CHAPTER I

INTRODUCTION

Today, gonorrhea is one of the most commonly reported communicable diseases that has been on the rise throughout in several areas of the world. In Thailand for the past twenty years, the incidence of gonorrhea has been climbing steadily. There are 236,544 cases of gonococcal infection (57%)recognized in 1986 by the Division of Venereal Diseases (1), the Ministry of Public Health, and the government must provide much money for the treatment and prevention of the disease each year.

<u>Neisseria gonorrhoeae</u>, or Gonococcus , is the etiological agent of gonorrhea, and the isolation and identification of this causative organism is essential for the diagnosis of the disease. The gram stained smears of exudates and culture are the standard diagnostic tests for this disease. The sensitivity of gram stain and culture in symptomatic men were 98% (2,3) and 95% (2) respectively. Thus, these methods are useful and reliable in evaluating male patients. In females, however , the usefulness of gram stain and culture are limited , their sensitivities ranging from 31-75.6 % (2,3) and 16-85 % (2,4,5,6) respectively. Hence the sensitivity is lower than that in male patients.

Moreover, the gram-stained smears of endocervic and vaginal material from females are often difficult to interpret, because of the numerous gram negative cocci present in the vaginal flora (4).

Several problems with culture for isolation of N. gonorrhoeae and subsequent confirmatory methods have been reported including loss of viable organisms because of suboptimal incubation conditions and/or transport, lack of sensitivity after initiation of antibiotic therapy,failure to culture vancomycin-sensitive strains or other strains with fastidious growth characteristics , a time requirement ,and proficiency level of laboratory personel (4,5,7-19).

Efforts to obviate these problems, in particular the prolonged time requirement, have given impetus to new technologies for detecting gonococcal enzyme, endotoxin, nucleic acid in genital secretion and antibodies in serum. The tests have included the limulus lysate assay for endotoxin (20-25), quantitation of 1,2-propanediol oxidoreductase (26), test for gonococcal DNA by genetic transformation of a mutant of N.gonorrhoeae (27-29) , radio immunoassay (RIA), immunofluorescent, indirect haemagglutina tion test (IHA), complement fixation test and enzyme linked immunosorbent assay (ELISA) to detect antibodies to various cellular components of N.gonorrhoeae. (30-44). In general, diagnostic methods based on detection of antibodies pose 2

disadvantages : delayed production of detectable levels of antibodies, and the persistence of antibodies from a past infection which may lead to false diagnosis (45). In addition, test for enzyme 1,2-propanediol oxidoreductase and endotoxin are not specific to gonococci since this enzyme could be found in Acinetobacter species (26), and this toxin could cross react with other gram negative bacteria(23,24,25).

In addition, indirect fluorescent antibody test, RIA, and ELISA have also been established to detect gonococcal antigens such as whole cell, Lipopolysaccharide and outer membrane proteins in clinical specimens (2,7,15,19,45-54). These techniques are highly sensitive but they cross react with other Neisseria species (15,45,47,49,50,51), for example, N. meningitidis, N. lactamica, N. pharyngitis, N.flava, N.sicca, N.flavescens, B.catarrhalis, and S.aureus. Therefore, a sensitive immunological test against a specific antigen might offer a better chance for diagnosis of gonococcal disease.

Previously, Kellogg et al.(55,56) reported that the virulence of gonococci was associated with specific colonial morphology. For instance Type 1 or 2 colony morphology are virulent whereas Type 3 or 4 are avirulent. In addition, Sparling and Yobs, and Kellogg et al. also found that gonococci initially isolated from clinical material of gonorrheal patients were mostly types 1 or 2 colony in more

than 90 % (55,57). When the surface of each colony types were examined, only types 1 and 2 possess pili (58-61).

Later, several investigators have presented evidences to explain that pili may be a major virulent factor of gonococci for mediating the attachment to tissue cells (62-66), and be an antiphagocytic factor to polymorpho nuclear leukocytes and macrophages (66-68).

Therefore, it seems reasonable that gonococcal pilus is a much more specific antigen for pathogenic gonococcal strains and might be a good candidate antigen for a specific diagnostic test. In Thailand, no study concerning with gonococcal pili from local strains has been reported. Therefore, in this study, we attempt to elucidate the morphology, physical property, antigenic and immunogenic characteristic of gonococcal pili from local strains, and the possibility to develop a technique for determining pili antigen. A sensitive immunological method such as ELISA using antigonococcal pili antiserum to detect gonococcal pili, may be beneficial for the diagnosis of gonococcal infection in women, and disseminated infection in both sexes. Moreover, this assay may be useful for screening large numbers of asymptomatic carriers to prevent the spread of the disease, the method of which is not applicable by conventional cultural procedure.

LITERATURE REVIEW

The increasing incidence of gonorrheal disease has resulted in a number of studies concerning better diagnostic methodology and virulence (2,6,7,13,15,19,20-58,). Hitherto, several immunological tests utilizing either whole gonococcal bacteria or certain surface antigens have been developed (2,7,15,19,45-54). Nevertheless, cross reactivity has always intervened. Pili, one of the surface appendages proposed to be associated with virulence (55,57-68) might be a potential antigen for a specific diagnostic test in gonorrheal disease, especialy in the late, disseminated type.

Due to the complexity of the surface components involved, a study of the biology of gonococcal pili will be extremely difficult without a thorough basic and current knowledge of gonococci in this regard. It is therefore the aim of this review to cover the surface structures of *Neisseria gonorrhoeae* with greater emphasis placed on the various aspects of gonococcal pili, including its properties, function and activity.

SURFACE STRUCTURE OF NEISSERIA GONORRHOEAE

1. Capsule

The presence or absence of capsules on *N.gonorrhoeae*

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had been debated for many years. Hendley et al.(69,70), James and Swanson (71), and the others (72,73) had reported the presence of gonococcal capsules of both virulent and avirulent colonial types; most capsules were easily seen on recent clinical isolates, but were also evident on laboratory strains (71). In vivo passage of gonococci in subcutaneous chambers in guinea pig enhanced capsule production (73). Capsule synthesis in vitro was apparently dependent upon the present of acid-hydrolysed casein in medium and the growth phase of the organism (70). Richardson and Sadoff (72) reported that capsule production was most apparent when gonococci were cultivated on a glucose containing agar medium in the presence of viridans streptococci. The reason for this stimulatory effect was not known but may be caused by a decrease in the pH of the medium resulting from the excretion of lactic and acetic acids by the streptococci (74). Morse and Hebeler reported that growth at an acidic pH markedly increased the total carbohydrate content of gonococci (75).

The chemical structure of the gonococcal capsule has not yet been determined. However, using positive staining with Alcian blue indicated that the capsule may be polysaccharide in nature (71). James and Swanson showed that the capsule was resistant to trypsin, chymotrypsin, lysozyme, hyaluronidase, neuraminidase and glucuronidase, and was also antiphagocytic (71,70,76).

2. Peptidoglycan

The chemical composition of the purified gonococcal peptidoglycan was initially reported by Hebeler and Young (77) and was confirmed by Wolf-Watz et al. (78). These investigators found that the peptidoglycan consisted of muramic acid, glutamic acid, alanine, meso-diaminopimelic acid and glucosamine in approximate molar ratios of 1:1:2:1:1, respectively. Analysis of peptidoglycan revealed that more than 96% of the total weight of the wall could be accounted for as amino acids and amino sugars (79). Trace amounts of aspartic acid, glycine and threonine were also observed and presented approximately 2% of the weight of the wall (79). Hebeler and Young determined that peptidoglycan represents 1 to 2% of the dry weight of the cells and there were no differences between the various colony types (79). The average chain length of the glycan backbone was between 80 and 110 disaccharide units and degree of cross-linking has not been examined.

N.gonorrhoeae does not survive for long periods after the cessation of growth. The decrease in viability is often correlated to cellular lysis (autolysis) (80). When cells were suspended in appropriate buffers, the rate of autolysis ranging from 23 to 35 minutes, and was dependent upon variables such as temperature, and the presence of cation. Whole cells autolysis was enhanced by K^+ and was markedly

reduced by divalent cations for example Mg^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+}, Cu^{2+}. The buffers such as Tris, that chelate divalent cations, yield faster rates of autolysis than buffers, such as HEPES which do not chelate. Whole cells autolysis is optimum at pH 9.5 and at 40°C, and is enzyme-mediated (77).

Peptidoglycan hydrolysis per se does not account for autolysis (81). Divalent cations, such as Mg^{2+} and Ca^{2+} reversible stabilize cell suspensions but do not inhibit peptidoglycan hydrolysis. The hydrolysis of peptidoglycan is enzyme-mediated, as suggested by its temperature dependence and inhibition by Hg^{2+}. The mechanism by which divalent cations prevent autolysis, as well as the mechanism of autolysis, remains unknown.

Activation of autolysis may produce cells with damaged envelopes which may remain viable due to stabilization with divalent cations (82). The damaged cell envelopes could undergo repair and resume growth under appropriate conditions. Autolytic activity may enhance the conversion of gonococci to L-forms(83).

Recently, McGee and co-workers (84) have shown that peptidoglycan fragments appear to be toxic to ciliated cells of fallopian tube and induce ciliostasis.

3. Lipopolysaccharide (LPS)

Gonococcal LPS containing lipid A and core disaccharide, is apparently located on the outer membrane and associated with endotoxic activity, as well as other gram negative bacteria.

Perry et al.(85), and Wiseman and Caird (86) determined that LPS from virulent type possessed the O polysaccharide side chains with structurally different side chains among strains (S-type) whereas that LPS from avirulent strains have not (R-type). In addition, LPS of virulent and avirulent colony types shared a common core oligosaccharide and S-type LPS, sometimes associated with R-type LPS.

In contrast, Stead et al.(87) reported that no significant differences between LPS of virulent and avirulent types and the LPS from both colonial variants lacked the O polysaccharide.

The reported differences may be dependent upon the variation in culture medium , therefore these discrepancies are still controversary.

The biological properties of the LPS are receptors for pyocins (88) which recognized core LPS sugar residues, are a target for bactericidal actions of antibody and

complement (89). In addition, MeGee et al. (84) and Gregg et al. (90) had shown that gonococci induce ciliostasis, followed by sloughing of ciliated cell from mucosa, in a tissue culture model of the fallopian tube. This appearently was the result of a toxic factor believed to be LPS that was elaborated from intact gonococcal surface in the form of outer membrane bleb; the lipid A portion of the LPS is probably critical for cytotoxicity (90,91).

4. Outer Membrane Proteins

The cell envelope of gram negative bacteria is usually composed of macromolecule, namely outer membrane proteins which comprise three major categories of proteins as classified by their quantitative abundance, molecular weight, and their modified migration by either heat or reducing agents in SDS-PAGE (92).

The first class of proteins, designated as Protein I, the principal outer membrane proteins, is usually the most abundant, accounted for over 60 % of the total outer membrane proteins with molecular weight between 36,000 and 32,000 daltons (91,92). Neither heat nor reducing agents affect its migration in SDS-PAGE. It acts as porins, because of its ability to form channels in lipid membranes (92).

Swanson (93) showed that protein I can be grouped

into 3 types on the basis of their migration in SDS-PAGE, and Blake et al.(94) demonstrated that the three classes of protein I show differences in susceptibility to proteolytic enzymes. They also proposed that protein I subunits exist in outer membranes in a hairpin configuration in which the carboxyl terminus is exposed on the inner surface of the membrane, the amino terminus is buried in the membrane's lipid phospholipid matrix, and each terminus is connected to hydrophobic portions, which are in turn connected by a region at or near the external surface of the organism (94).

Protein I appeared to be the major determining factor (antigen) in the diversity of gonococci serotypes, therefore the antigenic differences of protein I may be more adequately defined in the immunological serotyping systems (91). Blake and Gotschlich showed that protein I may insert into human erythrocytes membranes (92). It is possible that a similar insertion may promote internalization of gonococci by epithelial cells but this process is unclear.

Hildebrandt et al.(95) examined the relationship of outer membrane proteins to resistance to the complement dependent bactericidal activity of normal human serum. He showed that there are antigenic changes in the principal outer membrane proteins of the serum resistant strains. That finding implies that protein I is an important target for action of antibody and complement.

The second, designated Protein II, are usually less abundant than protein I and are heat modifiable protein that usually comprise a group of related proteins of apparent molecular weight of 27,000 to 30,000 daltons in SDS-PAGE (92). A variety of distinct protein II may be found within colony variants of a single strain and almost certainly occurs in vivo from different sites of infection in male and female sexual partners, for instance, some strains have been shown to be capable of expressing six different species of protein II (96,97).

Expression of protein II has been closely associated with the opacity of colony phenotype, and is independent of the state of piliation (92). Swanson showed that protein II exhibit an opaque colony morphology, whereas strains lacking this protein are transparent (98). The possession of different protein II may be of significance in different stages of gonococcal infection and differences in colony opacity of clinical isolates correlate with difference in the site of isolation or type of infection (99). The differences in protein II of some variants sometimes show differences in serum resistance(100). In addition, the expression of protein II has been shown to enhance adherence to a variety of cell types, including neutrophils, HeLa cells and buccal epithelial cells (100) and also increases intergonococcal association, resulting in clumps of organisms(98). Furthermore, protein II were associated with

enhancement of not only susceptibility to killing by proteolytic enzymes but also resistance to normal human serum and antimicrobial agents (92,96,99).

The production of protein II is genetically highly unstable, with frequency of switching for colonial opacity of about $1x10^{-3}$ or more per cell per generation (101). The genetic basis of protein II variation may also involved chromosomal rearrangements(102).

Protein III, which show variable migration in SDS-PAGE in the presence of reducing agents. This protein has a molecular weight of 30,000 to 31,000 daltons that depend on the presence or absence of reducing agent (92).

It has been demonstrated that protein III appear to be identical by peptide mapping in all strains. Using the Western blot technique, Wong and Johnston also found that antiserum raised against protein III of one strain cross reacted with protein III of all strains tested regardless of serotype (92). It was concluded that protein III appear to be a species specific protein and be identical or common to all strains of N.gonorrhoeae.

McDade and Johnston (103), and Jones et al.(104) found that protein III and protein I are closely associated in the membrane, i.e. protein III may closely bind to protein I. They also suggested that protein III is exposed

on the surface outer membrane and at least the majority of the protein III, if not all, is either buried deeply within the membrane or located on the periplasmic side of the outer membrane. Actually, how this structure is arranged in the outer membrane is unknown.

5. Pili

Pili were first reported on gonococci in 1971 by Jephcott, Reyn and Birch-Anderson, and independently by Swanson, Kraus and Gotschlich (59,60). They are present on both pathogenic and nonpathogenic Neisseria species (105,106). Short pili (175-210 nm in length) were seen only on non-pathogenic species, whereas long pili (upto 4,300 nm) were seen on both nonpathogenic and pathogenic species (105). Pili may be involved in surface translocation by twitching motility (70-72).

Several functions for pili have been extensively studied and proposed to explain the greater virulence of piliated gonococci. It has been demonstrated that piliated gonococci were more virulent than nonpiliated organisms for human and laboratory animal models (55,56,107). There is a considerable amount of evidence that gonococcal pili play a vital role in virulence by promoting attachment of organism to host cells (62-66,84,108-111). This attachment is probably the initial events of infection, which subsequently

lead to penetration and multiplication of gonococci within epithelial cells. In addition, the interaction between pili and host cells may involve a specific receptor, since the greatest adherence of pili is towards cells that are most histologically similar to those at actual sites of gonococcal infection.

The association of piliation with gonococcal virulence stimulated efforts to isolate and purify gonococcal pili in order to focus study on immunochemistry and pathogenic role of pili as described below.

These surface structures are shown in Figure 1.

The Study of Gonococcal Pili

1. Structure and Physicochemical Properties of Gonococcal Pili

Pili are nonflagellar, proteinaceous appendages extending from the cell surface. The gonococcal pilus is an assembly of up to 10,000 identical protein subunits, termed pilin, that polymerize to form a filamentous structures approximately 6 nm in diameter and 1,000 to 4,000 nm in length, and has an aggregate molecular weight of 1 to 20 million daltons (60,107,113).

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Pili purified from different gonococcal strains possess subunit molecular weights which vary between 17,500 and 21,000 as estimated by SDS-PAGE (112,113). The subunit molecular weight of pili from isogenic opaque (Op) and transparent (Tr) opacity variants may also differ. The molecular weight of pilin from intra-and inter-strain are shown in Table 1 . Similarly, inter - and intra-strain differences can be demonstrated by isoelectric focusing, isopycnic centrifugation and amino acid analysis.

Using isoelectric focusing, pilus subunits have acidic isoelectric points. Robertson and co-workers showed that the pI value of purified gonococcal pili from strain P9 is 4.9 and 5.3 (114). Buchanan (107) also reported a pI value in the region of 5.0 for purified pili preparations, and Lambden et al. (115,116) demonstrated the two types of gonococcal pili from variants strain P9 showing differences in pI (pI 5.2 and 4.3).

The density of purified gonococcal pili after centrifugation on a cesium chloride density gradient has been observed by different investigators (107,112,114,115). Its density ranges from 1.29 to 1.33 g/ml.

The amino acid composition and minimal molecular weight of gonococcal pilin from several strains, such as strains P9,201,33,F62,B,7122, have been determined and found

to be similar in their amino acid composition but not identical ; about 40-60 % of the residues are nonpolar amino acids(114,117). Approximately 1 to 2 carbohydrate (1.3-2 % w/w) and 1 to 2 phosphate have been detected per pilus subunit (114,118). The N-terminal amino acid sequence was determined to be identical through the 59 th residue for gonococcal pilin from Op and Tr variants of two strains (Table 2) and through the 29 th residue for four antigenically different pili types from N.gonorrhoeae and for pili from a single strain of N.meningitidis sequenced by Hermodson and coleaques (117). The amino terminal sequence is unusual amino acid N-methyl phenylalanine and the first 20 residues are hydrophobic. Moreover, its sequence is highly homologous with the amino terminal sequence of pili from Moraxella nonliquefaciens and Pseudomonas aeruginosa but entirely different from the N-terminal amino acid sequence of Escherichia coli pili. Schoolnik et al. (119) compared the entire pilin sequence of gonococcal strain MS11 and P. aeruginosa and showed that these sequences disclosed very little homology beyond residue 30 from amino terminus. Moreover, neither pilin sequence of E.coli was found to be homologous with the gonococcal or pseudomonad pilin sequence. The highly hydrophobic nature of the amino terminal sequence suggests that it is buried deep inside the subunit or involved in subunit-subunit interactions, and the remainder of the molecule has a rather hydrophilic character that probably contains the antigenic determinants.

Furthermore, Tryptic peptide maps of pili from the isogenic transparent variants of two gonococcal strains (MS 11 and R10) showed that of the 22 peptides from MS11(Tr) pili, 14 were identical to R10 pilus peptides, the others (30% of peptides) however were different. No significant differences were noted between the tryptic peptides of pili from the isogenic transparent and opaque clones of the same strains (113,120). These observations indicate that differences in the primary structure of gonococcal pili exist and may be responsible for their physical and serological diversity.

Recently, because aspects of the quaternary structure of lower eukaryotic actins and gonococcal pili are similar : both exist as filamentous structure of approximately the same diameter and both are composed of identical subunits that polymerize to form helical strands(119). Schoolnik et al. (119) proposed the model of gonococcal pilin according to the two-dimensional helical-wheel, which is similar to that model of lower eukaryotic actins, in which the amino acid side chains are separeted by 100 degree of arc. Further, the amino acids comprise a hydrophobic face on one side of the helix, suggesting that this stretch interacts with other subunits to stabilize polymeric structure.

Afterwards, Billyard et al.(121) further predicted

that the pilin molecule consists of a bundle of four antiparallel alpha helices aligned along the long axis of a roughly 25x25x70 angstrom molecule that is similar to the topology of tobacco mosaic virus coat protein.

2. Pili and Colony Forms

As early as 1904, differences were noted in the colonial appearance of N.gonorrhoeae. Hill initially reported this observations regarding the size, colour, elevation, opacity and consistency of gonococcal colonies (122). More recently, Kellogg et al. (55) defined four colonial forms for gonococci propagated in vitro and related those to virulence in human volunteers. Four morphologically distinct colonial types were designated types 1,2,3 and 4 (Table 3,Fig. 2). Subsequently, Jephcott and Reyn (123) reported a fifth colonial type (T_5) (Fig.2) after which another colonial type, designated T_1', had recently been described by Chan and Wiseman (124).

Sparling and Yobs (57), and Kellogg et al. (55) inferentially substantiated those virulence by demonstration that organisms initially isolated from male and female clinical material are almost exclusively types 1 or 2, about 6-10 % of which were noted small rough colonies (57,125).

In regard to the differences in the ultrastructure

of five distinct colony morphology; Elmros et al. (58) by means of scanning electron microscopy, observed the presence of intercellular filamentous strands among T_1 and T_2 and relative absence among T_3, T_4 and T_5. Jephcott, Reyn and Birch-Anderson (59) also found fibrils, sometimes seen in bundle, in preparations of T_1 and T_2 gonococci. Swanson et al. (60) and Kraus and Glassman (61) found pili on all preparations of T_1, T_2 gonococci and absence of pili in all T_3 and T_4 organisms. Furthermore, type 1 'possessed pili whereas type5 did not.

3. Phase Variation

On the basis of colonial morphology, T_1, T_2 and T_1' are piliated, whereas T_3, T_4 and T_5 are nonpiliated organisms. The piliated gonococci can change to nonpiliated one or vice versa, and be determined by their changes in colony morphology on solid media. Chan and Wiseman reported that the typical patterns of colonial variation which they observed were $T_1 \longrightarrow T_4$, $T_2 \longrightarrow T_3$, and $T_1' \longrightarrow T_5$ (124).

The frequency of piliated to nonpiliated transitions occurring in vitro are about 10^{-3} to 10^{-4} or 10^{-2} to 10^{-3} / cell / generation ; the reverse switch from nonpiliated to piliated occur at the frequency from more than 10^{-2} to undetectable (121,126,127).

On the genetic level, the genome of gonococcus contains two active expression loci, termed pil E1 and pil E2, that carry complete pilus structural genes, and loci for non-expression sequences, termed silent loci or pil S, which contains structural pilus gene information but lacks pilin promotor sequence and does not express pilin product (Fig.3) (128,129).

Phase variation appear to be regulated, at least in part, by recombination and rearrangement of DNA sequences related to pilin production (127-130). The regulation of expression of gonococcal pili is relatively more compilcated than that of E.coli type 1 pili (127).

Earlier study of gonococcal chromosomal DNA, revealed that DNA rearrangement with a deletion of all or part of pil gene, including promotor and 5' regions of the pilin gene, from both expression sites resulted in a nonreverting pilus$^-$ phenotype (P$^-$n). The reversion from this nonpiliated (P$^-$) to piliated (P$^+$) was accompanied by reconstitution of the deletion in one or both of the pilin expression sites (128,131). This result suggested that pil DNA had been reacquired in that site from information located elsewhere in the chromosome, probably in one of the silent pilin loci. Some P$^-$ cell with deletions in both expression sites revert to P$^+$ cell at low frequency (131), others with deletions in both expression sites may be unable to revert to P$^+$ cell (132).

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Recently,Swanson et al. and Bergstrom et al. reported two types of highly unstable P⁻ variants without obvious deletions of the pilin expression sites (126,132). One class synthesized pilin mRNA and apparently normal amounts of pilin protein, but failed either to export, assembly, or anchor these subunits as mature pili. The other class also produced pilin mRNA but elaborated no detectable pilin protein. These two classes of highly unstable P⁻ cells can result from intragenic recombination of partially homologous DNA sequences from a partial pilin gene (in silent, storage form) into the expression locus' complete pilin gene. Therefore, the former were thought to contain an "assembly missense" mutation of their expressed pilin gene on mRNA , and their pilin gene products may be not compatible with some aspect of pilus assembly. The later also contained a "nonsense" mutation and encoded a markedly truncated, immunologically undetected pilin. The results suggested that intragenic recombination between the structural gene and nonexpressed pilin gene homologous sequences can generate defective pilin gene products, resulting in a nonpiliated phenotype. These conclusions are depicted in Fig.4.

Segal et al. (131) and Hagblom et al. (133), recently, speculated that there might be a repressor, in addition to DNA rearrangement, that turns off transcription from the pilin gene, that is the "off" mode in P⁺ cells, but

in the "on" mode in P^- cells. There are some evidences that support this idea, for instance, crude extracts from P^- cell but not P^+ cell bind to a DNA fragment that contain the pilin promotor and in addition, a recombinant plasmid with a gonococcal insert from a P^- gene bank produces a protein that acts negatively on a pilin promotor present on another plasmid in the same E.coli cell (127).

Hence, the genetic process that controls piliation in gonococci could be depicted as pilin roulette in which a variety of outcomes in piliation status and pilin subunit expression can eventuate from independent analogous recombinational events involving pilin gene DNA.

4. Antigenic Relationship

The antigenic relationships between pili from different strains of N.gonorrhoeae have been investigated by using antibodies to purified or partially purified pili, or whole gonococci.

By using different assay systems, including direct agglutination of piliated gonococci (65), agglutination of laterally aggregated bundles of pili observable by dark field microscopy (134), ouchterlony immunodiffusion (65), two-dimensional immunoelectrophoresis(107), immune electron microscopy (135), radioactive antigen binding assay (30),

radioimmunoassay (65,136), haemagglutination inhibition (65), inhibition of attachment of isolated pili to human cells(110), and enzyme linked immunosorbent assay(134), pili have shown to vary considerably among strains of N. gonorrhoeae and also exhibit extensive intrastrain antigenic heterogeneity. Moreover, the variants of a gonococcal strain isolated from different anatomical locations express antigenically distinct pili of different subunit molecular weights(112,137). The occurrence of a widespread antigenic shift in pilus expression may play an important role in selective adherence to different mucosal surfaces and escape from specific immune surveillance directed against pili.

In addition to pili antigenic variation,common regions of pili do exist. To quantitate the degree of shared antigens among pili, Buchanan and Pearce reported that shared antigen generally accounted for less than 1 % of the weight of each pili type examined (65). The highest shared antigenicity was approximately 2.5 % (65,136).

5. Antigenic Variation

Buchanan, Novotny and Turner, and others demonstrated the antigenic variation of pili from different strains by radioimmunoassay, immune electron microscopy, and amino acid analysis (65,116,120,134-136,138). Because few serological cross-reactions occurred among pili of different strains,

the titre of antibodies to heterologous pili appeared to be lower than that against the homologous strain (115,134-136,).

In addition to the antigenic diversity of pili in different strains, there is also a variation in the pilin species expressed by different variants of a single gonococcal strain and results in variation in adherence to several types of host cells (112,115,116). Heckels' group had isolated four variants of one strain that produce different pilus, with subunit molecular weights varying from 18,000 to 21,000 and they differ in efficiency of adherence to different types of host tissues (115,116). Swanson had observed that a single gonococcal strain may be capable of producing as many as 12 different pilin species (139). Duckworth et al. (137) reported that pili from different anatomical sites (from cervix and urethra of female, and from urethra of male partner) during the course of natural infection are usually also different in subunit molecular weights.

In order to identify regions on the gonococcal subunit for antigenic diversity, Schoolnik et al. prepared cyanogen bromide fragments (CNBr) of gonococcal pilin (as indicated in Fig.5), namely : CNBr-1 (N-terminal 7 residues), CNBr-2 (residues 8 to ca.84), CNBr-3 (residues ca.85 to 160, the carboxy terminal portion of the molecule) (113). According to these investigators, anti-CNBr-3 antibody bound

only to the pilus antigen from which the CNBr-3 immunogen was prepared, but did not bind heterologous CNBr-3 fragment. Tryptic peptide maps and amino acid composition of CNBr-3 from different strains also indicated signifcant differences in the primary structure of the carboxy terminal region of the pilin. Therefore, CNBr-3 contains a variable region which determines type-specific antigenicity, and the serological diversity (immunodominant) of gonococcal pili (Fig.6). Thereafter, Rothbard et al. focused on the position of amino acid sequence that located on the variable region of CNBr-3 fragment (140). According to these investigators, the amino acid sequence of residues 121 to 151,corresponding to a part of CNBr-3 may contribute strain-specificity.

At DNA level, Hagblom et al.,recently, have shown that a pilin structural gene can be divided into 3 regions : a constant region (C), a central semivariable region (SV), and a hypervariable region (HV) as shown in Fig.7 (133). In silent loci, SV and HV domains are dissected into small variable regions so called "minicassettes", that are surrounded by short, strictly conserved regions (141). Silent constant and silent variable pilin gene segments (minicassetts) are separated and distinct from each other (142).

Antigenic variation of gonococcal pili appears to be due to the donation of variant DNA from the silent pil loci

into one or both of the pil expression loci. Two different genetic mechanisms may be involved. In the first, recombination presumably occurs between any of the several silent (incomplete) pil gene segments and the one present in an intact expression site, such as pil S1 and pil E1 or pil E2, to assemble a new gene for pilin variant. In the second, antigenic variation occurs by gene conversion (donation of DNA from a silent locus to an expression site without reciprocal exchange). It is theoretically possible for multiple recombination to occur any time between any two silent variable pilin sequences (minicassettes), i.e. mixing and matching of SV and HV gene segments by recombination, or between such minicassette from silent copies and expression gene in the chromosome, resulting in the generation of new pilin sequences and ultimately new pilin epitopes (133,141,142). Therefore, the capacity of N.gonorrhoeae to undergo pilus antigenic variation may be quite extensive.

6. Common Antigenic Determinant

As described above, evidence has been presented that other than type-specific determinant, gonococcal pili possess the common or conserved antigenic determinant.

To ascertain the structural basis for antigenic and serologic properties of pili, rabbit antibody was prepared to heterologous undenatured pili and to their cyanogen

bromide fragments (113,140). Schoolnik et al. reported that anti-CNBr-2 antisera could bind homologous and heterologous CNBr-2 fragments and also the homologous and heterologous native pilus antigens (113). In addition, tryptic peptide mapping study had shown that CNBr-2 fragments from different pilin species shared many common peptides (87 %) (113). Therefore, CNBr-2 encompasses a highly conserved region which mediates receptor binding, and contains an antigenic determinant common to all gonococcal pili. The common determinant of unmodified gonococcal pili is antigenic but is normally immunorecessive (Fig.6). It becomes immunodominant only when CNBr-2 fragment is cleaved from the pilin.

Rothbard et al. indicated that the precise location of epitope common to pili exist between amino acid residues 48 - 60 and that the immunogenicity of this region is determined by interactions present in intact pili but absent from CNBr-2. Another common determinant, corresponding to residues 69-84, is exposed when CNBr-2 is used as immunogen (140).

7. Haemagglutinating Activity

Direct bacterial haemagglutination was first described in 1902 by Kraus and Ludwig, who observed coarse clumping of erythrocytes with isolated colony from vibrios

and staphylococci. The mechanism of this observation was not elucidated until pili were demonstrated under electron microscopy by Anderson, Houwink, and Houwink and Van Iterson (143). This haemagglutinating phenomenon was also found in other piliated organisms (144-146).

Punsalang and Sawyer, Waikins, and Chan and Wiseman reported that piliated gonococci more readily attach to many mammalian and nonmammalian erythrocytes than nonpiliated organisms (66,124,147).

Koransky et al. and the others demonstrated that human erythrocytes could be also agglutinated by piliated gonococci(124,143,147,). Buchaman and Pearce, and Buchanan et al.showed that isolated purified gonococcal pili alone are capable of causing direct agglutination of human red blood cells, and this hemagglutination required pili at concentrations of 0.2 to 82.5 ug/ml (65,110,120). No difference in hemagglutinating activity was observed using any of the eight major human blood types (ABO-Rh) (120,143). Furthermore, Buchanan and Pearce also demonstrated that antiserum to purified gonococcal pili could inhibit the above hemagglutination, suggesting pili as a mediator of the attachment of piliated gonococci to human erythrocytes.

Previous study by Punsalang and Sawyer, and Koransky et al. also showed that the hemagglutination was not

inhibited by simple sugars includings D-glucose, maltose, D-mannitol, dulcitol, D-sorbital, raffinose, saccharose, lactose, D-fructose, D-galactose, D-mannose, and inulin (66,143). Therefore, the pilus receptors on red blood cells are not similar to those of type-1 pili of Enterobacteriaceae namely D-mannose, as described below.

8. Receptor Binding Domain

Structural analysis on cyanogen bromide fragments (Fig.5) were performed in order to determine the region on gonococcal pilin responsible for erythrocyte binding. Schoolnik et al. noted that both CNBr-2 and CNBr-3 fragments could not cause hemagglutination (113,120). However, only CNBr-2 but not CNBr-3, inhibited hemagglutination by homologous and heterologous native pili, but could not inhibit hemagglutination by _M_. _nonliquefaciens_ or _P_. _aeruginosa_ pili (113,120). These results suggested the following : first, the CNBr-2 fragment contain the amino acid sequence that recognized and bound to the erythrocyte surface receptor for native gonococcal pili. Secondly, pili from different gonococcal strains bound to the same erythrocyte receptor, which was different from the receptor for Moraxella or Pseudomonas pili, even if the N-terminal amino acid sequences of pili from these three species were highly homologous (113,120). Thirdly, the receptor binding domain of CNBr-2 was highly conserved for pili from

different gonococcal strains, and finally, each pilus subunit possessed a receptor binding domain, which, if expressed in the assembled state, results in a polyvalent ligand with a linear array of binding regions along the longitudinal exist of the intact pilus (Fig.8).

Similarly, tryptic cleavage of pilus showed that the receptor binding region that bind to human endocervical cells, exist in tryptic peptide-2 (TC-2) fragment (residues 31-111) which encompassed an amino acid sequence of CNBr-2 (119).

Recently, Rothbard et al. identified amino acid segments (or peptides) corresponding to receptor binding region by blocking bacterial adherence to endometrial cells by synthetic peptide antisera (148). This finding indicated that the amino acid residues 41-50 and residues 69-84 were involved in receptor binding.

In contrast to previous studies, Virji and Heckels (149) showed that variable domains of gonococcal pili play a role in binding to human epithelial cells, since strain-specific but not cross-reacting monoclonal antibodies, block the attachment. The observations may either due to the variable region itself containing the epithelial cell receptor or the tertiary structure of pili bringing the

binding site close enough to the variable region for the type specific antibodies to hinder attachment. However, the location of their epitopes were not determined.

9. Function of Gonococcal Pili

9.1. Pili Relationships to Virulence

On the basis of Kellogg's observation,colonial types 1 and 2 exhibited greater virulence than remaining colonial variants following urethral challenge in human volunteers (55,56). Swanson and colleagues noted morphologic differences between virulent and avirulent colony types under electron microscopy. Organisms of virulent types possessed surface pili and showed intercellular zones of adhesion ; avirulent types lacked these structural feature in vitro (60). In addition, piliated gonococci predominated in clinical isolates from infected sites in fresh culture, and were found more virulent than nonpiliated gonococci in chick embryo and chimpanzees (107). This suggested that pili present in virulent colonial types but lost on subculture, were important virulent factor. In other words, the loss of virulence on laboratory subculture was accompanied by a concomitant loss of pilus expression.

Interestingly, pili have rarely been observed during electron microscope studies of gonorrhea, although

the presence of pilus antibodies in patient sera indicated that the antigen is present in the natural infection (30,39, 40,44,138). However, few studies were able to show gonococci bearing morphologically recognizable pili in urethral exudates by negative staining and transmission electron microscopy (150,151). This association of piliation with virulence was thought, in part, to be due to the important role of pili to facilitate bacteria attachment to cells as mention later.

Furthermore, the virulent colony types were more resistant to phagocytosis than avirulent types by polymorphonuclear leukocytes from human, rabbit and by mouse macrophages (66-68). The resistance to phagocytosis was correlated with the presence of surface pili. Antibody to pili had been demonstrated to increase phagocytosis of piliated gonococci by human PMN and by mouse macrophages (66,118,152). These findings indicated that pili were responsible for the antiphagocytic activity of virulent _N. gonorrhoeae_.

9.2. The Role of Pili in Attachment

Many functions for pili have been proposed to explain the greater virulence of piliated gonococci. These include the attachment of gonococci to eukaryotic cells

(62-66,84,108-110,143-147,153-157).

Abundant evidences for attachment role for gonococcal pili are strong; many investigators have found that pilus bearing organisms adhere more avidly to human cells than nonpiliated organisms (62-66,143,147).

Direct hemagglutination caused by piliated gonococci, as described previously, had been reported to be correlated with the presence of pili, suggesting that pili are as structure or mediator for attachment to red blood cells (65,66,143,147).

Swanson,and Swanson et al. noted that piliated gonococci attach more readily than nonpiliated cells to human amnion cells, human foreskin cells, and HeLa cells (62,153). Pili were visualized by electron microscopy and appeared in some pictures to be responsible for holding the visualized gonococci to amnion tissue cultures (62). These studies again suggested that pili facilitated attachment by anchoring gonococci to tissue culture cells. Also demonstrable was enhanced attachment of piliated gonococci to urethral mucosal cells and human buccal epithelial cells (66,110,115,116,154). Mardh and Westrom demonstrated that freshly isolated gonococci (piliated cells) adhere more frequently to human vaginal epithelial cells than that which had been passed on artificial media for more than ten years

(nonpiliated cells), and subsequently, Mardh and associate also noted enhanced attachment to uroepithelial cells by piliated gonococci (108). Ward et al. and McGee et al. using elegant human fallopian tube perfusion models, demonstrated enhanced attachment of piliated gonococci to fallopian tube epithelium as compared to pilus-lacking organisms (84, 109). Using scanning and transmission electron microscopy of the human fallopian tube perfused with gonococci in vitro showed bacteria enchored to the mucosal surface by bundles of pili (109). This finding suggested that pili were involved in the initial attachment of gonococci to fallopian tube epithelium. Tebbutt et al. confirmed that piliated gonococci adhere better than nonpiliated organisms to fallopian tube mucosa as well as human endocervix and ectocervix (155). This same adherence advantage for piliated gonococci was not present for guinea pig epithelial surfaces or for human bronchial mucosa. Moreover, reduction of piliation by treatment with chemical, mechanical or enzymes, result in decreased attachment by gonococci (64,66).

Furthermore, James-Holmquest et al. and James et al.reported enhanced attachment of piliated gonococci to human sperm (63,64). This enhanced attachment was reduced by treatment of piliated organisms with enzymes, such as trypsin, alpha chymotrypsin, lysozyme, which destroy pili as seen by electron microscopy (64). The observation, therefore,suggested that pili were responsible for the enhanced attachment.

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The direct evidences for the adherence function of gonococci pili are provided by the observation that radiolabeled gonococcal pili were able to attach to human erythrocytes, buccal cells, cervical-vaginal epithelial cells, fallopian tube, sperm, HeLa cells, and endocervical cells (65,110). In addition, either anti-pili antibody in urogenital secretion or in serum inhibited the attachment of piliated or purified pili to these epithelial cells (65,110,143,156,157).

These indirect and direct evidences, therefore, implied that pili as mediator of attachment for N. gonorrhoeae, may involve in the first event of pathogenesis of gonococcal infection. Nevertheless, the mechanism by which gonococcal pili approach and adhere to host cell membranes is not entirely understood but have been proposed.

9.2.1. Pili and Surface Charge

Heckels et al. determined that the isoelectric point (pI) of N.gonorrhoeae was 5.3 (158). Thus, like other bacteria, gonococci are negatively charged at physiological pH. The negative charge on the bacterial surface should present an electrostatic barrier to the attachment to negatively charged surface of human cells. Modification of gonococcal surface indicated that attachment was decreased when the negative charge on the gonococcal

surface was increase, and attachment was increased when the bacterial cell surface became positively charged (158). However, this increased attachmant is not explained by nonspecific electrostatic attraction due to positively charged groups on the gonococcus since the enhanced attachment of the gonococcus to the cell membrane remained after both amino and carboxyl groups of the gonococcus had been blocked (electrostatically neutral) (158). These results led them to the postulation that pili is important in overcoming these electrostatic repulsive forces between negatively charged gonococcus and host cell surfaces (158).

Robertson et al. (114) demonstrated purified gonococcal pili have an isoelectric point identical to that of the whole piliated cell (pI 5.3) and slightly less than that of the nonpiliated cell of the same strain (pI 5.6). This finding suggested that pili may dominate the surface charge of the gonococcus and in themselves would not be more electrostatically attractive to the host cell surface than other gonococcal cell surface components. In addition, they also suggested that the presence of large amounts of nonpolar amino acids in pili may give rise to a less charge density than that of other gonococcal surface components. A reduction in charge density together with a small surface area may facilitate attachment by reducing the electrostatic repulsion of the pili compared with the whole gonococcus cell surface (114).

9.2.2. Hydrophobic Interactions

Buchanan et al.(118) and Hermodson et al. (117) demonstrated that the 22 of the fisrt 24 residues in the amino terminus of pili are hydrophobic in nature. Moreover, the amino acid composition of pili shows a high content of hydrophobic amino acids (nonpolar) approximately 25 to 46 % (114,115,117,118). The high proportion of hydrophobic amino acids can explain the ability of pili to bind to each other and suggest that they could attach to mucosal surfaces by embedding in the hydrophobic interior of the host cell membranes, as well as, Salmonella typhimurium of which the surface hydrophobicity is an important determinant in the attachment to HeLa cells (114).

9.2.3. Specific Membrane Receptors for Gonococcal Pili

Pili from a wide variety of bacteria agglutinate red blood cells. This adhesion in the case of E.coli can be selectively inhibited by D-mannose and methyl manoside, indicating that the pilus attaches to a specific carbohydrate, namely mannose, on the erythrocyte membrane. However, mannose and many simple sugars as described by Punsalang and Sawyer could not block the attachment of piliated gonococci to red cells (66). This finding suggested that the gonococcus pilus receptors are not composed of simple

sugar ; they may be composed of more complex carbohydrate moieties.

To determine the chemical nature of the pilus receptor, pretreatment of buccal epithelial cells with a mixture of neuraminidase and exoglycosidase inhibited binding of both piliated gonococci and purified pili, suggesting that the buccal cell receptors may be an oligosaccharide (sialic acid) (159). Moreover, the compounds frequently found on human surfaces, capable of inhibiting pilus attachment to human buccal cells had been found including gangliosides, synovial fluid, human serum, human cell surfaces, and extrated plasma membranes (119,159). These observations are consistent with a carbohydrate receptor for gonococcal pilus somewhat resembling the carbohydrate moiety found on gangliosides. Indeed, the chemical composition of gonococcal pilus receptor has yet to be fully characterized.