-OM specific serum. From these data, a list of predominat antigenic differences and similarities between PII^+ and PII^- OMs was constructed (Table 4.1).

Antiserum prepared against PII^+ OM vesicles showed cross-reactivity for PII antigens within a strain as well as PIIs between strain (Figure 4.4). For example, rabbit serum, anti-F62 PIIb OMs, showed reactiv-ity for PII variants b and d in strain F62 and PII variants a, b, and c in strain FA1090. Reactivites for PIIs d and e were difficult to distinguish because they coincided with PIII reactions. There were also cross-reactivities for PI, 42, 58, and 65 Kdalton antigens. A 46 Kdalton protein was detected only in OMs from strain F62 (PIIa and PIIb) (Figure 4.4).

When several antisera were tested and compared, variability of immune response between rabbits for OM antigens was found. In general, the rabbits made antibodies against PII ,PI, PIII, 58 Kdalton, but not necessarily other proteins.

During the studies of antigenic differences, it was noticed that some antisera contained antibodies cross-reactive for different PIIs; other antisera did not contain cross-reactive antibodies. This phenomenon was futher investigated. PIIa of strain F62 was purified and used as an immunogen. The antiserum was characterized by Western blot and it reacted with all the PIIs in strain F62 (Figure 4.5) as well as those in strain FA1090 (data not shown). These results suggested areas of antigenic similarity in PIIs.

The PIIa specific antiserum was used to investigate the antigenic similarities of peptide fragments from several PIIs (Figure 4.6). Peptides from each PII molecule could be detected by the antiserum. The fragments carrying the "common antigenic determinant(s)" were generally larger than 14 Kdaltons. ^{125}I peptide maps were compared with Western blot peptide maps and the results indicated that there were areas of structural and antigenic homology as well as diversity (Figure 4.7). Protein fragments of molecular weight lower than 14Kdalton could not be detected by antisera reactions or by ^{125}I peptide maps (data not shown). The PIIs were labeled in situ in OMs and thus, the "extrinsic" labelling of only the surface portion of the PII molecules probably accounted for the lack of the detection of the small fragments. These fragments may have been unlabelled or were from portions of the molecule located deep within the membrane and not available for iodination. The detection of most peptide fragments of PIIb from strain FA1090 by PIIa specific antiserum indicated that there were areas of antigenic homology between PIIs from different strains. Collectively, these observations suggested antigenically variable regions and constant regions exist for the PIIs.

DISCUSSION

In this Chapter, evidence of the antigenic similarities of PIIs within a strain and between strains was presented. The immunologic approach confirmed the structural information determined from peptide mapping studies presented in Chapter 2. PIIs contained areas of structurally homology and diversity. In addition, studies were designed

to detect OM proteins which varied with PII in presence and abundance. A 58 Kdalton surface protein which appears to vary in quantity with PII was detected. Also found were two proteins associated with the Tr phenotype, the 32 and 65 Kdalton proteins were detected prominently in PII$^-$ organisms. However, these antigens were detected only with antisera prepared from whole gonococci, and their surface location could not be confirmed.

In the present studies, PII was very immunogenic in rabbits when presented in whole organisms, OM vesicles, and in purified form. Antiserum, raised against PII variants contained in OM vesicles, showed cross-reactivity when characterized by Western blot assay. Previous studies using whole organisms with immunoprecipitation (60) and ELISA inhibition techniques (125) to characterize cross reactive PII antigens, have demonstrated very little cross-reactivity. One explanation for this discrepancy is the Western blot technique wherein the molecule is denatured in the presence of SDS. By this technique more of the common antigenic determinants would be exposed than in whole organisms and thus, available for reaction with antibody. Thus, the antisera of the previous studies may have contained cross-reactive antibodies but these would not have been detected by the techniques used.

Heckles has shown that the peptides on the surface of the PII molecule are unique (142). Subsequent studies by Diaz and Heckles have confirmed that the antigenic determinants of the surface region of PIIs are heterogeneous, and that the antibodies have little cross-reactivity (125). The results of the present studies also indicate that there were antigenically unique peptides in each PII. In the western blot peptide mapping studies there were fragment in PIIs not detected by heterologous

serum.

The interesting observations of these investigators and the data from the Western blot peptide mapping studies indicate that within the PII molecule there must be variable as well as constant regions. Preliminary evidence from PII gene sequencing studies indicates that this is true for PII (Magdalene So, personal communication). One could envision a constant region of the PII molecule which is buried in the membrane and an exposed, variable region of PII which is accessable to the external milieu. The results in this Chapter and those in Chapter 2 support such a model of PII structure and orientation. Both studies show structurally conserved and variable regions. What cannot be determined from these studies, is whether or not there are conserved, surface regions of PII. If PIIs have a common function of attachment, then it seems likely that such a region might be structurally conserved. It is possible, however, that such a region might contain determinant which were immunorecessive or poor immunogens. This phenomenon has been reported for gonococcal pili (64). The immunodominant region of the pilin molecule is structurally hypervariable and antigenically heterogeneous; the domain involved in binding is immunorecessive and specific antibodies are poorly stimulated when whole pili are used as immunogens. This condition may also exist in PII. There may be functional domains which are structurally conserved and other domains which contribute to the antigenic diversity of this molecule.

One difficulty encountered in these studies was the variation in immune response by different rabbits. Most rabbits responded to PI, PIII, and PII antigens; however, the rabbits tended to respond to other surface antigens in a variable manner. The proteins such as the 32, 39,

42, 44, 58, 65 Kdalton bands were not detected by some immune sera. Swanson has reported a similar variability in rabbit antisera (60).

Another difficulty encountered during these studies was the lack of effect on Western blot patterns when P++Op specific antiserum was exhaustively absorbed with organisms from P-Tr colonies (Figure 4.3A, panel B). There was very little decrease in the antibody reactivities for the proteins present in both the PII^- and PII^+ OMs. There are several possible explanations for this observation: i) the organism used to absorb the serum could have greatly changed its antigenic characteristics during in vitro subculture between the year that the antiserum was prepared and the studies performed; ii) the OM antigens detected in the Western blot reactions were not exposed at the surface of organisms, and thus, the P-Tr organisms do not absorb the reactive antibodies, and iii) some of the antigens contained in the OMs could have been cytosol or periplasmic space constituents which were trapped inside the vesicles as they formed. These antigens would be detected in Western blot reactions of OMs by serum specific for whole gonococci, but whole gonococci could not absorb the reactive antibodies. The latter explanation seems the most possible.

The impression gained from the antigenic studies was that the outer membrane of the gonococcus is a dynamic system. Several constituents are antigenically constant within a strain and several are antigenically variable. Results also indicated that several outer membrane proteins could vary in abundance between colony phenotypes i.e., PII, 65, 58, 45 Kdalton proteins. The implications for the virulence function of the components of the gonococcal surface are great. Subtle shifts in quantities of any "virulence determinant" could presumably alter

pathogenic outcome, extent of immune response, and perhaps infectivity. Such a dynamic mosaic of surface components confers flexibility and adaptability to the organisms and may be an explanation in part for the success of the gonococcus as a human pathogen.

Figure 4.1 The SDS-PAGE protein gel patterns of whole cell lysates of N. gonorrhoeae strain F62 colony phenotypes. The samples from each variant were solubilized at 37°C and 100°C. The heat modifiable PII in each strain is indicated with an arrow (➢) except in the PII⁻ variant. Lanes 1,2 PIIa; lanes 3,4 PIIb; lane 5,6 PIIc; lane 8,9 original PII⁻ organisms; lane 10,11 PII⁻ revertants from PIIb positive colonies; lanes 13,14 PIId; lanes 7,12 contain molecular weight standards of 92, 66, 45, 31, 21, 14.4 Kdaltons. Notice the difference in quantity of PII in each strain.

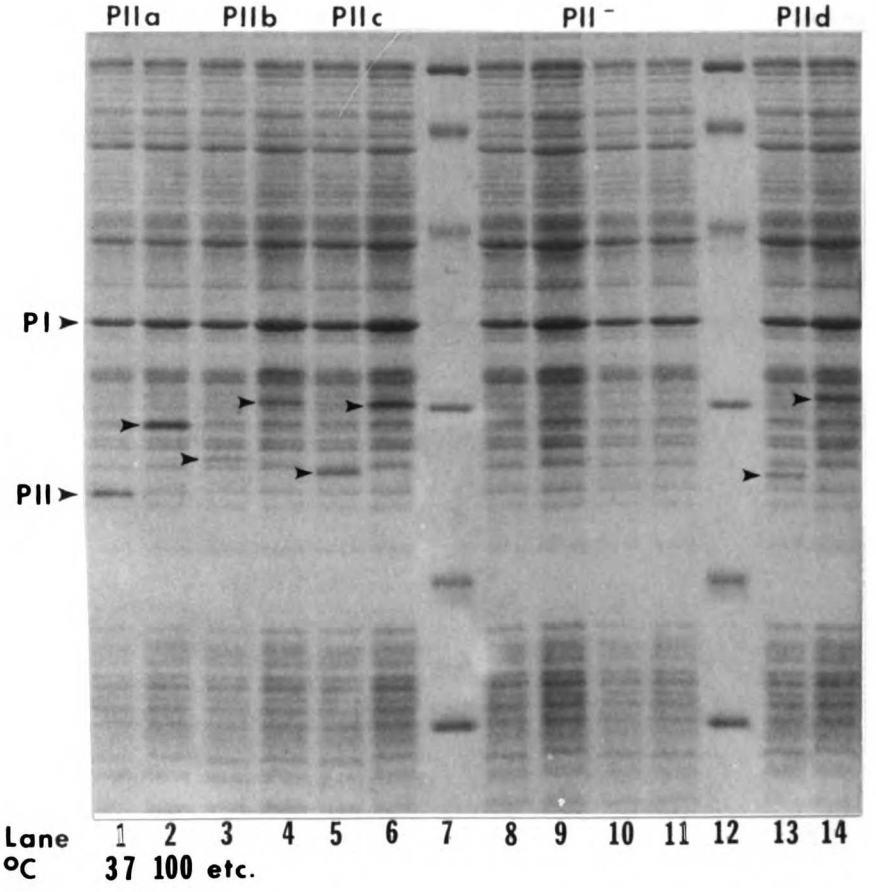


Figure 4.2A A silver stained SDS-PAGE gel of outer membrane vesicles from N. gonorrhoeae strain F62 and FA1090 phenotype variants. Samples were prepared at 100°C before electrophoresis. Lane 1-5 contain outer membranes from strain F62 with PII⁻, PIIa, b,c, or d positive samples, respectively. Lanes 6-9 contain outer membranes from strain FA1090 with PII⁻, PIIa,b,or d positve samples, respectively. Protein II is the prominent difference between the variants, however, there are subtle differences between variants in the presence and absence of high molecular weight proteins.

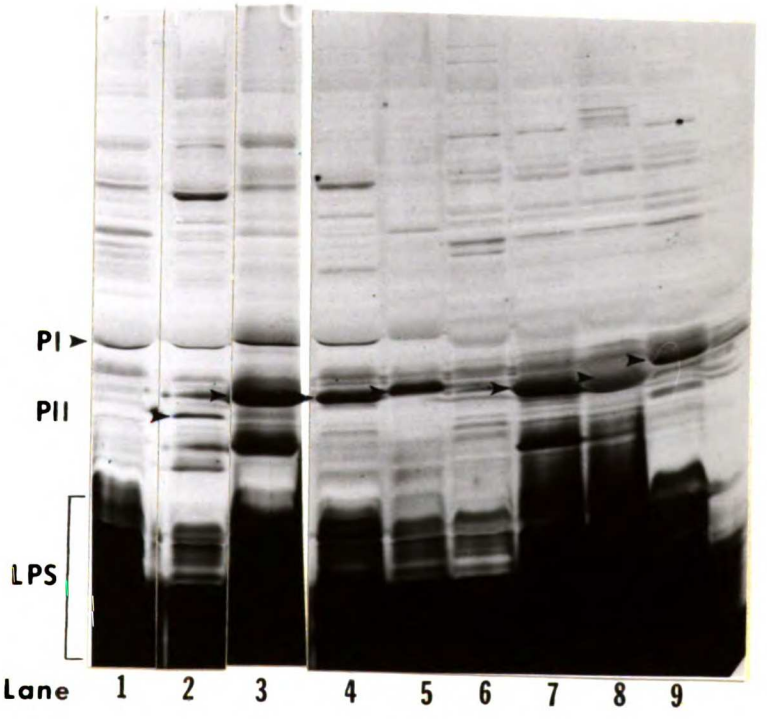
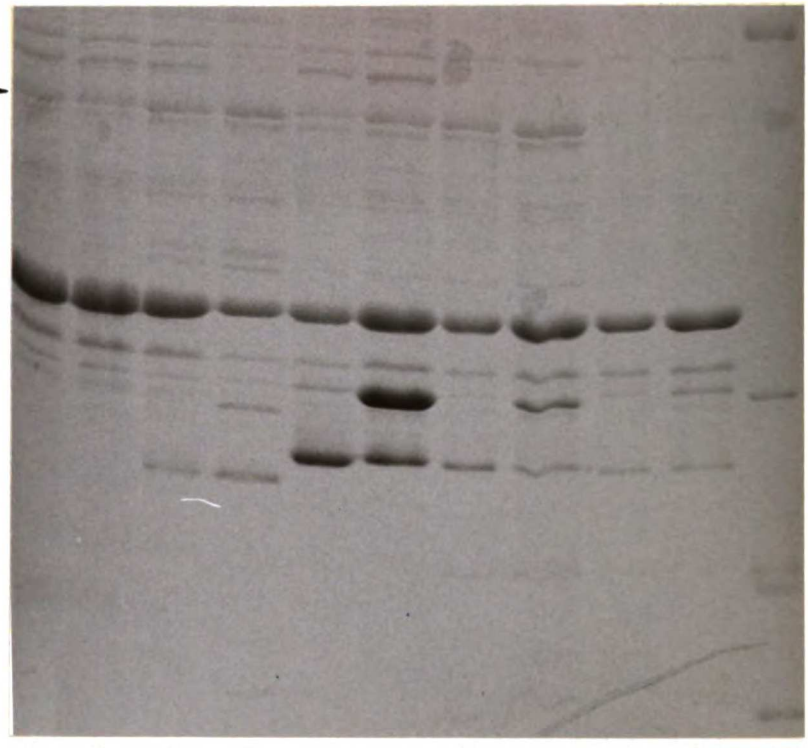


Figure 4.2B A Coomassie brilliant blue stained SDS-PAGE gel of N. gonorrhoeae proteins in outer membrane vesicles prepared from colony phenotype variants. Samples from each variant were solubilized at 37°C and 100°C. The first lane of each pair is the 37°C sample. A 58 Kdalton protein (►) is present in both PII^+ and PII^- outer membranes and does not seem to vary greatly in abundance. Panel A contains OMs with PII variants from strain F62: lane 1,2 PII^-; lane 3,4 PIIa; lane 5,6 PIIb; lane 7,8 PIIc; lane 9,10 PIId, lane 11 standards. Panel B contains OMs with PII variants from strain FA1090: lane 1,2 PII^-; lane 3,4 PIIa; lane 5,6 PIIb; lane 7,8 PIIc,e; lane 9,10 PIId; lane 11 standards. Molecular weight standards include (from the top) 92, 68, 45, 31, 21, 14.4 Kdaltons.

Panel A. F62 ►



lane 1 2 3 4 5 6 7 8 9 10 STD

Figure 4.2 B

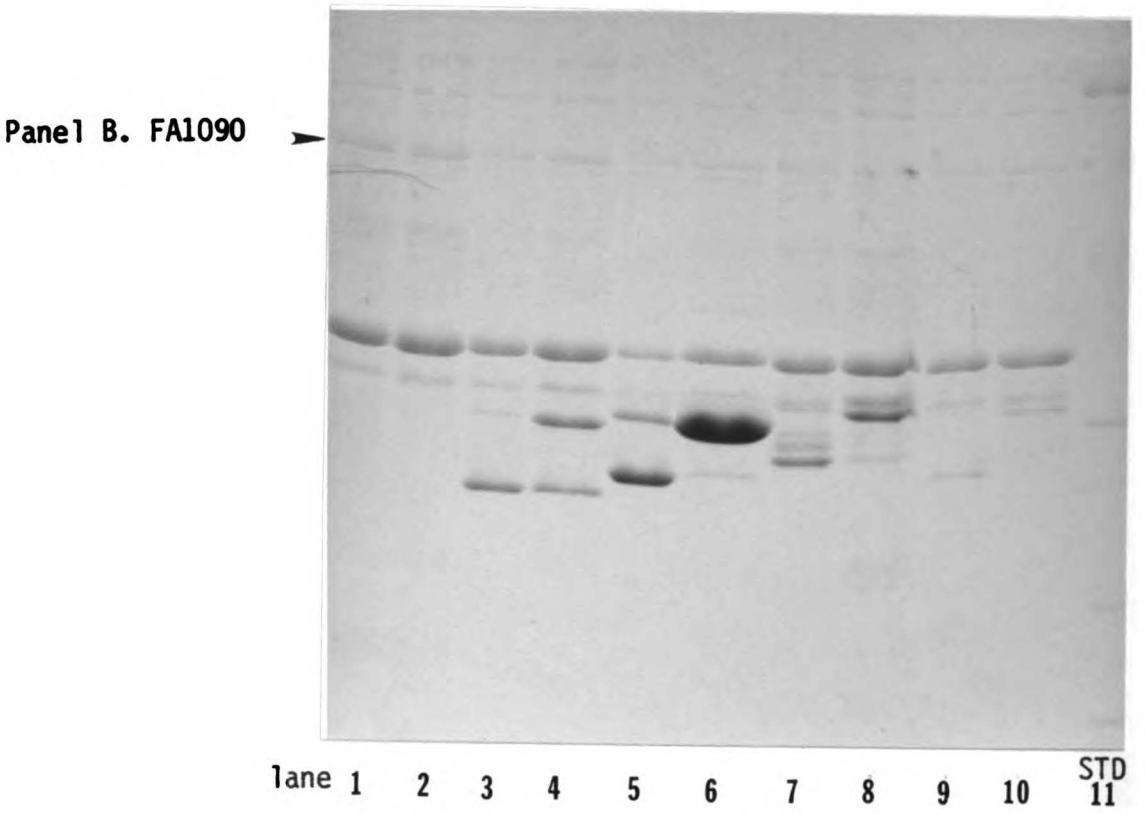


Figure 4.3A Autoradiograms of Western blot reactions of rabbit antisera specific for whole N. gonorrhoeae of either P++Tr or P++Op colony phenotype reacted with outer membrane vesicles of organisms with P-Tr or P-Op colony phenotypes. In each experiment, the serum was preabsorbed with organisms of the contrasting phenotype and the blot reactions compared to those of unabsorbed serum. Panel A: Serum (anti-P++Tr), absorbed with P-Op organisms, reacted with blots of P-Tr (lane 1,3) and P-Op (lane 2,4) OMs. Panel B: Serum (anti-P++Op), absorbed with P-Tr organisms, reacted with blots of OMs ordered as in panel A. In each panel, lanes 1 and 2 are reactions with unabsorbed serum and lanes 3 and 4 are with absorbed serum.

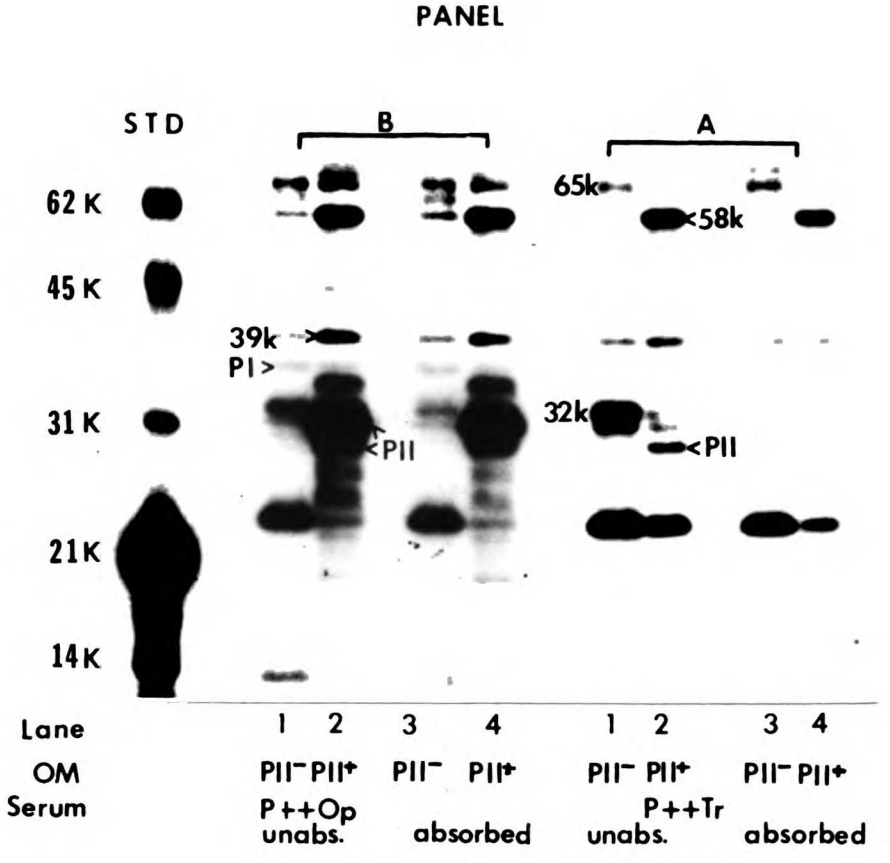


Figure 4.3B Autoradiograms of Western blot reations of rabbit antisera specific for outer membranes of nonpiliated N. gonorrhoeae strain F62, containing PII variants reacted with F62 OMs of organisms with P-Tr (PII^-) or P-Op (PII^+) colony phenotypes. Panel A: serum (anti-OM PIIa), absorbed with PII^- isogenic organisms, reacted with blots of PII^- OMs (lane 1,3) and PII^+ OMs (lane 2,4). Panel B: serum (anti-OM PIIb), absorbed with PII^- isogenic organisms, reacted with blots of OMs ordered as in panel A. In each panel, lanes 1 and 2 are reactions with unabsorbed serum and lanes 3 and 4 are with absorbed serum.

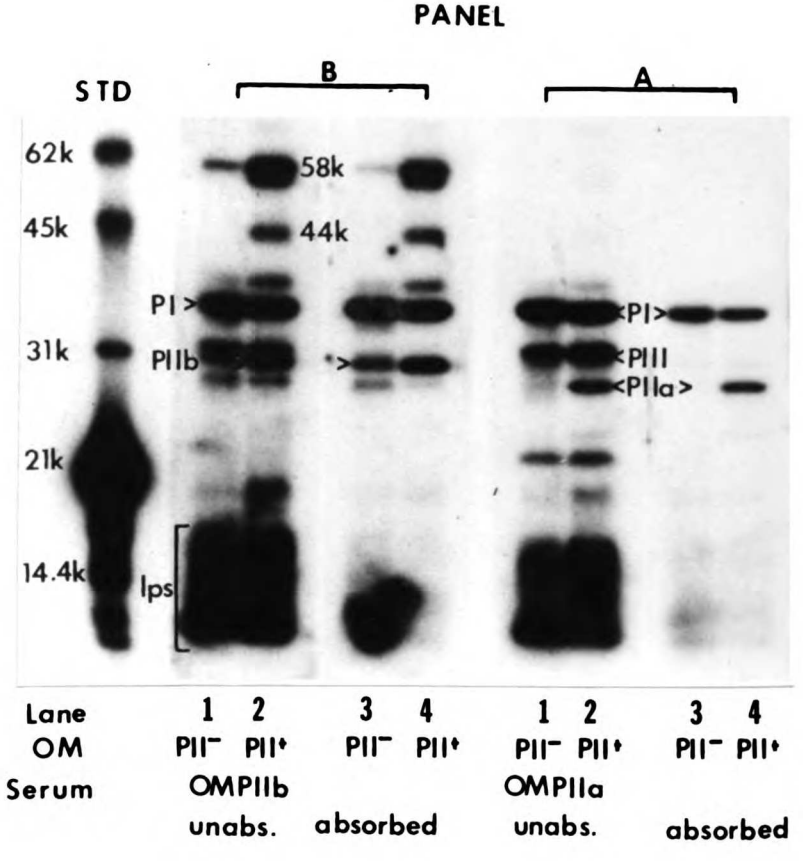


Table 4.1 A summary of antigens detected in PII^+ and PII^- outer membrane vesicles by rabbit antisera in Western blot reactions. Rabbits were immunized with either whole *N. gonorrhoeae* or outer membrane vesicles.

Antigen (Kdalton)	PII^+ OMs	PII^- OMs
65	-	+
58	+ (4+)	+ (1+)
44	+	+/-
42	+	+
39	+	+
36 (PI)	+	+
32	+/-	+
31 (PIII)	+	+
29 (PII)	+	-
22	+/-	+/-

Figure 4.4 Autoradiogram of a Western blot reaction between rabbit antiserum specific for gonococcal outer membrane vesicles from PIIb$^+$ organisms (strain F62) and preparations of outer membrane vesicles from colony phenotype variants in strain F62 and strain FA1090. The serum was diluted 1:100 and antigen-antibody reactions were detected with ^{125}I-labelled protein A. There are cross-reactions between PII antigens within a strain and between strains.

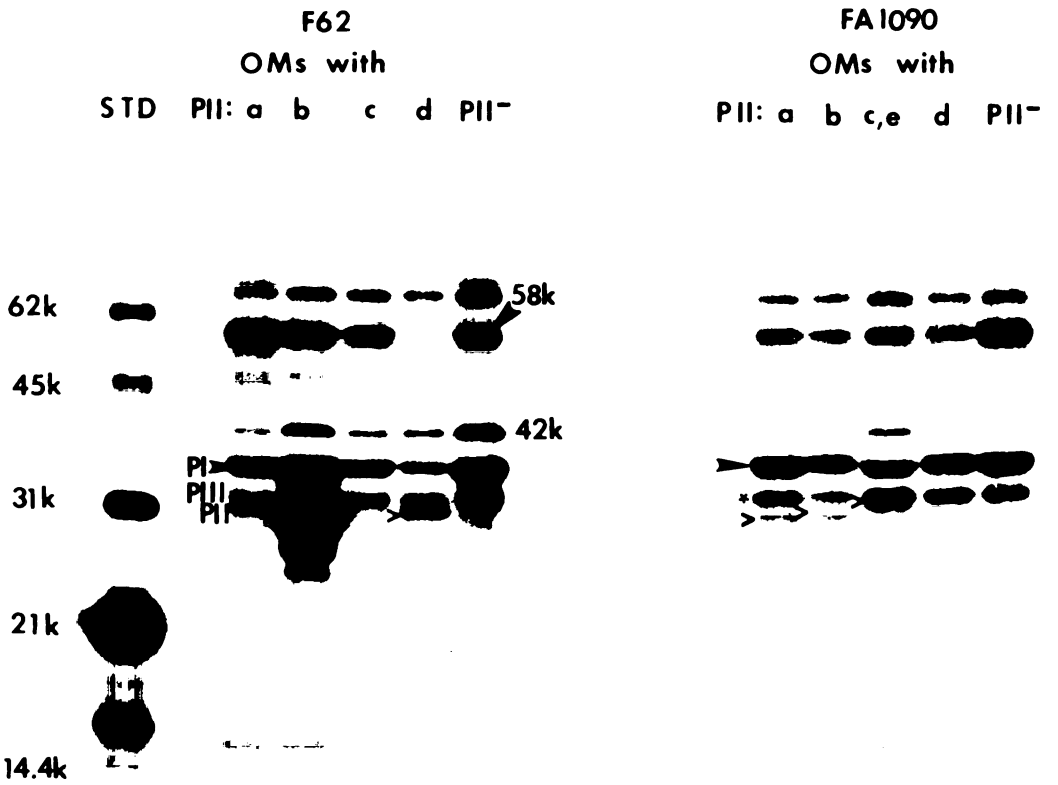


Figure 4.5 Autoradiogram of a Western blot reaction between rabbit serum specific for purified protein IIa from N. gonorrhoeae strain F62 and whole organisms or outer membranes from organisms containing PIIa, b, c, or d. The serum detected homologous antigen PIIa (lane 1,2, and 6) and there was cross reactivity between the PIIs and a slight cross reactivity for PI.

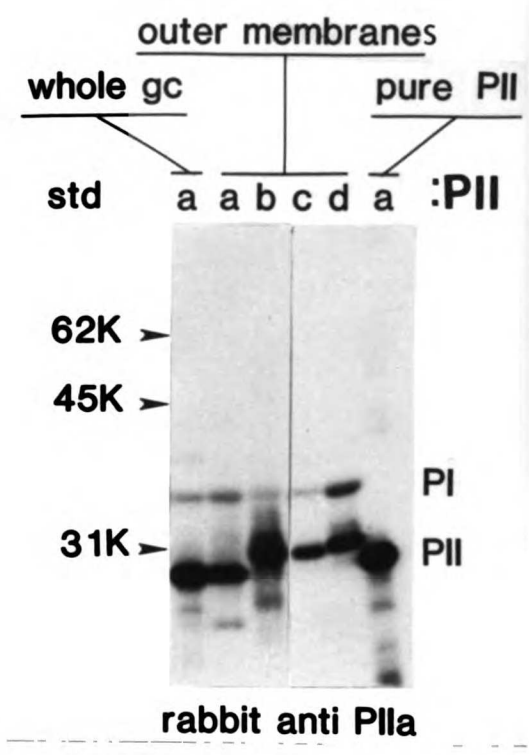


Figure 4.6 Autoradiogram of Western blot peptide mapping. Immune serum (anti-purified PIIa from strain F62) was reacted with V8 protease peptide fragments of PIIs a,b,or c from strain F62 and fragments of PIIb in strain FA1090. Antigen-antibody reactions were detected with ^{125}I protein A. All PIIs contained cross-reactive peptides to PIIa of F62.

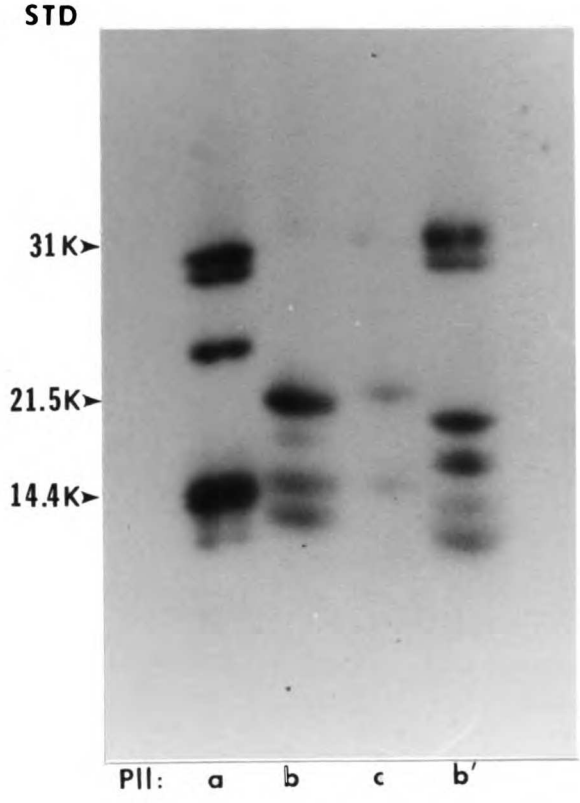
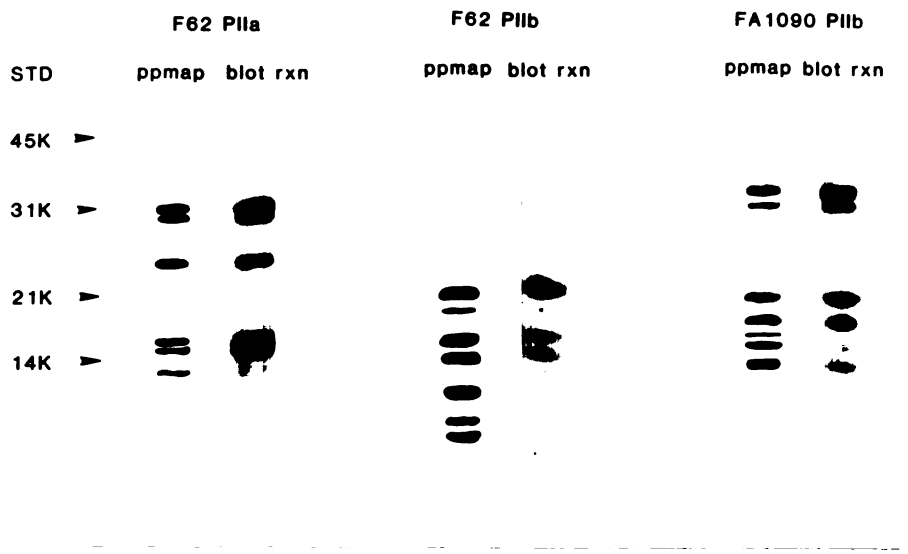


Figure 4.7 A comparison of Western blot peptide mapping patterns with ^{125}I peptide maps of PIIs from strain F62 and FA1090. The western blot technique detected most of the cross reactive peptides in the three PIIs tested. In PIIb of strain F62 there were fragments which were not detected with PIIa specific antiserum. These fragments may contain antigenicly unique regions of the PII.



Chapter 5

Summary and Disscussion

The previous sections of this dissertation presented data on the structural diversity of gonococcal OM protein II, on PII antigenic diversity, and on the role of PII in attachment of N. gonorrhoeae to human cells. In this Chapter, these findings are summarized and integrated into the current concepts of the pathogenesis of gonococcal infection.

The current model of gonococcal disease assumes that attachment is the first event. The host mucosal surface is the site of this process and it is generally believed to be a selective process. The gonococcus displays a remarkably limited host range and a predilection for "colonization" of human genital tissues. The attachment mechanism appears to be highly efficient, for as little as 100 organisms, when inoculated intraurethrally, can cause disease (174).

The gonococcal surface structures involved in attachment include pili and possibly PII. The process appears to be complex and there may be other attachment factors. The attachment phenomenon is divided into at least two processes, the initial attachment initiated by pili, and the secondary mechanism which binds the gonococcal membrane to the host membrane (Figure 3.1). This latter phenomenon is rapidly followed by "phagocytosis" of the organism by the epithelial cell of the mucosa. It is not known what triggers this "non-professional phagocytosis" by columnar epithelial cells (44). The gonococcal components which mediate

the secondary binding may also initiate the process of engulfment. The overlapping complexity of these two processes has made it difficult to identify the components of the secondary binding mechanism.

The primary goals of the research presented in this dissertation were to define the role of PII in gonococcal attachment, and to determine if PIIs are directly or indirectly involved in binding. Previous studies have used whole organisms with and without PII to measure attachment (61,127,150). These studies indicated that PII^+ organisms attach in greater numbers than PII^- organisms and that PII was associated with enhanced attachment to human cells. One antibody inhibition study has indirectly supported a ligand role for PII (126). However, direct evidence of PII as a ligand has been lacking.

It was reasoned that purified PII should be used in the assays and that its direct adherence to human cells should be demonstrated. To accomplish this goal, an improved technique was needed for measuring attachment. Other experiments were designed to provide supporting evidence by demonstrating that purified PII could successfully block adherence of PII^+ whole organism and PII^+ outer membranes. The demonstration of attachment inhibition by specific antibody would also provide strong supporting evidence for PII as a gonococcal ligand.

Several purification systems for PII were tested. There were many problems, including poor yield, denaturation, and poor solubilization and none of the procedures were found suitable. A new procedure was designed with the non-ionic detergent octyl glucoside. This detergent has been used successfully to purify membrane proteins in a functional form and the detergent could be removed by dialysis. The developed procedure was simple to perform. Unfortunately, when octyl glucoside was

removed from purified PII, the membrane protein precipitated. If the detergent were left in the preparation, then the membranes of the tissue culture cells were solubilized during assay. For these reasons, the results of assays using purified PIIs, with detergent, could not be interpreted. The problem of purified PII solubility and its use in biological systems is a difficult one. A solution to the problem is mandatory if functional studies of PII are to be carried out efficiently and successfully.

Alternative approaches were required to study PII function in attachment. Assays were designed to use OM vesicles. This type of assay system gave similar results to those with whole organism and was more sensitive and reproducible. In these assays, there was a high degree of correlation of PII content with variation in attachment of outer membrane vesicles. The studies were expanded to include comparisons of PII variants in OMs and results also indicated variation in adherence corresponding to variation in content of PII (Chapter 3). OMs containing certain PIIs demonstrated increased levels of attachment to HeLa 229 cells when compared to OMs lacking PIIs.

The OM assay was used to characterize the secondary binding process. The mechanism was sensitive to pH and maximum binding occurred at alkaline pHs, minimum binding occurred at pH 6.5-7.0. The binding parameters of reversibility, equilibrium, and saturation were studied. It was discovered that OM binding was not totally reversible, and that the ability of the system to reach equilibrium was pH dependent. At pH 6.0, a steady-state was reached; at pH 7.38, equilibrium was not achieved and the OMs continued to bind. In saturation studies, it was determined that the number of binding sites was also pH dependent. One

interpretation of these results was that there were several binding system and that one mechanism contained a pH sensitive.

Inhibition of PII^+ OM binding by PII specific antibody was tested in the assay and the results were inconclusive. Only 20% specific inhibition could be demonstrated. This result has borderline significance and is difficult to interpret. It is possible that the PII specific antibody had low specificity, or avidity, or the antibody did not recognize the binding domain of PII. The binding domain of PII could have had poor immunogenicity and other regions of PII could be more antigenicly dominant. Alternatively, one explanation could be that PII is not directly involved in attachment. There is, however, too much evidence supporting some attachment role for PII to state that it is not involved at all (48,61,126).

It should also be recognized that the procedures used in this study and in previous studies may not have the specificity and sensitivity to detect whether or not PII is a ligand. It is very probable that the maximum amount of information which can be generated from such techniques has been obtained. Future studies will need alternative approaches, such as functional studies of purified PII peptides.

In the following paragraphs, I review the results of some experiments in this dissertation, and by other workers, to suggest a mechanism that explains gonococcal attachment in the absence of pili, but which indirectly involves PII.

The results of nitrocellulose blot assays of HeLa proteins to which ^{125}I-OM vesicles bound were promising. There was a clear cut band with a M_r of 15,000 to which both PII^+ and PII^- vesicles bound. This finding indicates that the same binding mechanism may be operating for adherence

of these membrane types. These results of the HeLa cell receptor are only preliminary and more work is needed to determine the significance of the findings. However, the observations do provide a foundation for future experiments to identify the receptor(s) of the secondary binding process.

Experiments were designed to distinguish the nature of the OM interaction with the HeLa receptor. HeLa cells were pretreated with a variety of substances to block or destroy the receptor. These results did not clearly distinguish between PII^- and PII^+ mechanisms. However, pretreatment of the substrate cells with proteases, glycosidases, metal ions, or charged molecules yielded results which generally indicated that proteins, hydrophobicity and negative charges were important in the interaction (Chapter 3).

It is important to recognize that non-piliated, PII^- organisms also attach to human cells. The attachment is not efficient in the absence of pili, but it does occur (James et al 1983). What is this mechanism of attachment and what is the ligand? The technique used to answer this question was the selective binding assay. This procedure has been successful in identifying mycoplasma ligands (151).

The data of the selective binding assay showed that PI and PII were bound by glutaraldehyde fixed HeLa cells and remained bound after extensive washing with detergents. These results indicate that both PI and PII may have characteristics of "stickiness" and it is possible that both are involved in adherence. Because the experimental conditions are so extreme, (detergent, fixatives), the results should be interpreted cautiously. If the findings are valid, however, the adherence of PI provides a mechanism for PII^- attachment.

The results of the HeLa selective binding experiments are reminiscent of the membrane transfer experiments of Blake et al. performed with PI and red blood cell membranes (45). These studies have shown that PI may be transferred from whole N. gonorrhoeae into human red blood cells and inserted into the rbc membrane. Cook et al. have demonstrated that the transfer of intrinsic membrane proteins between artificial phospholipid vesicles and human erythrocytes can occur (175). The investigators have shown that the transfer is facilitated by recipient membranes being more fluid than donor membranes. In the gonococcus, Blake has performed studies that indicate that PII^+ membranes are less fluid than PII^- membranes (45). PII could influence the rigidity of the membrane, and thus, the transfer of PI into the eukaryotic membrane. Protein transfer could be the mechanism and the basis of the observed attachment and provide an alternative explanation for results of my studies. PII would not be involved directly as the ligand (although the data of the selective binding study suggest that PII might also be transferred) but involved as the promoter. If this mechanism does operate then PII antibody inhibition would not necessarily be expected to inhibit transfer. Surface bound immunoglobulin might sterically prevent PI transfer or it might also decrease membrane fluidity by cross-linking PII molecules in the gonococcal membrane. The latter event would promote more transfer of PI. PII structural diversity could provide a means by which membrane fluidity was subtly altered. The abundant PII variants could interact differently with each other or the lipids of the membrane, then membrane fluidity might be altered. "Hydrophobic" PIIs might interact strongly with each other and with the lipids and promote rigid areas of the

membrane.

These exciting possibilities need investigation and will no doubt be the focus of future research. The elucidation of the secondary attachment mechanism and the trigger of gonococcal engulfment will have great impact on the designs of immunoprophylaxis. If a vaccine cannot be successfully designed to prevent pilus-mediated attachment (primary), then the next event to be blocked would be the secondary mechanism. Efficient blocking measures cannot be designed until the mechanisms involved in this process are known.

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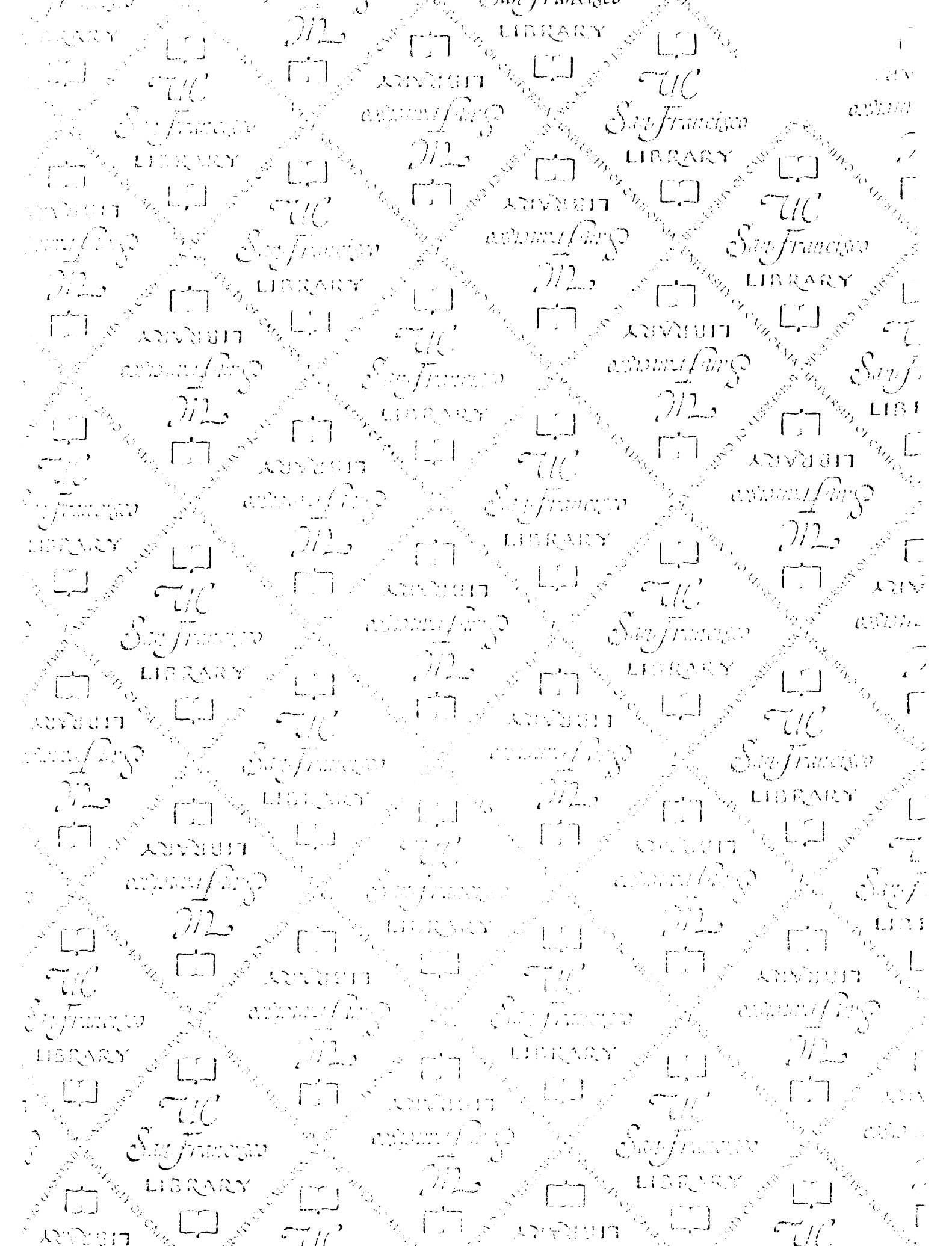
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