1991

Relationship of outer membrane proteins II of Neisseria gonorrhoeae [sic] to colony opacity and serum killing

Jing Chen
The University of Montana

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The Relationship of Outer Membrane Proteins II
of Neisseria gonorrhoeae to Colony
Opacity and Serum Killing

BY JING CHEN
B.S., Sichuan Medical College 1983
Sichan, P.R.China
Presented in Partial Fulfillment
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1991

Approved by
Chair, Board of Examiners
Dean, Graduate School
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The relationship of outer membrane protein II of *Neisseria gonorrhoeae* to colony opacity and serum killing (86pp.)

Director: Dr. Ralph C. Judd

Outer membrane protein II (PII) variants in seven gonococcal strains were isolated with the indication of colony opacity and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. High frequency switching in PII expression of organisms was indicated by variation in colony opacity. These extensive changes of PII expression possibly help organisms evade host immune response and increase the ability of bacteria to adjust to different internal environments. Gonococci forming opaque colonies posses a group of PIIs that are lacking in organisms which form the transparent colonies. In experimental strain FA899, the opaque phenotype colonies were observed despite the absence of PIIs in those colonies, which implies the involvement of other surface structures the colony opacity.

The serum sensitivity of opacity variants was examined with microassay. The opaque variants tend to be more resistant to normal human serum (NHS) than isogeneic transparent colonies. These results suggest that expression of PIIs enhance bacterial survival in the host.

Unlike other serum assays, serum killing in this microassay is likely mediated by a group of heat stable low-molecular-weight cationic proteins other than complement.
ACKNOWLEDGEMENTS

My special thanks goes to my advisor, Dr. Ralph C. Judd for his guidance, encouragement, and much patience during the course of this study. I would like to thank Dr. Tomth North and Dr. Willard O. Granath who offered guidance and served as the committee members in my project.

I can not adequately express my appreciation to my parents. I have relied heavily upon their perspective, love, encouragement just as I have throughout my life.

I would also like to express my gratitude to my fellow graduate students for their encouragement throughout this program.
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CHAPTER I

INTRODUCTION

Neisseria gonorrhoea (GC), the pathogenic agent of gonorrhea, is one of the most common sexually transmitted diseases in humans (7). It causes diseases ranging from uncomplicated genital infections to complicated infections including pelvic inflammatory disease and disseminated gonococcal infection (DGI) (7, 50). Human mucosae, such as the urethra, rectum, pharynx conjunctiva and endocervix (7) are most commonly infected. GC infects men, women, infants, even the unborn. In recent years, antibiotic-resistant strains of GC have appeared, causing an increasing in cases not responsive to treatments of antibiotics (15). GC infects individuals worldwide at any time of the year, not just in seasonal epidemics or in localized areas. Despite considerable efforts to control its infection, gonococcal disease still remains a common public health problem. Approximately 375 cases per 100,000 population were reported in the United States in 1987 (11).

Structural variation of surface components is one effective way used by many pathogens to evade the host immune response. GC uses this method to evade host immune responses. The antigenic determinants on the membrane
surface of GC, such as outer membrane proteins (OMPs) and lipopolysaccharide (LPS), appear to play a major role in pathogenesis. Those surface factors can change rapidly, making it possible for this gram-negative diplococcus to evade the host immune defense, conferring significant advantages in bacteria survival, and allowing GC to adapt to changes in the host environment. Antigenic variation (12, 41, 77, 78) and natural transformation (73) are thought to be responsible for those extensive variations. The variability of outer membrane (OM) structures has contributed to our general failure to develop a vaccine and probably account for multiple and recurrent gonococcal infections (95).

Pili are hairlike structures present on surface of GC. They consist of identical multiple pilin subunits. Pili range in molecular mass from about 19,000 dalton 19K to 25K (64, 71). Pili are one of the predominant variable factors of the OM and play an extremely important role in the GC pathogenesis (83).

Specific gonococcal colony forms were correlated with GC virulence by Kellogg, et al (42, 43, 83). Four colony types are observed T1, T2, T3, T4. Each colony type was morphologically different (8). Type 1 and type 2 (virulent) produced typical acute venereal infection whereas type 3 and type 4 (avirulent) caused only mild, transient symptoms without establishment of infection (42, 43). Pili have been
found only on the cells of type 1 and 2 and are absent from type 3 and 4 (3, 82). Data by J. Swanson and others (9, 24, 60, 83) suggested that piliated small colonies of GC are the invasive form of the species. This suggests a correlation between the virulence of GC and the presence of pili. Chromosomal rearrangements, such as deletions of pilus genes and intragenic recombination cause phase and antigenic variation. This enhances bacterial survival and virulence (18, 22, 55, 74). It has been shown that pili are involved in the first of a two-stage attachment process of GC infection. They help overcome the electrostatic barrier which exists between the negative charged surfaces of gonococcus and the host cell (24). Purified gonococcal pili attach to the cervical-vaginal and buccal epithelial cells (normal host cells) much better than they attach to the erythrocytes, leukocytes or fibroblast (59). This finding suggests that a specific relationship exists between GC and their host cells. Receptors on the host cells can specifically bind to the receptor-binding domain of the pilus molecule has been found. This site consists of 80 amino acid residues from 31 to 111 of pilus molecule (71). Antibodies against pili specifically block this binding, dramatically reducing the attachment to the host cells. This characteristic has made pili a potential candidate for a vaccine. However, indicative efforts to develop a pilus vaccines have not been successful, suggesting that the
extensive changes of pilus molecules caused by the phase and antigenic variation (76) may make pili unsuitable for a vaccine.

Outer membrane protein I (PI), which is present in all gonococci examined, is the major OMP of GC with a molecular mass varying from 34K to 38K (1, 36). It varies in subunit molecular mass among strains but is identical for all intrastrain variants (1) and accounts for approximately 60% of the protein in the OM (31). Two major groups of PIs, PIA (34-36KDa) and PIB (36-38KDa), have been recognized in the OM of GC and can be further subdivided by using monoclonal antibodies (MAbs) (44). PIA and PIB appear to be orientated differently in the OM. The PIA's have their N-terminal 15-20 amino acids exposed on the surface (1), while PIBs have both termini buried in the membrane, with a central region exposed on the surface (1, 38, 40). These two subclasses are structurally and electrophoretically distinct. Certain biological characteristics have been correlated with the PI types that the organisms bear. PIs affect the inhibition of bacterial growth by proteolytic enzymes, and influence sensitivity to serum killing (28). The strains isolated from the blood stream of patients with DGI are usually resistant to the bactericidal action of normal human serum (NHS) and tend to belong to the PIA group. Strains isolated from uncomplicated genitourinary disease tend to belong to group PIB and are often serum sensitive (39, 70). Because
of the distinctive exposure of PI subclass molecules in the OM, PIAs are resistant to in situ cleavage by α-chymotrypsin, trypsin and proteinase K, while PIBs are much more susceptible to those enzymes. The molecular mass of PI has been correlated with serum sensitivity, with strains bearing PI of the higher molecular mass more sensitive to NHS killing (9, 29). PIAs also serve as porins which form hydrophilic channels across OM and are the major determinants for serotypic specificity in the membrane (31). Although the PIA in GC strains WS3 and WS5 are identical, a slight alteration of exposure of PIAs present in these two strains was correlated with altered LPS and/or H.8 antigen structure found in those two strains. Many more alterations of exposure of PIBs were found in strains WS2 and WS4 which also correlated with the variations in LPS and/or H.8 antigen structure (41). It is possible that PI with LPS and/or H.8 antigen form a complex on the OM. Since PI appears to be invariant within a strain, this implies the organism may have developed a mechanism to protect this molecule from the immunological attack of the host by changing the other OM components (41).

Outer membrane Proteins II (PII), a group of heat-modifiable proteins on the surface of gonococci, may be important virulence associated OMP. They range in apparent molecular mass from 24KDa to 30KDa and are highly exposed on the surface of the bacteria (6, 25, 26, 65, 86). Since they
correlate with difference in colony opacity, they are also
called opacity-associated proteins (25, 31, 45, 88, 90, 96).
Although PII molecules show a high degree of sequence
conservation in their primary structure, they are function­
ally, serologically, and structurally distinguishable (89).
The difference has been seen in primary structure, antigenic
determinants, and migration in SDS-PAGE gels (14, 27, 37,
86, 88). A single organism can express from zero to four
PII species with different combinations, with a single
strain (MS11) able to produce as many as 11 electrophoretic­
ally distinct PII variants (2, 4, 14, 72, 88).

Protein IIIs are not structurally related to any of the
other OMPs (37). The members of the PII family share about
60% homology when compared by peptide mapping of
α-chymotrypsin digests of purified proteins. The other 40%
of the peptide are unique to each PII and represent the
portion of the molecule which is exposed on the surface of
bacteria (27, 37, 86). Antisera from purified PIIs of three
strains react with PIIs of all heterologous strains, but the
strongest reactions are with homologous PIIs (54, 72).
However MAbs are very specific for each PII, showing little
cross reactivity with other PIIs (4). Confirming PII
peptide mapping, the DNA sequence of two cloned PII genes
shows that they are very similar in large regions (80%
homology) with difference only in two short hypervariable
(HV) regions and a semivariable region which encodes the
N-terminal amino acids (77). Those two short hypervariable regions are responsible for most of the differences of PIIs (12). The restriction fragments containing only the HV regions cloned into an expression vector demonstrated that the epitopes recognized by specific PIIs monoclonal antibodies were completely encoded by either HV₁ or HV₂ (13).

PIIs are subject to extensive phase and antigenic variation (76, 77, 78). The genes are switched on and off nonrandomly at very high frequency. The average rate of change is about $2 \times 10^{-3}$ per cell per generation (48). This is too high to be explained by random mutation and reversion. This rate is not affected by the change in growth temperature, pH, amount of oxygen present, or state of piliation (48), but some substances such as pyruvate may induce the phenotypic change of GC (56, 57).

The expression of PIIs is controlled by a group of genes spread over throughout the gonococcal genome (77). At least nine expression loci (opa E) are involved in production of a large number of serologically distinct phenotypic variants. No correlation was found between the number of proteins produced and the number of opa loci; each locus may not be active in the production of a protein (93). The DNA sequence rearrangement that controls Salmonella phase variation may also contribute the rapid reverse phenotype changes in gonococci (12, 96). The recombination
among PII genes generates new coding sequences and contributes to the increase of structural variability in the PII family (12). Also, the genetic transformation of PII genes from a donor into a recipient was detected by MAbs (73). The DNA sequence rearrangement, PII genes recombination, and genetic transformations are thought to be responsible for the antigenic variation. Regulation of PII phase variation commonly occurs at the translation level (73, 77). A striking finding has been made when analyzing the opa-genes. All the opa-related genes appear to have a common 5'-coding sequence which includes a repetitive sequence, termed the coding repeat (CR). CR composed of pentameric CTCTT units. The number of the repeating units that code for the hydrophobic part of a leader or signal peptide is variable while all expression loci in a single cell are constitutively transcribed. However, the production of functional opacity proteins can only occur if the translational start codon ATG is in frame. The reading frame is controlled by the repeat sequence at translational level. Such repeat sequences can be spontaneously added or lost maneuvering the PII structural genes into or out of frame with ATG initiation codon so that either full-sized PII protein or a truncated protein is synthesized (77, 78). CR variation is thought to be the basic mechanism involved in phase and antigenic variation of PIIs expression (53, 58). Insertion and deletion of CTCTT repeats probably
involves slipped-strand mispairing of the DNA which occur during DNA replication or non-replication duplex. Changes could also result from unequal crossing-over among the signal peptide coding regions of different PII genes in the chromosome (53). Phenotypic changes of GC associated with variable PII expression occur not only in vitro but also in vivo during the course of gonococcal infection (60, 72, 89). Cultures from male urethrae tend to contain more opaque or (PII-bearing) colonies than those from female cervices (30). It was also found that cultures isolated at different anatomical locations have different predominant PII phenotypes. Even the cultures taken from the same location at different time intervals show different PII phenotypes. The phenotype of cultures taken from cervices depends on the stage of the menstrual cycle and whether or not the contraceptives have been used (30, 85, 90).

The function of PIIs has not been definitively determined. But this group of proteins has been characterized by:

1. Heat-modifiability: It is a very specific characteristic of PIIs and has been used to recognize PII bands on an SDS-PAGE gel. After heat-modification (solubilized sample at 100°C), PIIs migrate more slowly in SDS-PAGE gel. The PII bands, appeared on the SDS-PAGE gel with the cells solubilized at 37°C disappeared when solubilized the cells at 100°C, and the new bands with larger molecular mass
appear instead (88).

2. Relationship to colony opacity: In general the colonies are transparent when the organism lacks expression of PIIs. Different PII types have been correlated with distinctive degrees of colony opacities (88), however some PIIs do not show a discernible increase in colony opacity when present alone or in combination with other opacity-associated PIIs (30, 84, 85). Colony opacity is also thought to be a very reliable criteria used to select the different PII variants.

3. Virulence association: Mucosal isolates tend to have PIIs, while isolates from the blood always lack PIIs (46). The organisms expressing PIIs have been shown to be less virulent in the chicken embryo-model than those lacking PIIs (66).

4. Sensitivity to protease: PIIs are more sensitive to hydrolysis by trypsin than the major outer membrane protein (PI) of GC (85). It is of interest that the other outer membrane proteins in organisms containing PIIs are more sensitive to hydrolysis by trypsin than in those organisms which do not contain PIIs (5, 30, 84, 85).

5. Interaction with neutrophils: Most but not all PIIs can stimulate neutrophil respiratory burst and phagocytic killing. This ability of protein II bearing gonococci is mediated by lectin-like components, including a number of simple monosaccharides and sugars (62). PII-lacking GC adhered poorly to neutrophils and the GC were not killed at
the same rate as PII-bearing bacteria (16).

6. Ability to adhere to diverse types of eucaryotic cells: PIIIs appear to be important in attachment to the diverse eucaryotic cells (16, 45, 65). Colonies possessing PIIIs tend to clump together while colonies not expressing PIIIs are less adherent to each other (84). MAbs against PII can specifically inhibit the attachment of GC to Hela cells (81) helping to prevent infection. The interaction of PII with host cell surface components has been studied, showing that the predominant binding interactions were with proteins rather than carbohydrates. The chemical nature of interaction is very complex, involving more than purely ionic or hydrophobic forces (3).

7. Change serum resistance: increased sensitivity to killing by normal human serum (NHS) has been shown to correlate with PII expression (29), but P. R. Lambden reported the opposite results in his experiments in 1979 (46).

It is likely that expression of distinct PIIIs in variants of GC helps them to evade host responses and to survive in different micro-environments.

Outer membrane PIII, a surface exposed protein which is present in all GC strains examined (87). It appears to be 30KDa to 31KDa daltons and to be biochemically and immunologically identical in all strains studied (36, 63). In marked contrast to the PI and PIIIs, no phase or antigenic variation has been noted in PIII. So it is likely that PIII
is a cross reactive antigen among all GC, but the antisera raised against whole JS1 could not immunoprecipitate PIII of JS3. This suggests that PIII is either non-immunogenic or non-antigenic in intact organisms (36). PIII is only present in outer membranes of fresh isolates and is labelled with \(^{125}\)I in outer membrane preparations (26). The functions of PIII are not known, however it is possible that this protein serves as a target for blocking antibodies found in NHS which block the bactericidal activity of NHS (35, 63). Protein III is closely associated with PI and may, therefore, contribute to the function of the OM pore formed by PI (63).

H.8 antigen, another antigenic determinant on the outer membrane of GC, is a lipoprotein with an unusual amino acid sequence (20, 79). It appears to be invariable in intrastrain variants (80) and it stains poorly with the common stains used for visualizing the proteins on SDS-PAGE (Silver and Coomassie Brilliant Blue) (10). These staining characteristics can be explained by its unusual amino acid sequence, rich in alanine and proline, and the lack of aromatic and sulfur-containing amino acids. It is protease-sensitive, heat-modifiable but is not modified by 2-ME (10). The functions of this antigen are not clear. It is only detected in pathogenic gonococci and meningococci, but it is not found in nonpathogenic Neisseria species. This suggests that H.8 antigen may be an important virulence factor of
Lipopolysaccharide (LPS) is an antigenic determinant found on the surface of GC and another variable factor on the OM (19, 51, 75). It appears to have a critical role in determining the sensitivity of gonococcus to NHS (21, 75). LPS interacts with membrane proteins and appears to influence the exposure of PI on the surface of bacteria (41). LPS varies in size, structure, and antigenic expression both among strains and within strains. The chemical composition and structure of GC LPS are largely unknown. Three antigenic determinants have been indicated on the LPS molecules of GC: the common, variable and serotype antigens (51). The common antigen is present on all LPS molecules of GC, whereas the variable antigen may or may not be present. At least six serotype antigens are found on the LPS of GC. Unlike the LPS in other gram-negative bacteria, the LPS of GC has very short or no "O"-antigenic side chains (51).

Resistance to killing by NHS is a common phenomenon of gram-negative microorganisms (91, 94) and an important determinant of virulence in at least some infections due to gram-negative bacteria; although much of the literature does tend to show a relationship between sensitivities to serum and the ability to cause infection. The precise role and relative importance of serum resistance at any stage of the infection process is not clear at present. The majority of
GC strains isolated from patients with DGI are resistant to serum-killing, whereas strains isolated from mucosal sides are often sensitive to serum-killing (67). Two distinct forms of serum resistance have been observed (49). The first (stable) form of serum resistance persists during prolonged repeat passages on agar and was lost only when colony morphology changed. The second (acquired) form of serum resistance was discovered by selecting survivors in serum containing media. The acquired form of resistance was rapidly lost during repeated passage on agar without any changes in colony type. The factor in the serum which appeared to be stable to heating at 56°C was thought to be responsible for the acquired form of resistance. After dialysis, resistance-enhancing activity was found in the low molecular weight (12KDa to 14KDa) diffusate of serum. This resistance was greatly reduced by prolonged or low temperature (8°C) incubation and enhanced by lowered PH (94).

Bactericidal activity of NHS against gram-negative microorganisms involves both antibody and complement, but surprisingly little is known about the target antigens and how the serum resistant strains evade the host defense system (93). Antigonococcal AB in NHS may result from the presence of commensal gram-negative bacteria or with nonpathogenic Neisseria found in the respiratory tract. This is supported by the observation that AB can be removed
by absorption with some gram-negative organisms (19). The result of AB binding to GC is the activation of complement cascade. The resultant C5b-9 membrane attack complex (MAC) causes the lysis of cells. Alternative activation pathways may also be involved. C4 is essential for alternative pathway activation. However, the C4-deficient serum can still activate complement, but at a slower rate (68). No difference exists in the amount of complement activated nor in the MAC formation between the serum sensitive and resistant strains (23, 32, 33). However, the MAC fails to insert properly into the membrane of serum-resistant strains (17). Joiner, et al (34), demonstrated that C5b-9 forms high molecular weight complexes with outer membrane constituents of the serum-resistant strains, but not with serum-sensitive strains. These high molecular weight complexes may be responsible for the failure of the MAC to insert into the OM properly in resistant strains. Recent research has shown that blocking ABs for some surface components, especially PIII (63), which protect those surface components from attack by the specific bactericidal antibodies, may also contribute to the resistance of killing by NHS.

LPS is the main target antigen for ABs in the NHS (19, 68, 69, 75). For many years, researchers have tried to establish the relationship between LPS and sensitivity to killing by NHS. Several characteristics of LPS, such as the
molecular weight variation and the difference in sugar composition, have been associated with serum-sensitivity, but no consistent correlation with a single variant has been found (68). The 3.6KDa LPS component is commonly expressed by serum-resistant strains in varying amounts. The antibody specific for this 3.6KDa LPS is not found in the NHS, but when this antibody is added to NHS, it will initiate the lysis of bacteria mediated by human complements (9). This might mean 3.6 KDa LPS is although immunogenic, but it is not antigenic.

PIAs are associated with serum-resistance (39). The higher the molecular masses of the PIAs are, the more sensitive the bacteria are to the killing by NHS. It is interesting that among bacteria having the same PI, transparent colonies (organisms bearing no PII) appear to be more resistant to serum killing than opaque bacteria. Bacteria which survive in the presence of NHS tend to shut off PII expression and revert to transparent colonies (29). A genetic locus (sac-1) which has an effect on the level of serum resistance of GC was identified by Cannon, et al (9). This locus is closely linked to, but not identical with, a second locus (nmp-2) that affects PI.

Many surface components of GC are involved in the serum sensitivity. The contributions of each of these components to the total serum sensitivity is still unclear.
Overall Objective:

As mentioned above, the PIIs are important OM proteins of GC. They are associated with variation in virulence properties such as susceptibility to the bactericidal activity of NHS, attachment to diverse eucaryotic cells, susceptibility to phagocytosis and protease. Therefore, they may play an important role in GC infections.

This study proposes to study seven well characterized strains of GC (WS1, WS2, WS3, WS4, WS5, FA102, FA899) to elucidate the pattern of their PII expression. I will try to determine which PIIs are expressed and how many PIIs are produced in each strain. Since some strains (FA102 and FA899) are parent strains of others, it will be of interest to find out the relationship of PII expression in the parental and their derivative strains. I will then attempt to determine the relationship of PII expression to resistance and sensitivity to killing by NHS in WS1, WS2, WS3, WS4, WS5, FA102, and FA899. Results should help us to understand the role that PIIs play in serum resistance and propagation of GC infection in general.

Research Goal

1. Determination of PII types expressed in a set of seven transformant strains of *N. gonorrhoea*.

2. Comparison of the expression of PII of a parent stain with that of its derivative strains of *N. gonorrhoea*.
3. Correlation of PII expression with PI and LPS.

CHAPTER II

MATERIALS AND METHODS

**Bacteria:** Seven strains of *N. gonorrhoea*, which have been well characterized in our lab, were used in the experiments. Table 1 details the origins and serum killing phenotypes of these strains (Results from Robin K. Pettit's experiments in our lab). The reason that I chose those particular seven strains, is that they have been well characterized in our lab and they are all genetically inter-related (transformants). FA102 and FA899 are general parent strains of five other strains WS1, WS2, WS3, WS4, WS5. Four out of seven (FA102, WS1, WS3, WS5) have PIA and the other three (FA899, WS2, WS4) have PIB. The pictures of their PII expression will give us clear information about genetic events that happened when different PIIs were expressed. These characterized strains could be used to establish the role of PII molecules in a variety of cellular functions.

PI is an important factor that affects the sensitivity to serum killing, but it is not the only factor. The other surface components such as LPS and PIIs also change the serum sensitivity. Table II (on page 21) shows protein I type, migration of LPS, and H.8 antigen of these seven
strains (41).

It is interesting to compare the variants which have the same PII expression with different PI backgrounds, and also the variants having the same PI background, but with different PIIs expressed.

The strains were grown on the modified clear typing agar as previously described (84) at 37°C in 5% CO₂ atmosphere. Single colonies were selected and passed each day to ensure that pure cultures of each colony type were used. Colonies were chosen on the basis of color and opacity characterized by the criteria of J. Swanson (88) and verified by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Electrophoresis:** Colonies with various opacities which reflect PII-types, were harvested from solid medium into 1.5ml of complete Dulbecco phosphate buffered saline pH 7.4 (dPBS) and the absorbance was read with Coleman Junior II spectrophotometer. The suspensions were adjusted to optical density (OD) 0.68 at 600nm, and centrifuged at 10,000 R.P.M for 1 min. The pellets were solubilized in SDS solubilizing solution containing 4% of 2-mercaptoethanol at 37°C and 100°C as previously described (12). The whole cell lysates were separated by electrophoresis on an SDS-PAGE (12.5% acrylamide) for about 2 h at 10W constant power. The gels were then fixed in 7%
acetic acid -25% isopropyl alcohol (IPOH) overnight and stained with 0.2% Coomassie Brilliant Blue (CBB). The molecular mass of each PI and PII was determined on the gel by comparison with molecular weight markers (Bio-Rad low MW marker standards kit). In addition, silver staining was performed on 15% acrylamide with double cross linkage (1:16) SDS gel, as described Tsai and Frash (92) to show the LPS patterns of different variants in each strain.

**Microscopy:** A stereo microscope (Stereo Zoom 7 Bausch & Lomb, Rochester NY) equipped with a substage reflector having both a diffusing surface and a plane polished mirror was used for discerning the color and opacity characteristics of gonococcal colonies.

**Serum:** Pooled normal human serum of healthy donors having no history of infection with GC (the generous gift from Dr. William M. Shafer, Department of microbiology and immunology, School of Medicine, Atlanta, GA. 30322) were stored in 1ml portions in micro-centrifuge tubes at -70°C until use. The serum was thawed at room temperature immediately before use and used only once.

**Isolation of PII variants:** Colonies showing varying opacity were restreaked and grown for 18-20h on clear typing medium. Colonies from those plates were harvested, solubilized, and separated by 12.5% of SDS-PAGE gel and stained for identification of PII expression. Bacteria from the same plate were used for the serum killing assays.
TABLE I:

<table>
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<tr>
<th>STRAIN</th>
<th>SOURCE</th>
<th>PERCENT KILLING ± S.E.M.</th>
<th>SERUM-KILLING PHENOTYPE</th>
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<td>FA899 W. Shafer1; FA889xFA19</td>
<td>2.4 ± 8.1</td>
<td>SR</td>
<td></td>
</tr>
<tr>
<td>FA102</td>
<td>:FA48xFA19</td>
<td>12.4 ± 6.8</td>
<td>SR</td>
</tr>
<tr>
<td>WS1</td>
<td>:spontaneous pyocinR</td>
<td>102.4 ± 0.0</td>
<td>SS</td>
</tr>
<tr>
<td></td>
<td>mutant of FA102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WS2</td>
<td>:FA899xWS1</td>
<td>98.4 ± 0.0</td>
<td>SS</td>
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<td>102.4 ± 0.0</td>
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<td>SR</td>
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</tbody>
</table>

1 - Dr. William M. Shafer, Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, and The Veterans Administration Medical Center, Decatur, GA 30033

2 - Percent decrease in cfu with 25% pooled normal human serum (PNHS) ± standard error of the mean (S.E.M.)

3 - Serum resistant (SR) - >50% survival in 25% PNHS; Serum sensitive (SS) - ≤50% survival in 25% PNHS
Bactericidal Microassay: The microbactericidal assay, a method to determine serum bactericidal activity, performed as described by Schneider and Griffiss 1982 (47, 67, 94), was used in this experiment to determine the serum sensitivity of all the PII variants in seven strains of GC. As mentioned before, the cells tended to adhere together when they express PII. We faced the problem of the cells sticking together to form the colonies on the plates while performing the dilution plate assay (a common and reliable method used to determine serum sensitivity of bacteria by many scientists). The accuracy of results was largely affected by this characteristic of PII-bearing cells. We tried several methods in order to solve this problem: I. To remove GC aggregates, the suspensions were homogenized by vortex mixing and then centrifuged at 500g for 10 min before performing the dilution plate assay which was suggested by P.R. Lambden (46). II. The sonicator was used to separate the cells. None of these methods worked very well. The first method did not separate the cells any better; the irregular shaped colonies which formed from more than one cell still appeared on the plates. Under the second method, if the suspensions were sonicated less the 5 min it did not affect the cell homogenate, but more than 5 minutes would largely reduce the cell counts, indicating some cells were killed in the process. The bactericidal microassay avoids the requirement for accurate counting of the colonies, so
**TABLE II:**

Protein I type, migration of LPS and H.8 antigen of seven strains of GC used

<table>
<thead>
<tr>
<th>Strain</th>
<th>P.I type</th>
<th>LPS migration</th>
<th>H.8 migration</th>
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<td>FA899</td>
<td>P.IB9</td>
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<td>Intermediate</td>
</tr>
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<td>FA102</td>
<td>P.IA1</td>
<td>Intermediate</td>
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<td>P.IA1</td>
<td>Fast</td>
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<tr>
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<td>P.IB9</td>
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<td>Fast</td>
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<tr>
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<td>P.IA1</td>
<td>Fast</td>
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</tr>
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<td>P.IB9</td>
<td>Slow</td>
<td>Slow</td>
</tr>
<tr>
<td>WS5</td>
<td>P.IA1</td>
<td>Slow</td>
<td>Slow</td>
</tr>
</tbody>
</table>


the results were not affected by the adhering characteristic of PII-expressing cells. In this particular experiment, the microassay method appears to be more appropriate than the other serum bactericidal assay. Also in our lab, Robin Pettit compared the dilution plate assay with the microassay. Although little correlation has been found between these two assays in her experiments, it has been shown that the microassay was still a rapid, repeatable and reliable method to determine serum bactericidal activity. So we used the serum microassay instead of the dilution plates assay for serum bactericidal experiments.

Eighteen hour cultures of bacteria were suspended in the clear typing medium lacking agar (76) and the organisms were dispersed by vortexing. The suspensions were adjusted to 0.30 optical density at 600 (about $3 \times 10^8$ CFU/ml) with Coleman Junior II Spectrophotometer. Serum (0.2 ml) was diluted in serial 2-fold dilutions (1:4 to 1:4096) with clear typing broth in a sterile, flat-bottomed 24 well tissue culture micro-titer plate (Corning Glass Works, Corning, NY). The serial two-fold dilutions of 0.2 ml of serum in clear typing broth were also made in sterile 24 well tissue culture microtiter plates (Corning Glass Works, Corning, NY). An equal volume of clear typing broth containing about $3 \times 10^6$ cfu/ml was added to each well. The plates were incubated at 37°C for 45 min before overlaid each well with 0.3 ml of molten agar equilibrated to 52°C.
All the test plates were then incubated at 37°C for about 20 hours. The bactericidal titers were determined by the last serum dilution which killed 100% of the bacteria. The controls included incubating the bacteria in both clear typing broth without serum and heat inactive NHS (HINHS).
CHAPTER III

RESULTS

General Comments:

A single transparent (non-opaque) colony of each strain lacking any detectable PII was selected and passed. During the passages, we observed that the majority of colonies in the culture had the characteristics of their parental colonies which were transparent and did not express any kind of PIIs (O'). However, there were occasional variants showing different colony opacity in the culture. Usually the colonies of those variants displayed from an intermediate (O') degree to a marked (O'') degree of opacity. These desired colonies with different opacity were selected and cultured. As previously stated, the colony opacities were often associated with different PII expression. A sample containing a mixture of opacity variants could contain organisms expressing different PII species. Also, organisms were capable of changing their colony opacity which generally indicated a change in the PII expression during the passages. Of course, the results from a heterogeneous PII population mixture culture would be difficult to interpret, as they possibly were contributed by
any PII variants or all of them within the mixed culture. For these reasons, the colonies of different variants were examined very carefully with a dissecting microscope, using the criteria of J. Swanson (88) to ensure the right colonies were picked and passed.

Obtaining pure cultures of opacity (PII) variants in each strain was very important and was considered to be the first step of our experiments.

**PII Variants in Seven Strains of GC:**

PII variants from seven transformant strains, FA102 FA899 WS1 WS2 WS3 WS4 WS5, were selected for this study. The pure cultures of all the opacity variants in each strain were harvested by scraping plates and solubilized in the solubilizing solution with 2-mercaptoethanol at 60°C and 100°C. SDS-PAGE was performed with all the solubilized samples to identify the PIIs expressed by each variant. The PII variants were maintained by selective passage on clear typing medium each day. In addition, more PII variants were continuously selected over a six month time period.

The molecular masses of PIs and PIIs of each strain were determined by separating whole cell (WC) lysates in 12.5% or 15% acrylamide gels and comparing the migration to standard molecular weight markers. Whole cell lysates of each strain were then arranged on the 12.5% and 15% SDS gel based on the increasing apparent molecular mass of the PIIs when whole cells were solubilized at 37°C. Gels were
stained with 0.2% of CBB or silver, as described by Tsai and Frash (92).

The CBB stained 12.5% gel of the whole cell lysates of transparent colonies revealed that PIs in strains WS1, WS3, WS5, and FA102 migrated identically, having a molecular mass of about 36 Kd. The PIs in strains WS2, WS4, and FA899 also showed the identical migrations on the 12.5% CBB stained SDS gel with an the apparent molecular mass of around 37 Kd (Fig. 1).

The majority of the population in our stock cultures were transparent and, therefore, did not express PIIs. However, the opaque colonies occasionally arose from the transparent parents. Usually one or two distinct opacity variants were regularly selected among the progeny of transparent PII' colonies of each strain. The differences in colony opacities were due to the different PII expressed in these colonies, as illustrated in table III.

In the seven experimental strains, the transparent colony phenotype usually correlated with a PII' variant. The only exception was in the strain FA899, where no truly transparent phenotype colonies could be found within the experimental period of more than a half year. Therefore, a PII' variant in FA899 was selected from the colonies which had intermediate opacity (O+). In all other strains, the intermediate (O+) colony opacity was associated with a single PII that had a molecular mass of about 25.2 Kd. But
the variant expressing the 25.2 Kd PII protein in strain FA899 appeared to have marked (O++) colony opacity. The rest of the PIIs selected from seven transformant strains imparted marked opacity (O++) to the colonies. Unfortunately, it was not possible to predict the particular PII types based on the O++ phenotypes. Therefore, several O++ colonies were selected, solubilized, and separated on the SDS-PAGE gel in order to determine the PII types present in each colonial isolate. The expression of different PIIs was verified in those colonies that appeared to have the O++ phenotype by the SDS-PAGE.

The following results were obtained for each GC strain used in this study:

Strain WS1: Fig. 2 show the different PIIs in strain WS1 in a 12.5% SDS gel stained with CBB. The LPS type of each variant was determined by separating the whole cell lysates in the 15% double crosslinker SDS gel and staining with Silver.

Fig. 2 A is a CBB stained gel of lysates of strain WS1. The PI of each variant appears to be identical on the gel. We isolated six PII variants in WS1. One had no PIIs (lane 1); three of them possessed only a single PII protein (lanes 2, 3, 4); and the other two expressed two PII proteins each (lane 5, 6). The molecular masses of the PIIs were estimated by comparison with standard molecular weight markers. Among the variants, we noticed PII protein bands
of four distinct electrophoretic mobilities. They are 24.8, 25.2, 25.6, 26 Kd PIIs. All of the PII proteins were heat modifiable. At 100°C, the PII bands seen at 60°C (left) disappeared and the new bands appeared with higher molecular mass around 30 Kd. Lane 1: no PII band was seen. Lane 2: one PII band with a molecular mass of about 25.2 Kd appeared. Lane 3: the PII of this variant was about 25.6 Kd. Lane 4: the PII of this variant was about 26 Kd. Lane 5: two PII bands appeared. One was 25.6 Kd, the same as in lane 3, and the second had molecular mass of about 24.8 Kd. When heated at 100°C this 24.8 Kd PII band disappeared and a new band with molecular mass 28 Kd appeared. Lane 6: had two PII bands also. They were 26 and 25.6 Kd (same as lane 4, and lane 3).

In WS1, we found that the transparent colonies did not express any PIIs. The intermediated (O+) colony opacity was associated with 25.2 Kd PII. The rest of the PII variants showed the same marked (O+++) colony opacity. No obvious differences in colony opacity were observed among these marked (O+++) colonies which were verified to express different PIIs on the SDS-PAGE gel.

Fig. 2 B shows the LPS pattern of PII variants in WS1. No differences were found in LPS among the PII variants.

WS2: Like WS1, four distinctive PII protein bands were detected by 12.5% SDS gel with CBB staining (Fig. 3 A). At 60°C, from left to right. Lane 1: no PII. Lane 2: 24.8 Kd
PII. Lane 3: 25.2 Kd PII. Lane 4: 25.6 Kd PII. Lane 5: 26 Kd PII. Lane 6: two PIIs 26 Kd and 25.6 Kd. All PIIs were heat modifiable in WS2. The 24.8 Kd PII band at 60°C moved to 28 Kd area at 100°C. Other PIIs moved to around 30 Kd.

In WS2, the transparent colonies did not express any PIIs. Expression of the 25.2 Kd PII displayed intermediate colony opacity and the remaining PIIs -24.8, 26- Kd had marked colony opacity.

No differences in LPS were detected among the PII variants in WS2 (Fig. 3. B).

WS3: Five PII variants were selected with different PIIs expressed. One had no PIIs. Three of them expressed a single PII protein while another one expressed two PIIs at same time. In sum, three PII proteins (24.8, 25.2 and 26 Kd) were found in WS3.

Non-PII expressing colonies were transparent while the expression of a 25.2 Kd PII resulted in intermediate colony opacity. Expression of the other two PIIs (24.8 and 26 Kd) resulted in marked colony opacity. No difference in opacity could be distinguished between them, but 12.5% SDS gel with CBB staining verified that different PIIs were expressed in these two variants.

No differences in the LPS were found among the PII variants of WS3 in a 15% double crosslinker SDS gel with silver stain.

WS4: Four distinctive PII bands were found with
molecular masses around 25.2, 25.6, 26 and 26.7 Kd in strain WS4 (Fig. 5 A). As with the other strains, transparent colonies expressed no PIIs. The expression of the 25.2 Kd PII resulted in an intermediate colony opacity. The other PII-expressing colonies had marked colony opacity.

A 15% double crosslinker SDS gel, stained with silver, revealed unexpected differences in the LPS expressed among the PII variants. Lane 1 and lane 4 were non-PII and 26 Kd PII expressing variants. Respectively, the LPS of these two variants migrated more slowly than the LPS of the rest of the PII variants on the gel.

To further confirm the observed LPS differences in this strain, the new cell isolates were selected from the stock culture that expressed the same PIIs. The new samples from these isolates were analyzed by SDS-PAGE. The results were the same, as shown in Fig. 5 B.

WS5: Fig. 6 A shows that only two PIIs types were expressed by WS5. The apparent molecular masses of these two PII are about 25.2 Kd (Lane 2) and 26 Kd (Lane 3).

The LPS among the two PII variants in WS5 appeared to be the same on the 15% double crosslinker SDS gel stained with silver (Fig. 6 B).

FA102: Four types of PII variants were selected from FA102 (Fig. 7 A). One did not express any PIIs, the others expressed either the 24.8 Kd, 25.2 Kd, or 26 Kd PIIs. As demonstrated in the other strains, the PIIs in FA102 were
also heat modifiable. The 24.8 Kd PII band (at 60°C) moved to 28 Kd (at 100°C). The other PII bands moved to the area around 30 Kd (at 100°C).

In FA102, transparent colonies did not express any PIIs. The 25.2 Kd PII-expressing colonies had intermediate (O') colony opacity and the 26 Kd PII was associated with marked (O'') colony opacity. No differences in LPS were detected among the PII variants in FA102 on the 15% double crosslinker silver stained gel (Fig. 7 B).

FA899: Six PII variants were found in strain FA899 (Fig. 8 A), but only two colony opacities were found in this strain: the intermediate (O') and marked (O'') colony opacities. No transparent colonies were ever observed in our experiments within a time period of more than six months. The PII variant was selected from intermediate opacity colonies this was unusual, since the non-PII expressing colonies were transparent in the other six experimental strains. The other PII variants all showed marked colony opacity.

Four distinct PII bands were recognized on the CBB stained gel. These PIIs appeared in the variants in different combinations. Four variants possessed only one PII each, 25.2, 25.6, 26 and 26.7 Kd, while the other two variants expressed two PIIs in each variant at the same time. No difference was observed in the LPS among the variants in FA899 with silver stained 15% double crosslinker
SDS gel (Fig. 8 B).

Table III summarizes the PII-expression of the seven strains used in this study.
Figure 1:
Separation of WC lysates of seven strains of N. gonorrhoea in 12.5% polyacrylamide gel

This Coomassie Brilliant blue (CBB) stained 12.5% SDS gel shows the migration of the PIs in the seven experimental strains. The major outer proteins (PIs) from strains, WS1, WS3, WS5 and FA102 migrated to same position in the gel, having a molecular mass around 36 Kd. The PIs from strains WS2, WS4 and FA899 also migrated to the same position in the gel, having a molecular mass of about 37 Kd. Molecular mass markers (MW) are expressed in thousands of daltons.
FIGURE 2:
SDS-PAGE profiles of strain WS1 opacity variants

Whole cells of each PII variant were solubilized at either 60°C and 100°C and separated in 12.5% SDS gels in the order of increasing apparent molecular mass of PII molecules at 60°C.

A 12.5% SDS gel of 6 colony isolates (Lanes 1-6) were solubilized at either 60°C or 100°C and stained with Coomassie Brilliant blue (CBB) (Fig. 2 A). The major outer membrane protein PI and the outer protein IIs are identified as (PI) and (PII) on the gel.

A 15% double crosslinker SDS gel was stained with silver (SIL) to reveal Lipopolysaccharide (LPS) migration Fig. 2 B). Molecular mass markers (MW) are expressed in thousands of daltons.
FIGURE 3:

SDS-PAGE profiles of strain WS2 opacity variants

A 12.5% SDS gel stained with Coomassie Brilliant blue (BBC) shows the different PIIs in each PII variant (Fig. 3 A). The major outer membrane protein PI and outer membrane II are identified as (PI) and (PII) on the gel.

Whole cell lysates was separated in 15% double crosslinker SDS gel and stained with silver (SIL) to show the lipopolysaccharide (LPS) among the PII variants (Fig. 3 B). Molecular mass markers (MW) are expressed in thousands of daltons.
FIGURE 3

SDS-PAGE profiles of strain WS2 opacity variants

The whole cell lysates of each of the variant strains were
prepared and at two different temperatures, 60°C and
100°C, and then separated on the gel with a molecular
weight marker.
FIGURE 4:

SDS-PAGE profiles of strain WS3 opacity variants

The whole cell lysates of each PII variant were solubilized at two different temperatures (at 60°C and 100°C) and then separated on the 12.5% SDS gel (Fig 4 A). The gel was stained with Coomassie Brilliant blue (CBB). The major outer membrane PI and outer membrane II are identified as (PI) and (PII) on the gel.

The 15% double crosslinker SDS gel was used to separated the whole cell lysates in strain WS3 to show lipopolysaccharide (LPS) (Fig. 4 B). Gel was stained with silver (SIL). Molecular mass markers (MW) are expressed in thousands of daltons.
FIGURE 5:

SDS-PAGE profiles of strain WS4 opacity variants

The whole cell lysate of each PII variant, prepared at 60°C (on the left) and 100°C (on the right), was loaded to 12.5% SDS gel (Fig. 5 A). We visualized the protein bands on the gel by staining with Coomassie Brilliant blue (CBB). The major outer membrane protein PI and outer membrane protein II are identified as (PI) and (PII) on the gel.

The 15% SDS gel separated the whole cell lysates of the PII variants in strain WS4 with silver stain (SIL) to show the lipopolysaccharide (LPS) (Fig. 5 B). Molecular mass markers (MW) are expressed in thousands of daltons.
FIGURE 5
SDS-PAGE profiles of allele RSP quality variants.

The samples of each PI variant in strains 1-6 were separated on the 12.5% SDS gel (Fig. 5A and B).
FIGURE 6:
SDS-PAGE profiles of strain WS5 opacity variants.

The samples of each PII variant in strain WS5 were separated on the 12.5% SDS gel (Fig. 6 A). The gel was stained with 0.2% Coomassie Brilliant blue (CBB). The major outer membrane protein PI and outer membrane protein are identified on the gel as (PI) and (PII).

Lipopolysaccharide (LPS) showed on a 15% double crosslinker SDS gel with silver stain (SIL) (Fig. 6 B). No differences are seen among the PII variants in WS5. Molecular mass markers (MW) are identified in thousands of daltons.
FIGURE 6

Protein analysis of WS5 cells. Panel A shows SDS-PAGE analysis of cell lysates from WS5 cells incubated at 60°C and 100°C. Panel B shows a Western blot analysis of the same samples. The molecular weight (MW) markers are indicated at the left side of each panel. The bands at -94, -68, -44, -30, and -21 are labeled as PI, PI1, SIL, and LPS, respectively.
FIGURE 7:
SDS-PAGE profiles of strain FA102 opacity variants.

Coomassie Brilliant blue (CBB) stained 12.5% SDS gel show the different PII bands in each variant in the strain FA102 (Fig. 7 A). The abbreviations of (PI) and (PII) on the gel are identified as major outer membrane protein and outer membrane protein II.

A double crosslinker SDS-PAGE gel with silver stain (SIL) show the lipopolysaccharide (LPS) in each PII variant (Fig. 7 B). Molecular mass markers (MW) are expressed in thousands of daltons.
FIGURE 8:

SDS-PAGE profiles of strain FA899 opacity variants.

A Coomassie Brilliant blue (CBB) stained 12.5% SDS gel, showing the PIIIs in the PII variants in strain FA899 (Fig. 8 A). The abbreviation of major membrane protein and outer membrane II are identified as (PI) and (PII) in this Figure.

A silver stained (SIL) 15% double crosslinker SDS gel shows migration of lipopolysaccharide (LPS) in the PII variants (Fig. 8 B). Molecular mass markers (MW) are expressed in thousands of daltons.
Table III:
The capacity (PII) variants of seven strains of GC

FIGURE: 8
Table III:

The opacity (PII) variants of seven strains of GC

<table>
<thead>
<tr>
<th>PII variants Mol. MasPII</th>
<th>WS1</th>
<th>WS2</th>
<th>WS3</th>
<th>WS4</th>
<th>WS5</th>
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</table>

*: All the samples were prepared at 60°C and separated on a 12.5% of SDS gel with CBB stain. The molecular masses of PIIIs in each variant were estimated by comparison with standard molecular weight markers.

*: The variants express two PII types.
The serum sensitivity of PII variants in seven strains of GC

There is no single technique which has emerged as a standard assay for determination of serum activity. The bewildering array of assays made meaningful comparison among studies very difficult. Several assays have been generally applied to determine the serum sensitivity of gram-negative bacteria. One most commonly used method is dilution plate assay; another is the microbactericidal assay (microassay). In our study, we chose the microassay method, because of the advantages for our particular experiments with PIIs as discussed in the materials and methods section.

The NHS serum killing titers (the last serum dilutions that kills 100% of bacteria) for the seven strains used in this study were determined using the microassay in a 2-fold serum dilution system. The results of serum sensitivities for these transformant strains are shown in figure 9. Strain FA899 was the most serum resistant strain of all with a bactericidal titer 8 (100% bacteria was killed at 1:8 serum dilution). The WS5 appeared to be the most serum sensitive strain with a bactericidal titer 526. WS2 was quite serum sensitive with a titer 256. The rest of the strains were comparably serum resistant, FA102 and WS4 had the titer 128. The WS1, WS3 had the titer 64.

No differences were found in the serum sensitivities among the PII variants. As the results stated, the same titer was observed in all the PII variants as was seen in
the transparent colony types. No differences existed, which seemed unlikely, since previous studies by several groups (42, 71) had demonstrated the differences in serum killing that correlated with PII-expression.

The serum dilutions in the experiments were 2-fold. The differences in serum dilution between each well became much larger as the dilution increased. Serum dilution of the first well was 1:4 and was 1:8 in the second well; the difference in serum dilution between those two wells was 1:4. The difference between well 7 and 8 was 1:256, while the difference between wells 10 and 11 was 1:2048. The titers of most of our experimental strains, determined by microassay, were quite high. This meant that the difference in serum dilution between each well near the titer area was quite large. Therefore, modest differences in killing might not be seen using 2-fold serum dilution. To solve this problem, progressively smaller dilutions were made around the titer for all variants of each strain. A more accurate new titer was found each time. Using the new titers, we made dilutions around them even further. As a result, the differences in the serum sensitivity among the PII variants became apparent (Table IV).

With the individual serials smaller serum dilutions, the differences in the serum sensitivities among the PII variants have been shown (Table IV). These differences in the serum sensitivity are well correlated with the different
PIIs in each variants. The transparent colonies have the highest serum titer, and the different isogeneic opaque colonies have lower titers. These differences are only associated with PIIs expressing, not with the PIIs and LPS changes.

The HINHS, used as a control for all the experiments, was prepared by the standard method. The complement activity in the HINHS has been abrogated by heating to 56°C for 30 minutes. The results are shown on Figure 9.

In general, the bactericidal activities of NHS are mediated by the complement. In HINHS, the complement was inactivated by heating. The bactericidal activity of this serum should abrogate with the inactivation of the complement. The results from our serum test are quite unexpected. The HINHS has almost the same killing as the NHS (Fig. 9).
FIGURE 9:

Microtiter bactericidal assay results from seven gonorrheal strains using 2-fold serum dilutions. The titers are the reciprocal of the last dilution killing 100% of the test strains. The same titer was observed for all the PII variants in each strain with the 2-fold serum dilutions system. The titer for each PII variant appeared to be the same.

The bars are the mean of duplicated samples. The hatched bars indicates the pooled normal human serum and the solid bars indicate the heat-inactive pooled normal human serum (HINHS).
No differences have been found in serum sensitivities among the PII variants with the 2-fold serum dilution system. The smaller serum dilutions have been made around the titers that observed from 2-fold dilution for each strain.

Eleven smaller serum dilutions were made in WS1 around its bactericidal titer (64) from 1:36 to 1:116 with 1:8 serum dilution difference between each well. The new Titers for each PII variant in WS1 from the smaller serum dilution system are shown in table IV. From results, we have noticed that the non-PII expressing transparent colonies tend to have the highest titer (76), and the rest of variants have titers from 60 to 68. One PII variant with 25.2 Kd PII has the same titer (76) as the transparent colonies.

The bactericidal titer of WS2 using the microassay in 2-fold serum dilutions was 256 (Fig. 8). Eleven smaller serum dilutions were made around the titer from 1:128 to 1:562 with 1:35 difference between each dilution. Another new titer (268) was found, but still no differences have shown in serum sensitivity among the PII variants.

Another set of 11 smaller serum dilutions was made around the new titer (268) from 1:248 to 1:328. There was 1:8 serum dilution difference between each well. With this secondary smaller serum dilution, the differences in the serum sensitivities among the PII variants finally appeared (table IV). We have noticed in WS2 also that the
transparent colonies tend to have the highest titer (296) among the PII variants. It exhibited that the transparent colonies were killed at greater serum dilution than that of other PII variants which means the transparent colonies are less resistant to the NHS than the rest of the PII variants.

The serum titer for WS3 was 64 in the 2-fold serum dilution. Abundant growth was observed in the next well that had serum dilution 1:128. This suggested that the most accurate titers for PII variants could be located between 64 and 128, and closer to 64.

A set of 11 smaller serum dilutions was made from 1:32 to 1:116 with an equal dilution difference of 1:8 between each well. The titer for transparent (no PII) and 25.2 Kd PII variant was 76, and the other two PII variants was 68.

We tried to determine if more differences could be found among the PII variants in WS3. The eleven even smaller serum dilutions were made from 1:60 to 1:80 with only 1:2 serum dilution difference between each well. Differences in killing were observed (table IV). The titer for transparent colonies was 68, the other 25.2 Kd PII variant had the same titer as the transparent (no PII) colonies. The rest of PII variants had lower titers than the transparent colonies.

Eleven smaller serum dilutions were made in WS4. They were around titer 128 (Fig. 8) with the dilutions from 1:114
to 1:194 and 1:8 apart. Results are shown on the table IV. The trans-parent colonies have the highest serum titer (194) among the PII variants. The other PII variants have lower serum titers than the no PII expressing colonies. Further dilutions were also made, but no more differences have appeared.

For WS5, the titer in 2-fold dilution was 256. At this point, no difference in the serum sensitivity has been found among the PII variants. Therefore, eleven smaller dilutions have been made from 1:206 to 1:306. New titers for each variant are shown on table IV. Like other strains, the highest serum titer (286) has appeared in the transparent colonies in WS5.

The strain FA102 had the same bactericidal titer as strain WS4 (Fig. 8) with 2-fold serum dilution. The same secondary 11 smaller serum dilutions were made as in WS4. The titers are shown on table IV. The highest serum titer (154) was also with the transparent colonies.

Strain FA899 was very serum resistant in microasssy. The titer was 8 with 2-fold serum dilution. We made 8 secondary serum dilutions from 1:4 to 1:32 and 1:4 apart. Secondary titers were found and showed on table IV. The highest serum titer in this strain was 20. The transparent phenotype colonies and a 26.7 Kd expressing variant had the same titer 20, and the rest of PII variants had lower serum titers.

60
Table IV:
The serum sensitivities of PII variants to NHS

<table>
<thead>
<tr>
<th>PII VARIANTS</th>
<th>Mol. Wt</th>
<th>WS1</th>
<th>WS2</th>
<th>WS3</th>
<th>WS4</th>
<th>WS5</th>
<th>FA899</th>
<th>FA102</th>
</tr>
</thead>
<tbody>
<tr>
<td>No PII</td>
<td>76</td>
<td>296</td>
<td>68</td>
<td>194</td>
<td>286</td>
<td>20</td>
<td>154</td>
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<tr>
<td>24.8Kd</td>
<td></td>
<td>280</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>25.2Kd</td>
<td>76</td>
<td>280</td>
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<td></td>
</tr>
<tr>
<td>25.6Kd</td>
<td>68</td>
<td>264</td>
<td></td>
<td>66</td>
<td>186</td>
<td>8</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>26Kd</td>
<td>60</td>
<td>264</td>
<td></td>
<td>162</td>
<td>266</td>
<td>8</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>26.7Kd</td>
<td></td>
<td></td>
<td></td>
<td>66</td>
<td></td>
<td></td>
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<td>25.2Kd*</td>
<td>288</td>
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<td>12</td>
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<tr>
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<td>25.2Kd*</td>
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<tr>
<td>26.7Kd*</td>
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<td>25.6Kd*</td>
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<td>186</td>
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<tr>
<td>26Kd</td>
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</table>

^: The bactercidal titers of seven strains were not determined under a standard serum dilution system as Fig. 9. The serum dilutions were made differently for individual strains, but the same serum dilutions were used for all the variants within a single strain.

*: The variants express two PII types
CHAPTER IV

DISCUSSION

Outer membrane proteins II (PIIs) are dominant variable surface structures of GC (6, 25). The functions of this group of proteins have not been definitively determined, but they are associated with pathogenesis (29, 66) and believed to be an important colonization factor (16, 45, 65). PIIs correlate with colony-opacity, and are called opacity-associated proteins (25, 31, 88, 90, 96). The roles PIIs play in GC infection appear to be quite complex.

In general, bacteria with transparent phenotype do not express any PIIs. Expression of different PIIs is associated with distinctive colony morphologies and opacities. Colony opacity is believed to be one of the reliable criteria in PII selection. However, the PIIs are not always associated with colony opacity. In strain JS1 (88), one PII protein, called PIIa, was not correlated with any colony opacities. The colonies expressing this PII appeared to be as transparent as the non-PII expressing colonies.

In the study of seven transformant strains of GC, we have selected the non-PII expressing variants based on the
transparent phenotype. Only strain FA899 failed to express a transparent phenotype colonies despite the absence of any PIIs. The non-PII expressing variant in this strain was selected from the colonies which have an intermediate opacity, as indicated in the results section. This suggests that besides PIIs the other components in the membrane, such as LPS, may have contributed to the colony opacity in this strain. In majority of our experimental strains, the 25.2 Kd PII variant generally impacted an intermediate colony opacity, but appeared to impact a marked colony opacity in FA899.

The factor(s) that could possibly affect colony opacity are most likely the variable structures on the membrane. It is unlikely that the PI subclass contributed to the colony opacity. The PI proteins of each strain showed no variation either in migration or concentration, regardless of the PII variation occurring in each strain. This was true whether the strains expressed PIA (WS1, WS3, WS5, and FA102) or PIB (WS2, WS4, and FA899) (Table II). Therefore, the factor(s) other than PIIs must contribute to the colony opacity.

LPS, a variable membrane structure, could possibly play a role in colony opacity. To determine if LPS contributed to opacity variation, a 15% double crosslinker SDS-PAGE gel was strained with silver to examine LPS in all PII variants. No difference in LPS was revealed in six strains with the exception of strain WS4, in which the LPS patterns in two
PII variants were different (Fig. 5 B), with the LPS in the non-PII expressing colony (Lane 1) and 26 Kd PII (Lane 3) being different from others. The LPS in these two PII variants moved slower than the LPS in the rest of the variants in this strain. However, no correlation could be found between LPS differences and the difference in colony opacity. If LPS had affected the colony opacity, it would likely have shown up in the non-PII expressing transparent colonies. We also has not found any differences in LPS among the PII variants in FA899 which never did express the transparent colony opacity phenotype. No changes have been observed in colony opacity in these colonies, indicating that LPS did not influence colony opacity. Therefore, it seems that LPS did not influence colony opacity in our experiments. Since we did not do extensive studies to identify LPS variation within our strains, it is still possible that LPS variation could influence colony opacity.

In WS1, four distinctive PIIs are found. They are 24.8, 25.2, 25.6, 26 Kd. WS1 was a spontaneous pyocin\(^R\) mutant of FA102 (Table I). In FA102, except for the transparent non-PII expressing colony, three PIIs, (25.2, 25.6, 26 Kd) were found. Comparing the PIIs in these two strains, one additional (24.8 Kd) PII has appeared in WS1 that has not been found in FA102. Since FA102 was considered to be the only parent of WS1, we assumed that the DNA sequence code for 24.8 Kd PII probably also existed in the FA102.
However, we have not successfully selected this PII.

The PII gene could exist in the genome of FA102 in the form of an unexpressed gene or it might exist as a partial gene, which could not express itself except by recombining with homologous sequences in another complete genes (seminar of J. Swanson). The reasons that the 24.8 Kd PII was not found in FA102 could possibly be: We simply missed this PII while we selecting the PII variants; special conditions might be needed for expression of this PII protein; or promoter of this gene was inactivated, so the gene can no longer to be expressed. It is also possible that a recombination event occurred in the DNA sequence of WS1 along with the mutation in the gene which controls the pyocin resistance. If so, the 24.8 Kd PII gene might have been created during the process. This raises several unanswered questions about the stability and expression of PII genes that are beyond the scope of this study.

WS2 has two parent strains, FA899 and WS1. Each parental strain has four PIIs. Three of them were common to both parents and were also expressed in the daughter strain WS2 (25.2 Kd, 25.6 Kd and 26 Kd). The other two were unique in each parental strain, with WS1 expressing a 24.8 Kd PII and FA899 a 26.7 Kd PII. The (24.8 Kd) PII in WS1 also has been seen in the daughter strain WS2. But the 26.7 Kd PII that was expressed in another parent FA899 has not been found in WS2.
PII genes from both parents could be transferred to the daughter strain. It is possible that the DNA sequence for this 26.7 Kd PII was transferred from the parent FA899 to daughter WS2, but due to present of suppressor, loss of correct promoter or intervening sequence was not expressed. Conversely, the sequence may not has been successfully transferred to the daughter gamone. This could be tested by several methods; We have learned, when bacteria are grown under different stresses, the cell can change its gene expression, as the cell under heat shock. Suppressed genes could be turned on and the others could be turned off. We can put strain WS2 under different stresses to see whether or not this 26.7 Kd PII can be expressed. The positive result would confirm the existence of the gene, but the negative result does not necessarily mean the absence of this gene. Another new technique allows us sequencing of the PII genes in WS2 or specific probes can be used to detect the existence of the gene in the gamone.

All the PIIs expressed by WS2 were also expressed in its parental strains. No new PIIs were found in the progeny (WS2) that were not also found in the parental strains (FA899 or WS1).

WS3 has the same parents (WS1 and FA899) as WS2. In WS3, three PIIs have been selected. They are 25.2, 25.6, and 26.7 Kd. The 26.7 Kd PII expressed in parent FA899 that has not found in one daughter strain WS2 was shown up in
WS3. Two 26.7 Kd PII expression variants have been found in WS3, one express only 26.7 Kd PII and another express two PIIs, 26.7 and 25.2 Kd. It seems that 26.7 Kd PII was actively expressed in WS3.

Comparing the PII expression patterns in WS2 and WS3, the 26.7 and 24.8 Kd PIIs was not expressed in same strain. Another 26.7 Kd expression strain FA899, 24.8 Kd PII has also not found. The 24.8 Kd PII was expressed in WS4, 26.7 Kd PII was also not selected from this strain. This may imply a possible relationship between those two genes, expression of one gene inhibited the expression of another.

WS4 has WS2 and FA102 as the parental strains. In addition to the transparent variant (no PII), three (24.8, 25.2, 26 Kd) PIIs have been identified in WS4. All of them have also appeared in the parents.

WS5 has the same parents as WS4 as shown in Table I. Only two PIIs have been selected from this strain. Fewer PIIs were found in this strain than in either of its parents (WS2 and FA102).

Expression of PIIs is controlled by a group of opa genes, which are spread throughout the gonococcal genome (77). These genes can be turned on and off at a very high frequency depending on the number of CTCTT (RC) sequences in the leader sequence. The number of RC sequences causes a frameshift within the gene which can either put the PII gene in or out of frame with ATG initiation codon, so either a
functional PII protein or a truncated PII protein could be synthesized. The mechanisms that control the high frequency changes in the number of CTCTT sequences in PII mRNA are still poorly understood. They could possibly result from the slippage or stuttering of RNA polymerase as the genes are transcribed. The unequal crossing-over among the signal peptide coding regions of different PII genes that causes the insertion or deletion of RC repeat units could also be responsible for the changes in the number of RC sequences.

Using gene sequencing and common PII gene probe techniques, many more PII genes have been identified in the genome than the PIIs have expressed (77). This indicates a lot more PII genes remain in an inactive state in the genome. Different genetic movements have been detected among PII genes: Gene duplication and conversion generate new PII coding sequences and increase the structural variability in the proteins PII family (93); the genetic transformation of PII genes from donor to the recipient are confirmed by using MABs (73). These genetic movements could occur among genes within the genome, or between different cells, creating many opportunities for new PII coding sequences. However, our experiments did not identify any new PIIs expressed in the daughter strains that were not also expressed in parental strains. It is possible that new PII genes have been created but remain unexpressed in the genome. Those PII genes exist in the genome in an inactive
state (48) when the bacteria are grown under the certain conditions. The cells retain the ability to express those PIIIs when it becomes necessary. This could be confirmed by comparing PII expressing patterns of a single strain under different growth conditions. However, the mechanisms that prevent PII genes from being expressed under certain conditions and allow them to be expressed under others are still not well characterized.

From PIIIs expressing patterns of our experimental strains, we have noticed that almost all PIIIs expressed in daughter strains were also found in at least one of their parents. There were not as many as PIIIs expressed in each daughter strains, as we expected. It is likely that gene products from a particular gene could serve as suppressor preventing expression of another gene.

OM proteins II, the colony opacity associated protein on the surface of GC, are not the only structure that could contribute to the colony opacity. The possible involvement of LPS has not been confirmed in our experiments. The possibility of PII genes existing in an inactive state in the genome is proposed. Those PII genes can be expressed at different environmental conditions which could increase bacterial survival and help bacteria evade the host immune response. In efforts to evaluate whether many more PIIIs could be expressed in each strain, future experiments will observe the PII expression of strains under different growth
Serum resistance is a common phenomenon of gram-negative bacteria that has been recognized for many years. Surprisingly little is know about the molecular mechanisms involved. Like many gram-negative bacteria, differences in serum resistance have been observed among different strains of GC. Adding to the difficulties in determining molecular factors which contribute to serum resistance is the variability of results, depending on the bactericidal assay, the definition of serum resistance (indicated as 90% of survival, 80% of survival or 20% of survival), the serum concentration and the source of serum. As mentioned in the material and method section, we chose the microassay method instead of dilution plate assay method for the advantages of this method in our particular experiment.

Many studies have examined the bactericidal actions of NHS as a function of colony phenotypes (49, 60), OM protein I (29) and LPS (92), the important structures on the surface of the cell which have been associated with serum sensitivity. Our studies have been focused on another group of OM proteins (PIIs).

Previous research indicates that the proteins II on the surface of GC could affect the serum killing and might be one important factor in determining the serum sensitivity (29, 46). The serum sensitivities are determined by the presence of the bacterial surface structures which are
capable of interfering with the formation, attachment, and subsequent activity of the MAC formed by complements cascade (91). In our serum test, it seems that the bactericidal activity in the microassay measures both complement-mediated killing and a non-complement-mediated killing mechanism.

Differences in serum sensitivity of non-PII bearing bacteria has been determined in our seven transformant experimental strains (Fig. 9). Our data showed that strain WS5 was a very serum sensitive strain; it is counted as a serum resistant strain in table I (the results from dilution plate assay). WS4 and FA102 are serum resistant strains in table I, but appeared to be quite sensitive in our test. Those differences were caused by the different methods used to test the serum sensitivity. The serum concentration used and the time incubate bacteria with serum are quite different in these two methods.

Different serum titers have been obtained among the PII variants with the secondary smaller serum dilution. The transparent colonies (having no PII) tended to have higher killing titer than their isogeneic opaque colonies, indicating that transparent colonies were more sensitive to the NHS than opaque colonies. It suggests that expression of PIIs enhanced the ability of these strains to resist to killing by NHS.

Previous reports showed PI and LPS were both significant in determining the serum sensitivity (29, 75).
In our experimental strains, the PIs appeared to be invariable among the PII variants (identical migration on the SDS gel). The LPS shown on a 15% double crosslinker SDS gel with silver stain revealed no differences in LPS structure with the exception of strain WS4. In strain WS4, different LPS patterns were seen in non-PII expressing colonies and the variant express 26 Kd PII. However, these differences were not associated with differences in serum sensitivities. The transparent colonies were still the most serum sensitive among the PII variants. Therefore, LPS variation did not contribute to the differences in serum sensitivity in this strain. No conclusions can be made based on these LPS variations. As mentioned before, WS2 and WS3 have the same parents, a large difference in serum sensitivity has been observed with the same PII expression variants in those two strains. This suggests that PI or PI combining with LPS are important in affecting serum sensitivity among strains.

Our results demonstrated that variation in PII expression has contribute to the variation in serum sensitivity, and that PIIs rather than the LPS or PI-type contributed to the variations in serum killing within each strain.

The results from our microassay serum tests contradict a previous report which suggested the opaque colonies were less resistant to the NHS than the isogeneic transparent
colonies (29). However, our data supports another report which demonstrated that the expression of PII increased serum resistance (46). Results from our serum test indicated that the transparent colonies were more sensitive to the NHS than the isogeneic opaque colonies. We have found no relationship between a specific PII and the greatest serum resistance within our experimental strains.

We have used the heat-inactivated normal human serum (HINHS) as one of the controls in our experiment. Therefore, we did not expect to see significant killing with this serum with no complement activity, as we believed that the serum killing of gram-negative bacteria is mediated by the complement (68, 75, 91). The inactivation of the complement should abrogate the bactericidal activity of NHS.

The results from our serum tests with microassay indicated that HINHS was not only bactericidal to all the experimental strains, but also had almost as much killing as intact NHS (Fig. 9). In an effort to understand this phenomenon, a set of experiments were performed by using HINHS with human complement added back (data not shown). We hoped to determine if the complement played the same role in microassay as it did in other serum assays, or if other unknown mechanisms were involved in the killing in the microassay method. Surprisingly, no difference in serum killing has been observed using HINHS with human complement adding back, suggesting that the bactericidal activities in
microassay were mediated by mechanisms other than the activation of complement. The substance contributed to the serum killing in HINHS must be heat stable, since the bactericidal activity existed after heating the serum at 56°C for 30 minutes.

The same phenomena was also discovered by a colleague in our lab (61). She discussed two possibilities: The killing could be mediated by 1). toxic fatty acids or 2). specific Abs. The bactericidal capacity of HINHS (the c'-depleted serum) was not affected by doubling the amount of soluble starch in the medium which could bind to and neutralize toxic fatty acids, nor decreased by pre-absorbing the serum with the test strain of GC to get rid of specific Abs.

Besides the complement, toxic fatty acids and specific Abs, several other substances in the serum could possibly contribute the serum killing. A group of low-molecular-weight cationic proteins in the serum termed β-lysins possess lethal activity against gram-positive bacteria (91) and some gram-negative bacteria at very high concentration. β-lysins appear to be heat stable (52), and are released from platelets during the process of blood coagulation. It is believed that the primary site of action for β-lysins is the cytoplasmic membrane. No direct lethal action has been observed against gram-negative bacteria possessing an integral envelope structure. However, GC is
well know to have a "leaky" outer membrane structure; the outer membrane of GC was more permeable than the other members in the Enterobacteriaceae family (penicillin can penetrate OM and kill GC but not effect most gram-negative bacteria) (52). Perhaps, this explained our observation.

The data from our experiments provide direct evidence of the relationships among the PII variants and their isogeneic transparent colonies in the serum susceptibilities. Expressing of PIIs in the GC outer membrane could not only increase the attachment of bacteria to different host cells which help establish infection but also increase serum resistance which enhance bacterial survival in the internal environments. PIIs are important not only in GC infection but also in bacterial survival. Thus, serum killing in microassay appears unusual; potentially a group of heat stable low-molecular-weight cationic proteins in the serum may be involved.
SUMMARY

Outer membrane protein II (PII) of Neisseria gonorrhoeae is recognized by their ability to affect colony opacity. Our studies on expression of PII in seven strains indicate that PII is important in affecting colony opacity, but not the only factor. A variable surface structure, lipopolysaccharide (LPS) may play a role in colony opacity; however, result suggests that effects of LPS on colony opacity are minimal.

The serum sensitivities of different PII variants in each strain were examined. Despite the evidence that gonococcal resistance to normal human serum is multifactorial, the use of isogeneic variants shown OMP II is associated with considerable serum resistance.

The serum bactericidal microassay tests the different killing aspects of the normal human serum (NHS), it was revealed by using the complements abrogated serum (heat-inactivated normal human serum). This suggests that serum killing in the microassay method likely mediated by a group of heat stable low-molecular-weight cationic proteins termed β-lysins. These serum proteins released from platelets during process of blood coagulation.
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