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HOST-PARASITE INTERRELATIONS:  
BACTERIAL ADHERENCE TO AND INVASION OF EPITHELIAL CELLS

A thesis submitted to the Faculty of  
The Rockefeller University  
in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy

by

Debra Bessen

December, 1986



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To my family and friends who are a constant reminder of how good things really are.

## SUMMARY

Enteroinvasive Escherichia coli (EIEC) preferentially invaded multinucleated HeLa cells which arose naturally in monolayer cultures at a low frequency. Data suggests that enhancement of the penetration step accounts partly for increased invasion of multinucleated cells. Treatment of HeLa cell monolayers with polyethylene glycol (PEG) led to cell fusion, and invasion of PEG-treated monolayers by EIEC was increased by an average of 7-fold compared to untreated HeLa cells. Treatment of HeLa cells with PEG had no significant effect on the low level of cell-association displayed by noninvasive organisms and thus, PEG-treated HeLa cells appeared to retain their selectivity for EIEC. The microfilament disrupting agent cytochalasin B (CB) reduced EIEC invasion by more than 50-fold. Furthermore, CB depressed the extent of EIEC invasion of PEG-treated and untreated HeLa cells to equivalent levels. The large, multinucleated HeLa cells generated by PEG treatment revealed a striking accumulation of EIEC in cellular extensions. This study demonstrates that alteration of HeLa cell morphology results in increased invasion by EIEC.

Colonial variants of Neisseria gonorrhoeae differ in their interactions with eucaryotic cells. Gonococci giving rise to the opaque colony type possess one or more proteins II in their outer membrane. When gonococci were cultivated with HeLa cell monolayers, the opaque phenotype became increasingly dominant in the subpopulation of organisms which adhered to the HeLa cells. Once bound, opaque organisms displayed

very low levels of detachment. Adherent opaque gonococci exhibited generation times up to three-fold greater than cultures containing gonococci in the absence of HeLa cells. In addition, the progeny of adherent opaque organisms remained bound to the HeLa cell monolayer. Both piliated and transparent colony types attached to HeLa cells, but their progeny were retained less efficiently. Gonococci bound to HeLa cells were subjected to the bactericidal action of fresh rat serum and approximately 0.5% to 2.5% survived, irrespective of their opaque or piliation phenotype. Incubation with gentamicin resulted in a 10- to 50-fold further reduction in viability. Pretreatment of HeLa cell monolayers with CB diminished gonococcal survival in either serum or gentamicin by up to 8-fold. In contrast, CB treatment did not decrease the survival of the commensal organism Neisseria sicca. The data suggests that very few gonococci are completely interiorized, and a small proportion of adherent gonococci are partially protected from the soluble-phase environment by HeLa cells.

Binding of the opacity-associated Protein II (P.IIop), P.IIa, to eucaryotic macromolecules was studied. HeLa cell extracts were subjected to SDS-PAGE and transferred to nitrocellulose, and purified P.IIa bound to approximately 30 to 50 distinct molecular species. The binding of P.IIa to HeLa cell components was stable in high NaCl and nonionic detergent, and was not inhibited by monosaccharides. The binding behavior of P.IIa was compared to that of two model carbohydrate-binding proteins, wheat germ agglutinin (WGA) and concanavalin A (ConA). Glycoproteins (ovomucoid, fetuin, mucin, ovalbumin) inhibited binding by

P.IIa, WGA, and ConA to variable degrees. HeLa cell glycopeptides, generated by pronase digestion of chloroform:methanol-extracted cells, were tested for their ability to inhibit binding by P.IIa to Western blots of HeLa cell macromolecules. HeLa cell extracts inhibited P.IIa binding prior to pronase treatment, but inhibitory activity was lost as a result of pronase digestion. Direct binding to defined glycosylated and nonglycosylated proteins revealed that ConA and WGA bound only glycoproteins, whereas P.IIa bound to proteins lacking carbohydrate as well. The basic P.IIa was compared to another P.IIop whose overall net charge was significantly less (P.IIc). Both P.IIa and P.IIc displayed similar binding specificities and affinities. The predominant binding interactions of P.IIa and P.IIc were with protein rather than eucaryotic carbohydrate, and the chemical nature of the interactions was more complex than the involvement of purely ionic or purely hydrophobic forces.

Gonococci expressing P.IIa, P.IIc, neither or both were compared for colonial opacity, adherence to HeLa cells, and growth under stressful conditions. Both the quantity and type of P.II determined colonial opacity. Gonococcal adherence to HeLa cells correlated with both opacity and P.II content. Environmental factors distinguished between gonococci expressing distinct P.IIop molecules in a manner independent of opacity. The role of P.II as a determinant of tissue tropism is discussed.

## TABLE OF CONTENTS

CHAPTER 1	INTRODUCTION	2
1.1	ADHERENCE	4
1.1.1	Overview	4
1.1.2	<u>Neisseria gonorrhoeae</u>	7
1.2	INVASION	16
1.2.1	Overview	16
1.2.2	<u>Neisseria gonorrhoeae</u>	20
1.2.3	<u>Shigella flexneri</u> and Enteroinvasive <u>Escherichia coli</u>	24
CHAPTER 2	INVASION OF EPITHELIAL CELLS BY ENTEROINVASIVE <u>E. COLI</u>	27
2.1	MATERIALS AND METHODS	28
2.2	RESULTS	31
2.2.1	EIEC Invasion of Multinucleated HeLa Cells	31
2.2.2	Morphology of EIEC-Invaded, Multinucleated HeLa Cells	38
2.3	DISCUSSION	38
CHAPTER 3	INTERACTIONS OF GONOCOCCI WITH EPITHELIAL CELLS	42
3.1	MATERIALS AND METHODS	43
3.2	RESULTS	46
3.2.1	P.II Content of Gonococcal Colony Types	46
3.2.2	Net Distribution of Gonococcal Colony Types	49
3.2.3	Detachment of Gonococci	50
3.2.4	Multiplication of Gonococci	54
3.2.5	Nature of the Association with HeLa Cells	58

3.3	DISCUSSION	64
3.4	GENERAL DISCUSSION OF CHAPTERS 2 AND 3	70
CHAPTER 4	BINDING PROPERTIES OF GONOCOCCAL PROTEIN II	73
4.1	MATERIALS AND METHODS	75
4.2	RESULTS	81
4.2.1	Purity of P.IIa	81
4.2.2	Binding to HeLa Cell Protein/Glycoprotein: Role of Carbohydrate	81
4.2.3	P.IIa Binding to Proteins of Defined Composition	88
4.2.4	Covalent Modification of P.IIa and Effects on Binding	91
4.2.5	Comparative Study of Functionally Distinct P.IIops	93
4.2.6	Chemical Basis of P.IIa and P.IIc Binding to Protein	96
4.2.7	Effect of Ligands on Attachment by P.II-Bearing Gonococci	100
4.3	DISCUSSION	104
4.4	ADDENDUM	113
4.4.1	Structural Analysis of Two P.IIs from Strain MS11	113
CHAPTER 5	STRUCTURE-FUNCTION CORRELATES OF GONOCOCCAL PROTEIN IIS	115
5.1	RESULTS	116
5.1.1	Environmental Influences on P.IIa and P.IIc Expression	116
5.2	GENERAL DISCUSSION OF CHAPTERS 3, 4, AND 5	121

## ABBREVIATIONS

BSA	bovine serum albumin
BSM mucin	bovine submaxillary gland mucin
CB	cytochalasin B
CFU	colony-forming units
CM	carboxymethyl
ConA	concanavalin A
DEAE	diethylaminoethyl
DGI	disseminated gonococcal infection
DTT	dithiothreitol
EIEC	enteroinvasive <u>E. coli</u>
FITC	fluorescein isothiocyanate
FRS	fresh rat serum
FTOC	fallopian tube organ culture
GAMIg	goat anti-mouse immunoglobulin
GARIg	goat anti-rabbit immunoglobulin
HSA	human serum albumin
Ig	immunoglobulin
kDa	kilodalton
LPS	lipopolysaccharide
MBSA	carboxymethylated BSA
MDa	megadalton
MOI	multiplicity of infection
O <sup>-</sup>	transparent
O <sup>+</sup>	moderately opaque
O <sup>++</sup>	highly opaque
OmpA	outer membrane protein A
OMV	outer membrane vesicles
P <sup>+</sup>	piliated
P <sup>-</sup>	nonpiliated
P.I	Protein I
P.II	Protein II
P.IInop	nonopacity-associated P.II
P.IIop	opacity-associated P.II
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PMN	polymorphonuclear leukocyte
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
WGA	wheat germ agglutinin



## CHAPTER 1

### INTRODUCTION

Parasitism was defined by Theobald Smith in 1934 as having four basic requirements (Smith, 1934; Francis, 1952). The first is that the bacterium gains entrance to the host, and this most often occurs through an opening (oral, urogenital) which leads to a mucosal epithelial surface. Secondly, the organism adapts to its new environment and replicates. If this is achieved without damage to the host and the bacterium becomes part of the normal flora, it is considered to be a commensal. The organism which injures the host and causes disease is pathogenic. Although the initial contact is with the mucosal surface, many microorganisms go on to invade individual cells and deeper tissue. The bacterium must ward off host defenses such as the immune response. It must also compete for nutrients in order to grow. The third requirement for parasitism is that the organism exit the host, and lastly, that it be transmitted to a new host. This thesis is concerned with the adaptation and growth of pathogenic bacteria, and cultured epithelial cells are employed as an in vitro model.

The two bacterial pathogens under study are enteroinvasive Escherichia coli (EIEC), a causative agent of bacillary dysentery, and Neisseria gonorrhoeae, the causative agent of gonorrhea. The clinical disease caused by EIEC is indistinguishable from that of Shigella, taking place in the intestine where the organism invades cells of the epithelial lining, multiplies, and destroys the mucosa. The ability to enter epithelial cells (invasion) is a primary virulence factor of "shigellae-like" E. coli. Neisseria gonorrhoeae is another example of an invasive organism, however, it is less clear whether invasion is essential for gonococcal disease. The primary site of gonococcal infection is the urethra and cervix of men and women, respectively. The

gonococcus can also cause pharyngitis, conjunctivitis, and anorectal infection at mucosal sites, pelvic inflammatory disease (including salpingitis), bacteremia, and disseminated infection. While invasion seems to be associated with the more serious clinical manifestations, survival on the epithelial cell surface appears to be important in uncomplicated gonococcal infection.

## 1.1 ADHERENCE

### 1.1.1 Overview

Attachment to the epithelial cell surface is considered to be an important first step in tissue colonization (Beachey, 1981). The current view is that adherence is achieved through an "adhesin" which recognizes a molecular conformation on the epithelial cell in a lock-and-key fit (eg., carbohydrate), or by less defined means involving one or more bacterial surface components engaged in electrostatic and/or hydrophobic interactions. An early observation of the adherence properties of bacteria was the agglutination of red blood cells by E. coli (Guyot, 1908). Electron microscopy of hemagglutinating bacteria revealed filamentous appendages which were termed "fimbriae" (Duguid et al., 1955), and often referred to as "pili" (Brinton, 1959). Hemagglutination induced by certain bacteria could be blocked with mannose, and the fimbriae of these organisms are designated mannose-sensitive or type 1 fimbriae (Collier and de Miranda, 1955; Duguid and Gillies, 1957);

this probably represents the first demonstration of lectin-like binding activity by a bacterial adhesin. The importance of adherence in bacterial pathogenesis is illustrated with type 1 fimbriated E. coli, which colonizes to a lesser extent when administered in mouse bladders in the presence of  $\alpha$ -methylmannoside (Aronson et al., 1979). Similarly, experimental urinary tract infection caused by uropathogenic E. coli bearing P fimbriae, which recognize Gal $\alpha$ 1 $\rightarrow$ 4Gal-containing glycolipid (Bock et al., 1985), was diminished by administration of the ligand analogue globotetraose (Svanborg-Eden et al., 1982). Furthermore, the susceptibility of an individual to infection with P-fimbriated organisms correlates with P blood group type and the density of globoside in their tissue (Leffler et al., 1982). Thus, host range is determined in part by the availability of attachment sites, and colonization can be limited if the ability to adhere is impaired.

If a bacterial structure is to be identified as an "attachment factor", it should confer an adherence advantage on the organism. One example of an attachment factor is the pilus, a complex structure composed of hundreds of polymerized subunits of pilin, its major protein. Genetic manipulations on organisms bearing P fimbriae have demonstrated that a number of cistrons are required for adhesion, and that a single mutation can be made which diminishes adherence and leaves pilus fiber formation intact (Norgren et al., 1984). The finding that the pilin subunit in itself is not sufficient for adherence has reshaped some of our concepts on the role of the pilus in attachment. Definition of an "adhesin" in molecular terms requires direct involvement in a receptor-ligand relationship. To prove that a particular molecule is an adhesin requires identification of its ligand. Since it is probable

that one of the primary defensive roles of secretory IgA is to block attachment of microorganisms to the mucosal surface (Williams and Gibbons, 1972), identification of the molecule engaged in direct contact may be of practical importance in vaccine development.

There is substantial evidence which suggests that many adhesins specifically recognize carbohydrate in a lectin-like fashion, and not all of these interactions are mediated by fimbriae. Streptococcus mutans uses its glucosyltransferase and a surface glucan-binding protein to bind glucan, which in turn adsorbs to dental enamel (Christensen et al., 1985). Adherence mechanisms of a non-carbohydrate nature have also been proposed. Streptococcus pyogenes binding to buccal cells is mediated by the lipid moiety of lipoteichoic acid interacting with fibronectin present on the epithelial cell surface (Courtney et al., 1983). There are many attachment factors whose ligand specificity remains undefined. The overall hydrophobicity of an organism can correlate with its ability to adhere to other surfaces (Garber et al., 1985; Falkowski et al., 1986; Sobel and Obedeanu, 1984; Nesbitt et al., 1982; Speert et al., 1986), and the hydrophobic properties of fimbriae may serve to increase adherence, but this would not necessarily involve a precise stereochemical fit. It has been proposed that through the hydrophobic bridging of the bacterium to the epithelial cell, the interactions between lock-and-key complements are facilitated (Smyth et al., 1978; Sobel and Obedeanu, 1984).

### 1.1.2 Neisseria gonorrhoeae

Gonococci grown on solid agar medium give rise to colonial types which vary in size, edge sharpness, darkness, and opacity (Kellogg et al., 1963). Colonies can be viewed on a dissecting microscope with reflecting substages (diffuser and mirror). The variations in colony type represent major structural differences on the surface of gonococcus. Negative staining reveals that small colonies with sharp, refractile edges bear pili ( $P^+$ ) (Swanson et al., 1971; Jepcott et al., 1971). Improvements in the medium led to better growth of the organisms which give rise to dark, opaque colonies (Swanson, 1978a). Thin sections of dark, opaque colonies show individual cocci adjoined to one another at their outer membranes through numerous zones of adherence, which have an intercellular space of about 2 nm (Swanson, 1978a; Swanson et al., 1971). The intergonococcal adhesions account for the altered transmittance of light through opaque colonies. Transparent colonies appear light with the diffuser, and colorless to slightly blue with the mirror. They are shown to contain separate diplococcal units by electron microscopy (Swanson et al., 1971).

Colonial morphology is correlated with virulence of the gonococcus. Piliated organisms are generally virulent in human volunteers whereas nonpiliated gonococci are avirulent, and most fresh clinical isolates are piliated (Sparling and Yos, 1967; Kellogg et al., 1963). In contrast, clinical cultures vary widely in their degree of opacity. Male urethral isolates are predominantly dark and opaque as are pharyngeal and rectal isolates, whereas female cervical isolates are opaque near ovulation and largely transparent at or near the time of menses (James

and Swanson, 1978b). Isolates from the fallopian tube and from disseminated sites (blood, joints) are mostly transparent (Draper et al., 1980). The transparent colony types isolated from females near menses show increased resistance to trypsin, suggesting that environmental factors may influence the ability of opaque organisms to persist at particular anatomical sites (James and Swanson, 1978b).

Interactions of gonococci with other cells is related to colony phenotype. Pili provide the organism with enhanced ability to adhere to human amnion cells (Swanson, 1973). Organisms tend to attach above the perinuclear cisternae, and fibrils radiating from the bacterium surface are seen in contact with the amnion cell membrane. Piliated gonococci adhere more avidly to fallopian tube organ cultures (FTOC) than do non-piliated organisms, and bind specifically to nonciliated, columnar epithelial cells (Ward et al., 1974). In addition, pili-mediated attachment to FTOC is species-specific suggesting that gonococcal pili are determinants of host range, which is limited to humans (Johnson et al., 1977).

The cell-cell interactions associated with opaque colonies appear to involve a protease-sensitive molecule (Swanson, 1978a). Addition of trypsin to broth cultures of opaque gonococci leads to an rapid initial increase in colony-forming units, suggesting that the glue-like surface component of opaque colonies is proteinaceous and upon its digestion, the clumps of bacteria dissociate. Addition of trypsin at a position adjacent to opaque colonies growing on solid medium results in a more transparent appearance within 2 h. A family of outer membrane proteins associated with colonial opacity has been identified and designated

Protein II (P.II), although not all P.IIs give rise to opaque colonies (Heckels, 1977; Walstad et al., 1977; Swanson, 1977). The apparent molecular weight of P.IIs range from about 24,000 to 30,000 and all share the property of heat-modifiability, whereby their migration on sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) is retarded following solubilization at 100°C. P.IIs are susceptible to digestion by trypsin and killing of opaque colonies by trypsin correlates with P.II degradation (Swanson, 1978b). Association of gonococci with polymorphonuclear leukocytes (PMN) was found to be mediated by a trypsin-sensitive, nonpilus association factor (Swanson et al., 1974), later identified as Protein II, but this factor does not always give rise to colonial opacity (Swanson, 1977; Swanson and King, 1978). Gonococci giving rise to opaque colonies and bearing P.II on their surface display an increased association with buccal epithelial cells compared to P.II-deficient organisms and furthermore, the degree of adherence varies for each P.II molecular species (Lambden et al., 1979). In addition, opaque gonococci bind better than their transparent counterparts to HeLa (cervical epithelial) cells (James et al., 1980) and to Chang conjunctiva epithelial cells (Virji and Everson, 1981). The differential binding of P.II-bearing organisms to eucaryotic cells in vitro and the preponderance of opaque gonococci at certain anatomical sites, has led several investigators to suggest that P.II-mediated attachment is a determinant of tissue tropism.

In summary, most (but not all) P.II-bearing organisms are opaque and all opaque gonococci possess P.II. The only apparent exception to the latter statement are P.II-deficient light colonies which appear to have opaque centers and transparent rims and are quite distinct



(unpublished observations). All dark, opaque colonies display extensive intergonococcal aggregation through zones of adherence. Many organisms bearing opacity-associated P.II (P.II<sub>op</sub>) show increased adherence to epithelial cells. An increase in association with PMNs is attributed to P.IIs which may or may not give rise to opaque colonies (P.II<sub>nop</sub>, nonopacity-associated P.II). In conclusion, both pili and P.II qualify as attachment factors of the gonococcus.

The contribution of P.II and pili to surface hydrophobicity has been explored. No correlation is found between the binding of P.II-bearing gonococci to buccal epithelial cells and to hydrophobic substituted gels (Lambden et al., 1979). Two-phase partitioning of gonococci in polyethylene glycol and dextran show nonpiliated organisms to be slightly more hydrophobic than those which are piliated (Magnusson et al., 1980). On the contrary, colony size and convexity (doming) can be best explained by the hydrophobicity of pili, whereby pili would tend to associate with other hydrophobic surfaces (such as themselves) rather than with the aqueous agar surface (Swanson, 1977). Opaque colonies are sometimes noticeably smaller and have better defined edges than their transparent counterparts (unpublished observations). In addition, piliated gonococci (and opaque to a lesser extent) can be selected for by pellicle formation in static broth culture (M. Koomey, personal communication); pellicle formation correlates with increased surface hydrophobicity in other organisms (Speert et al., 1986). Opaque colonies are found to be more hydrophobic than transparent by measurement of the contact angle of a liquid droplet placed on top of the colony (James, 1986). It is difficult to draw conclusions on net surface hydrophobicity based on these incongruous findings. The

importance of hydrophobicity in gonococcal attachment is not established.

Protein II and pilin represent independent families of proteins which undergo antigenic and phase variation, whereby the synthesis of P.II or pilin is switched on and off at a high frequency (about  $10^{-3}$ ), and switching to different molecular forms occurs as part of this mechanism. Phase and antigenic variation of pili can be attributed to a single, nonreciprocal intragenic recombinational event, and it appears that the capacity for antigenic variation of pilin protein is tremendous (Koomey and Falkow, 1985; Hagblom et al., 1985; Bergstrom et al., 1986; Swanson et al., 1985; Meyer et al., 1982). The pilin subunit is approximately 20 kDa, and is comprised of an immunorecessive N-terminal portion which is believed to contain a conserved binding region, and an immunodominant, variable C-terminal portion (Schoolnik et al., 1984). Antibodies directed against peptides corresponding to conserved sequences of pilin can block the attachment of piliated gonococci to endometrial cells (Rothbard et al., 1985). The ligand recognized by pili in mediating attachment to epithelial cells remains undefined.

A single gonococcus can express multiple P.IIs (up to about 3) at a time, and 6 to 8 P.IIs of distinct molecular size have been identified in most strains examined (Heckels, 1977; Swanson, 1977; Black et al., 1984). This estimate roughly corresponds to the number of distinct P.II genes per organism (Connell et al., 1986). Whereas pilus phase variation often involves extensive genomic rearrangement, opacity phase variants show no heterogeneity in hybridization patterns with a probe corresponding to a large portion of the opacity structural gene (Stern

et al., 1984; Stern et al., 1985; Stern et al., 1986). The DNA sequence corresponding to the leader peptide contains anywhere from 7 to 28 repeats of the pentamer CTCTT; it is postulated that the number of repeats in the leader sequence determines whether the correct reading frame is available for translation of a functional protein, and thereby phase switching is regulated (Stern et al., 1986; Connell et al., 1986). It is not known what mechanism controls the number of copies of the CTCTT element. Antigenic variation of P.II appears to involve a gene conversion event based on differences in Southern hybridization patterns using probes corresponding only to variable portions of the opacity gene (Stern et al., 1986). Comparison of DNA sequences of P.IIs from strain MS11 reveals 3 major variable regions: a small region at the N-terminus, and larger regions mapping at the N-terminal side near the center of the mature molecule (HV1) and the other at one-third the distance from the C-terminus (HV2) (Connell et al., 1986; Stern et al., 1985; Stern et al., 1986). Peptide maps of chymotrypsin digests of P.IIops originating from different strains display a moderate degree of structural homology, as do P.IIops and leukocyte-association factors (Swanson, 1980a).

Cell surface-labeling by iodination and proteolytic treatment of intact gonococci demonstrate that P.IIs are accessible on the surface (Swanson, 1978b). Tryptic peptide maps of surface-labeled P.IIs demonstrate that the few peptides which are unique to an individual P.II are surface-exposed, and based on their chromatographic position are probably more hydrophilic than the other peptides (Heckels, 1981). Cyanogen bromide cleavage at a single site generates common and distinct halves of P.II molecules of strain P9, and both are accessible to

surface labeling (Heckels, 1981). Further studies on peptide maps of surface-labeled P.IIs reveals that unique peptides are located at both exposed and buried positions, and that several of the common peptides are exposed to the surface (Judd, 1985). When intact organisms undergo extensive digestion with trypsin, a 6,000 dalton fragment remains associated with the membrane, suggesting that as much as 75% of the molecule is not buried in the membrane (Blake et al., 1981).

The surface disposition of common and variable antigenic domains of P.II has been studied. Immunization of rabbits with P.II-bearing gonococci elicits antibodies directed largely at the homologous P.II used for immunization (Swanson and Barrera, 1983). Antibodies raised by immunization of rabbits with P.II-bearing outer membrane vesicles (OMV) and tested against OMVs bearing homologous and heterologous P.IIs display strong reactivity with the homologous P.II-bearing OMVs only (Diaz and Heckels, 1982). These results indicate that when P.IIs are presented on the intact gonococcal surface, the immunodominant regions are unique for that P.II species. However in other studies, whole gonococcus immunization elicits antibodies having strong reactivity to P.IIs of other strains as well (Blake and Gotschlich, 1984); the reason for the inconsistent findings is not known, but it may be that the surface-exposed common domains of P.II demonstrated by peptide mapping (Judd, 1985) are sufficiently immunogenic. Purified P.II administered in Freund's adjuvant elicits antibodies that are cross-reactive with most other P.II species from either the same or different strain, tested on immunoblots in their heat-modified form (Swanson and Barrera, 1983) and tested by enzyme-linked immunoabsorbant assay in their native form (Blake and Gotschlich, 1984). However, others observe that some

purified P.IIs (injected in Freund's adjuvant) elicit antibodies displaying little cross-reactivity with heterologous P.IIs, and thus it is not entirely clear whether the common domains of P.II are more immunogenic when presented in a purified and denatured form (Newhall et al., 1985). Monoclonal antibodies have been produced which are specific to individual P.II molecules (Newhall et al., 1985; Black et al., 1984), confirming that intrastrain antigenic variation occurs among P.IIs. It has been proposed that the exposure of distinct antigenic moieties on the gonococcal surface may contribute to the variability among P.IIs in their ability to adhere to a particular cell type (Swanson and Barrera, 1983).

Interactions between P.II-bearing gonococci and eucaryotic cells can be blocked with monoclonal antibodies directed against P.II. Attachment of opaque gonococci to HeLa cells is diminished following preincubation of the organisms with anti-P.II Fab fragments (Sugasawara et al., 1983). Similarly, gonococcal-mediated stimulation of chemiluminescence in neutrophils is blocked following preincubation with anti-P.II monoclonal antibodies specific for the P.II present on the gonococcal surface (Rest et al., 1985; Virji and Heckels, 1986). These findings strongly suggest that P.II is engaged in direct contact with eucaryotic cells. A role for surface carbohydrate in P.II-mediated adhesion has been addressed by several investigators. Attachment of P.II-bearing OMVs to buccal cells is not inhibited in the presence of a variety of sugars, but binding is diminished following treatment of the buccal cell surface with glycosidic enzymes (Heckels, 1982). It has been reported that stimulation of the neutrophil oxidative burst by P.II-bearing gonococci is blocked in the presence of 100 mM

beta-D-glucosamine, N-acetyl-D-neuraminic acid, and mannose (Rest et al., 1985). Purified P.IIop binds directly to purified gonococcal lipopolysaccharide (LPS), whereas P.IInop fails to bind; data indicates that it is the oligosaccharide portion of LPS which participates in this interaction and not lipid A (Blake, 1985) (M. Blake, personal communication). It is proposed that a receptor-ligand relationship between P.IIop and LPS underlies the basis for the intergonococcal adhesions which give rise to the opaque colony phenotype (Blake, 1985). Radiolabeled whole gonococci were tested for binding to a variety of glycosphingolipids separated on a thin layer chromatogram (Deal et al., 1986), a technique which has been used successfully to demonstrate digalactose-specific binding by uropathogenic E. coli (Bock et al., 1985). Gonococci bind to lactotriaosylceramide, and binding is independent of both piliation and the presence of P.II. Neither pili-dependent or P.II-dependent binding to any glycolipid is observed in these studies. The common adhesin is tentatively identified as a 22.5 kDa protein distinct from pilin.

In summary, both P.II and pili qualify as attachment factors. Inhibition of gonococcal attachment with antibodies directed against P.II or pilin provides strong evidence that each of these proteins act as adhesins engaged in direct contact with the adjacent cell surface. The contribution of pili and P.II to net surface hydrophobicity is not entirely clear. The ligands recognized in P.II-mediated and pili-mediated adherence have not been characterized. In Chapter 4, the nature of P.IIop interactions with other macromolecules is chemically defined.

## 1.2 INVASION

### 1.2.1 Overview

Histopathological examination of infected mammalian tissue by light microscopy shows penetration by some microorganisms into the lamina propria which underlies the mucosal epithelial lining (Harkness, 1948; LaBrec et al., 1964). Electron microscopy confirms that bacteria gain access to subepithelial tissue by passage in between cells of the mucosal epithelial lining, or by entry into the interior of an epithelial cell (Takeuchi, 1967; Takeuchi et al., 1965). The term "invasion" is often used loosely to describe both of these events, but I shall restrict its definition to the process by which a bacterium gains access to an intracellular locale and survives its new environment. Following adherence to the epithelial cell surface, invasive bacteria are internalized by endocytosis, maintained intracellularly within a membrane-bound vacuole or lie free in the cytoplasm, may or may not replicate at an intracellular site, and exit from the host cell by exocytosis or cell lysis. The intracellular environment of the host may be advantageous to the bacterium by providing a protective haven from the immune response or a nutrient-rich milieu favorable to growth. Passage through the epithelial cell and penetration into deeper tissue allows an organism to enter the bloodstream from which it can disseminate to distal sites throughout the body. Bacteria which are invasive include pathogens of the genus Shigella, Neisseria, Yersinia, Salmonella, Chlamydia, and Rickettsia.

Invasive bacteria appear to enter epithelial cells by a phagocytic mechanism. Phagocytosis has been best studied in those cells considered to be "professional" phagocytes: macrophage and PMNs. Phagocytosis by macrophage and PMNs is promoted through specific receptors (eg., IgG and complement), whereby an attached particle is enveloped by plasma membrane through sequential and circumferential interactions of receptors with ligand (Griffin et al., 1975). The initial surface stimulus which precedes the endocytic event is coupled to movements of ions across the plasma membrane (Korchak and Weissmann, 1978). Ingestion is an energy-dependent process, requiring microfilaments for pseudopod extension. A filamentous network of actin and myosin lies beneath the plasma membrane at the base of the endocytic cup; actin polymerization, gelation, and contraction, controlled in part by the local concentration of calcium, are believed to provide the driving force behind formation of the phagocytic vacuole (Hartwig and Stossel, 1976; Reaven and Axline, 1973). The fungal-derived drug cytochalasin B (CB) prevents phagocytosis by blocking actin polymerization, and this drug also affects cell shape and other cell movements such as locomotion and plasma membrane ruffling (Zigmond and Hirsch, 1972; Davies et al., 1973; Hartwig and Stossel, 1979). When the tips of the advancing pseudopods meet, the membranes fuse and the particle becomes contained within a membrane-bound vacuole. Nonopsonized particles are phagocytosed following recognition by other receptors, such as the mannose receptor which mediates uptake of yeast cell walls (Sung et al., 1983), or perhaps by mechanisms not requiring a particular receptor-ligand interaction. A "specific" uptake mechanism is one whereby ingestion of a certain particle proceeds at a rate substantially greater than that of nonspecific particles such as latex beads.



Epithelial cells are capable of phagocytosis and are considered to be "nonprofessional" phagocytes since their rate of particle ingestion is considerably less than that of PMNs and macrophage. Follicle-associated epithelial cells which overlay lymphoid follicles such as Peyer's patches, phagocytose bacteria and other particles on their microvillous surface and transport them within cytoplasmic vacuoles to the basolateral membrane where they are exocytosed (Bockman et al., 1983). Retinal pigment epithelial cells specifically phagocytose retinal rod outer segments, perhaps through a mannose receptor, and microvilli participate in this process as well (Mayerson and Hall, 1986). Evidence supporting a phagocytic mode of entry of invasive bacteria into epithelial cells includes temperature-dependence, sensitivity to CB, and enclosure within a membrane-bound vacuole (Ward et al., 1974; Hale et al., 1979; Kihlstrom and Nilsson, 1977; Hale and Bonventre, 1979; Ward and Murray, 1984; Friis, 1972; Walker and Winkler, 1978; Lee et al., 1977). Since epithelial cells ingest only select bacteria by specific uptake mechanisms, it is presumed that invasive bacteria provide the stimulus for their ingestion, a process often referred to as "induced" phagocytosis.

Chlamydia and Rickettsia spp. are obligate intracellular parasites which must invade eucaryotic cells in order to survive. The elementary body of chlamydiae binds to the host cell surface, induces phagocytosis by unknown means, divides within a giant cytoplasmic vacuole, and escapes the host cell by rupturing it (Schachter and Caldwell, 1980). Phagocytosis of chlamydiae by HeLa cells or fibroblasts (L cells) is 10 to 100 times greater than uptake of latex spheres (Byrne and Moulder, 1978). Chlamydiae are engulfed by microvilli on the HeLa cell membrane

and clathrin-coated pits are not associated with the endocytic vesicles, although coated pits are observed elsewhere in the cell; furthermore, endocytosis is blocked by cytochalasin D suggesting a dependence on microfilament function (Ward and Murray, 1984). Chlamydiae differ from rickettsiae (an arthropod-borne organism) in that the latter escape from the phagocytic vacuole and lie free in the cytoplasm where they replicate; it has been postulated that a phospholipase produced by rickettsiae is responsible for phagosome escape (Winkler and Miller, 1982).

Facultative intracellular parasites can grow and survive outside of an intracellular environment, however the ability to invade host cells has a profound influence on their pathogenicity. Salmonella penetrate the epithelial barrier of the intestine by passing through and occasionally in between cells, causing local inflammation and in some instances, gain access to the bloodstream (Takeuchi, 1967; Takeuchi and Sprinz, 1967). In the process of Salmonella typhimurium invasion, the microvilli of the brush border and the apical cytoplasm degenerate and bleb, and the bacterium becomes enclosed within a vacuole formed from the degenerated luminal surface or from the lateral plasmalemma of two adjoining cells. The enteric pathogen Yersinia enterocolitica is ingested by phagocytosis, remains within membrane-bound vacuoles in the cytoplasm, and does not replicate intracellularly (Lee et al., 1977; Devenish and Schiemann, 1981). Nonviable Yersinia killed by ultraviolet radiation are invasive for HeLa cells (Pedersen et al., 1979), thus distinguishing them from some of the other invasive pathogens. A single outer membrane protein responsible for Yersinia pseudotuberculosis invasion of HEP-2 cells has been identified and the cloned gene confers the ability to invade HEP-2 cells on the E. coli clone (Isberg and

Falkow, 1985). Separation of the attachment and invasion functions of the cloned gene product has not been achieved as yet.

In summary, invasive bacteria "induce" their uptake by nonprofessional phagocytic cells. Some adherent organisms become surrounded by microvillous projections on the epithelial cell surface (Ward et al., 1974; McGee et al., 1983), whereas others cause degeneration of microvilli (Takeuchi, 1967; Takeuchi et al., 1965; Takeuchi and Sprinz, 1967). At the intracellular site, bacteria are enclosed within a vacuole or lie free in the cytoplasm, and some replicate. A correlation between specific adherence mechanisms and invasion has not yet been drawn. Only recently have investigators begun to identify the bacterial elements essential for penetration and the intracellular life cycle (Isberg and Falkow, 1985; Hale et al., 1985; Maurelli et al., 1985; Sansonetti et al., 1986).

#### 1.2.2 Neisseria gonorrhoeae

Primary infection with Neisseria gonorrhoeae occurs most commonly in the columnar or transitional (squamocolumnar) epithelium of the genital tract (Harkness, 1948). Electron microscopy of infected tissue shows replicating gonococci on the surface of desquamated cervical cells (Evans, 1977) and on urethral cells (Ward et al., 1975), and clusters or sheets of gonococci are evident on exfoliated epithelial cells (Ward et al., 1975; Novotny and Short, 1977; Ward and Watt, 1972). Organisms attached to the epithelial cell surface often appear embedded or partially engulfed, surrounded by short pseudopodia. A major pathogenic

event following attachment is penetration of the mucosal lining, and organisms accumulate in the subepithelial connective tissue (Harkness, 1948). Gonococci appear to enter cells by a phagocytic mechanism, and bacteria can be seen surrounded by microvillous projections on the epithelial surface of FTOCs, and in membrane-bound vacuoles within the cytoplasm (Ward et al., 1974; McGee et al., 1983; Ward et al., 1975; Novotny and Short, 1977; Evans, 1977). Intracellular gonococci are observed within epithelial cells of the urethra, cervix, and fallopian tube.

The piliated, transparent colony type is presumed to be the highly invasive form of N. gonorrhoeae. This phenotype is isolated from disseminated sites, fallopian tubes, and the cervix during menses (Draper et al., 1980; James and Swanson, 1978b). While the incidence of disseminated gonococcal infection (DGI) is only about 1 %, penetration of the epithelial barrier is essential for gaining access to the bloodstream. About 10 to 15 % of infected women develop pelvic inflammatory disease involving infection of the fallopian tubes (salpingitis), with clinical onset usually occurring just at or after menstruation (McGee, 1984). The exact nature of the naturally occurring interaction of gonococci with fallopian tube epithelium is not entirely clear, nevertheless, the FTOC model has been used extensively for the in vitro study of gonococcal invasion (McGee and Horn, 1979; Ward et al., 1974; McGee et al., 1983). While much of our understanding of gonococcal invasion has been derived from study of FTOC, one should keep in mind our lack of clear understanding of the importance and nature of gonococcal invasion at other key anatomical sites, such as the stratified and pseudo-stratified columnar epithelium of the male urethra and the

transitional zone between the ecto- and endocervix (Ward and Watt, 1972; Harkness, 1948; Ward et al., 1975; Evans, 1977; Novotny and Short, 1977). It is at these sites where infection is most common (Harkness, 1948) and gonococci of the piliated, opaque phenotype predominate (James and Swanson, 1978b; Draper et al., 1980).

Explants of human fallopian tubes can be infected with gonococci and the course of infection followed by electron microscopy (Watt and Ward, 1977; Ward et al., 1974; McGee et al., 1983; McGee and Horn, 1979). Organisms attach almost exclusively to the microvillous surface of nonciliated cells. These cells are columnar, typically secrete mucus, and are adjoined to one another by tight junctions, thereby creating a barrier to the subepithelial tissue. At about 20 h after infection, the microvilli surrounding the attached gonococcus begin to retract, pulling the organism into a membrane-bound vacuole in a manner similar to that employed by professional phagocytes (McGee et al., 1983). Replication occurs at intracellular sites, and cytoplasmic vacuoles enlarge as a result of replication or from fusion with other vacuoles. Gonococci are transported to the base of the cell where they are eventually exocytosed into the subepithelial connective tissue. A similar mechanism for invasion is observed with Neisseria meningitidis cultivated on nasopharyngeal organ cultures (McGee et al., 1983; Stephens et al., 1983). Cultivation of gonococci on FTOC reveals that 1% of attached organisms penetrate by 8 h (Ward et al., 1975). A comparison of piliated and nonpiliated phenotypes show that while piliated attach more efficiently, penetration is equally effective for both types once attached (Ward et al., 1975; McGee et al., 1983).

Electron micrographs of gonococci cultivated with monolayers of tissue culture cells show organisms enclosed in membrane-bound vacuoles within the cytoplasm (Brodeur et al., 1977; Tyeryar et al., 1974; Ota et al., 1975; Waitkins and Flynn, 1973; Swanson, 1973). Cultivation on endometrial cell monolayers leads to attachment of gonococci at cell junctions at 6 h, deeper embedment by 8 h, internalization and escape from vacuoles at 12 h, and enmeshment in a matrix of cellular debris by 24 h (Shaw and Falkow, 1986); similar clusters of gonococci enmeshed in epithelial cell remnants has been observed in exudates from infected individuals (Novotny and Short, 1977). Furthermore, invasion of cultured endometrial cells is independent of piliation or P.II. Gonococcal DNA encoding those elements which give rise to the invasive phenotype can be cloned into E. coli, and the invasion genes encode a multitude of proteins of distinct molecular size which are currently being characterized (Shaw and Falkow, 1986).

The mechanism by which gonococci induce their phagocytosis by epithelial cells is not known. One speculation was that the IgA1 protease produced by gonococci serves to attach the organism to IgA1 present on the surface of secretory cells (Swanson, 1980b). Invasion of FTOC by an IgA1 protease-deficient mutant was compared to its wild type parent and no differences were found, suggesting that IgA1 protease does not play an essential role in invasion (Cooper et al., 1984). Another hypothesis concerning the mechanism of gonococcal invasion proposes that the porin (major outer membrane protein or Protein I, P.I) of the gonococcus inserts into the epithelial cell membrane, causing alterations in ion flow which trigger the phagocytic event (Blake and Gotschlich, 1983). The efficiency at which Neisseria spp. spontaneously transfer P.I into

planar lipid bilayers appears to correlate with invasive capacity, whereby N. meningitidis is extremely efficient, followed by DGI strains, gonococci from localized infections, and the commensal N. sicca (Lynch et al., 1984). The exact mechanism by which gonococci induce phagocytosis remains to be determined.

Chapter 3 examines several events which take place during the cultivation of gonococci on HeLa cell monolayers. The degree of invasion is measured and P.II-associated interactions are defined.

### 1.2.3 Shigella flexneri and Enteroinvasive Escherichia coli

Enteroinvasive E. coli (EIEC) penetrate the mucosal epithelial lining of the gut, producing a dysentery-like illness similar to that caused by Shigella flexneri (DuPont et al., 1971). Shigella flexneri, which has been studied more extensively than "Shigella-like" E. coli, has been orally administered to rhesus monkeys and the infected tissue examined (Takeuchi et al., 1968; LaBrec et al., 1964). The mucosa of the colon displays inflammation and micro-ulceration. Microvilli are damaged and organisms are found lying free in the cytoplasm or within membrane-bound vacuoles of epithelial cells, where they replicate and spread laterally to infect new cells. Both S. flexneri and EIEC fed to starved guinea pigs induce acute enteritis and organisms are observed within epithelial cells of the intestinal lumen (DuPont et al., 1971; Takeuchi et al., 1965; LaBrec et al., 1964).

Shigella flexneri attach to the surface of cultured epithelial cells (HeLa cells, Henle 407 cells) and stimulate plasma membrane ruffling, which is accompanied by ingestion of the bacterium and vesicle formation (Ogawa et al., 1968). Penetration of Henle cells by S. flexneri can be blocked by the microfilament-disrupting agent CB, suggesting that internalization occurs by a phagocytic mechanism; electron microscopy of thin sections show organisms surrounded by pseudopod like structures during the process of engulfment, and cytoskeletal elements in close proximity to membrane-enclosed vacuoles (Hale et al., 1979; Sansonetti et al., 1986; Hale et al., 1983). Internalized Shigellae are observed within membrane-bound vacuoles or lying free in the cytoplasm, and rupture of the phagosomal membrane strongly correlates with the ability to replicate intracellularly; phagosome rupture and intracellular growth has been observed within minutes following penetration (Hale et al., 1979; Takeuchi et al., 1968; LaBrec et al., 1964; Sansonetti et al., 1986). Phase-contrast microscopy has shown intracellular organisms to be pressing against the cytoplasmic side of the plasma membrane of the infected cell, causing a protrusion to emanate outward from the cell surface (Ogawa et al., 1968). It has been suggested that organisms escape the infected epithelial cell either by rupturing it or by a nonlytic mechanism and proceed to invade neighboring cells, thereby spreading the area of infection (Oaks et al., 1985; Takeuchi et al., 1968).

The molecular mechanisms by which S. flexneri and EIEC promote uptake by nonprofessional phagocytes is currently not understood. The 2 organisms have key virulence factors in common (Hale et al., 1983; Hale et al., 1985). High molecular weight plasmids are essential for



invasion by EIEC and S. flexneri, and provide each organism with the capacity to invade cultured epithelial cells in vitro (Sansonetti et al., 1982; Harris et al., 1982; Silva et al., 1982; Sansonetti et al., 1982). The 140 MDa plasmids of the organisms share a significant degree of DNA sequence homology, and encode for proteins of identical molecular size and antigenicity (Sansonetti et al., 1983; Hale et al., 1983; Hale et al., 1985). The 140 MDa plasmid can be transformed into anucleate minicells and the ability to invade HeLa cells is retained (Hale et al., 1985; Hale et al., 1983). Of the 7 polypeptides (a through g) synthesized by invasive minicells, 3 (e through g) have an extremely basic isoelectric point and may function as DNA regulatory proteins. The other virulence-associated proteins (a through d) are highly reactive with antisera from convalescing monkeys, and it appears that 2 or 3 of them are present on the bacterial cell surface in small quantities (Oaks et al., 1986; Hale et al., 1985). The large plasmid has been subcloned to a 37 kilobase segment which carries the minimum sequence necessary for invasion, and the plasmid-specific peptides "a through d" are produced (Maurelli et al., 1985). A 32 MDa subclone of the large plasmid encodes a hemolysin capable of lysing the membrane of the phagocytic vacuole, and rapid intracellular growth accompanies early and efficient lysis of the phagosome (Sansonetti et al., 1986). The precise function of the gene products which induce phagocytosis by epithelial cells remains to be defined. The contribution of the host cell to induced phagocytosis and intracellular growth is explored in Chapter 2.

## CHAPTER 2

N OF EPITHELIAL CELLS BY ENTEROINVASIVE E. COLI

The term invasion is used in reference to the overall infection process consisting of a penetration step, an intracellular stage, escape from the cell, and reinfection of new epithelial cells. The original intent in studying invasion of HeLa cell monolayers by EIEC was to provide a standard for comparison with a similar assay under development for gonococcal invasion. An observation was made with HeLa cell monolayers which contained a minor population (approximately 1.0 %) of well-spread, binucleated cells. When incubated with EIEC, intracellular E. coli were found to be disproportionately associated with the few binucleated cells present. The finding raised the question as to whether artificially fused HeLa cells could better support the invasion of EIEC. This chapter describes increased infection by EIEC of HeLa cell monolayers treated with the fusogen, polyethylene glycol (PEG), and the striking accumulation of EIEC in cellular extensions of multinucleated cells.

## 2.1 MATERIALS AND METHODS

Plasmid preparation: Plasmids were prepared by alkaline extraction (Birnboim and Doly, 1979), electrophoresed on 0.7% agarose gels using a Tris-borate-EDTA buffer system (Meyers et al., 1976), and stained with ethidium bromide.

Bacteria: Enteroinvasive Escherichia coli strain 5800-82 (0124) was obtained from Dr. S. Hirshfeld (University of California at San Francisco). Figure 1 shows the plasmid profile of the EIEC under study,

which harbored the 140 MDa plasmid associated with the ability to invade HeLa cells. E. coli strain HB101 (K-12 x B) lacked the plasmid.

Tissue culture: HeLa cells (ATCC CCL2) were derived from a cervical carcinoma of human origin and were epithelial-like in morphology. Monolayers were grown in 35 mm polystyrene wells (Flow Laboratories, McLean, VA) at 37°C with 6% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Flow) at pH 7.4 containing 10% heat-inactivated zeta D serum (AMF Biologicals, Seguin, TX), and antibiotics (zeta-DME).

Polyethylene glycol treatment of HeLa cell monolayers: One day prior to attaining confluence, HeLa cell monolayers were washed in phosphate-buffered saline (PBS) and incubated with prewarmed 40% PEG 1000 (T.J. Baker, Phillipsburg, NJ) in PBS (unless indicated otherwise) for 1 min at 37°C. The PEG was removed and cells washed extensively with PBS to remove residual PEG. Fresh growth medium (zeta-DME) lacking antibiotics was added and cells incubated for an additional 18 h before use in the invasion assay. This treatment generally led to fusion of greater than 75% of the cells.

Invasion assay: Bacteria were grown to stationary phase in LB broth at 37°C. HeLa cell monolayers (PEG-treated or untreated) were inoculated with stationary phase E. coli ranging from 1 to 10 bacteria per HeLa nucleus, and incubated for 2 to 3 h at 37°C. The inoculation medium consisted of brain-heart infusion broth containing bile salts and 20% zeta serum, prepared as specified (Mehlman et al., 1982). For CB (Sigma Chemical Company, St. Louis, MO) treatment, monolayers were subjected to 5 ug/ml of CB 30 min prior to addition of E. coli, and CB

was maintained throughout the experiment. After a 2 to 3 h incubation period, nonadherent bacteria were removed from the infected monolayers, HeLa cells were washed, and zeta-DME containing gentamicin (5 ug/ml) and lysozyme (300 ug/ml) (intracellular growth medium) was added. Incubation continued for an additional 2 to 3 h (intracellular growth period). During this time extracellular organisms were killed, while intracellular bacteria survived due to the exclusion of the antibiotic by the eucaryotic cell plasma membrane (Mehlman et al., 1982; Hale et al., 1979; Mandell, 1973). Monolayers were washed and HeLa cells were detached by 0.25% trypsin, centrifuged at 15,000 x g for 5 min, resuspended in zeta-DME, vigorously vortexed to release intracellular organisms, and plated on LB agar. Colony forming units (CFUs) were enumerated.

Light microscopy: Infected monolayers were prepared as described above, except that HeLa cells were grown on glass Lab-Tek chamber slides or on Thermanox coverslips (Miles Laboratories, Inc., Naperville, IL). At the end of the intracellular growth period, monolayers were washed in PBS, fixed 10 min in methanol, stained 10 min each with May-Grunwald and Giemsa stains, dehydrated and decolorized in acetone and xylene, and mounted with Permount (Fisher Scientific Company, Pittsburgh, PA). Individual cells were scored positive for invasion if 5 or more organisms remained associated with the cell (Mehlman et al., 1982).

## 2.2 RESULTS

### 2.2.1 EIEC Invasion of Multinucleated HeLa Cells

Multinucleated HeLa cells arose naturally at a low frequency in normal monolayers (approximately 1.0%). Table 1 indicates that EIEC 5800-82 (0124) preferentially invaded this minor population of cells. Enhancement of the penetration step appeared to account partially for increased invasion of multinucleated cells since increased cell surface area alone does not compensate for the difference. The observation raised the question whether artificially fused cells were better targets for invasion. Infection by EIEC was enhanced when HeLa cells were pre-treated with PEG (Table 2). The extent of enhancement ranged from none to over 20-fold, averaging approximately 7-fold. The level of HeLa cell-association by noninvasive HB101 showed no obvious net change in response to PEG treatment; thus, PEG does not appear to produce a general stimulation of endocytosis.

By modulating the PEG concentration, the percentage of HeLa cells which fused as well as the size of the fused cells could be controlled. HeLa cells were divided into three groups (mononucleated, bi- to tetranucleated, and larger) and the extent of fusion was assessed by measuring the proportion of total nuclei falling into each group at a given PEG concentration (Figure 2). Treatment of HeLa monolayers with up to 20% PEG led to fusion of less than 3% of the cells, which is comparable to that found in untreated monolayers. At 25% PEG, one-fifth of the cells had fused, and at 30% PEG a sharp increase in cell fusion

Figure 1: Agarose gel electrophoresis of plasmid DNA from E. coli. Lanes: (A) 5800-82 (0124), (B) HB101. The 140 MDa plasmid is indicated.

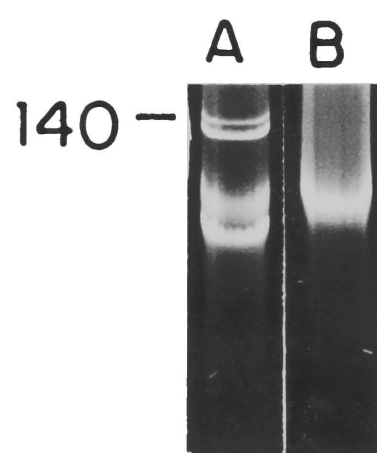




Table 1: Frequency of invasion of mononucleated and  
multinucleated HeLa cells.

(a) E. coli were incubated with HeLa cell monolayers for 2 to 3 h, and nonadherent organisms were removed and replaced with intracellular growth medium for an additional 2 to 3 h. Multiplicity of infection ranged from 1 to 10 bacteria per nucleus; experiments are listed in order of increased invasion. A single cell was scored as positive for invasion if 5 or more organisms were associated. The number of multinucleated cells scored is low because they were present as a minor population; multinucleated cells had 2 to 4 nuclei. Multinucleated cells were sought out and scored, and the mononucleated cells immediately surrounding multinucleated cells were scored as well. For mononucleated cells, 300 to 500 cells were scored. The percentage of cells invaded is indicated in parentheses.

Table 1: Frequency of invasion of mononucleated and  
multinucleated HeLa cells (a).

<u>Expt.</u>	<u>No. of cells invaded/no. of cells scored</u>		Chi-square
	<u>Mononucleated</u>	<u>Multinucleated</u>	<u>Analysis</u>
1	1/100 (1)	3/20 (15)	P < 0.005
2	1/100 (1)	3/17 (18)	P < 0.005
3	2/100 (2)	2/20 (10)	P < 0.100
4	2/100 (2)	6/20 (30)	P < 0.005
5	5/100 (5)	7/20 (35)	P < 0.005
6	21/100 (21)	11/20 (55)	P < 0.005
7	35/100 (35)	16/20 (80)	P < 0.005

Table 2: Effect of PEG treatment of HeLa cells on E. coli yield.

- (a) HeLa cells were untreated or treated with 30 to 40% PEG. Monolayers were incubated with E. coli as described in Table 1. CFUs were measured following an intracellular growth period of 2 to 3 h.
- (b) The increase (+) or decrease (-) in yield (CFUs) as a consequence of PEG-treatment of HeLa monolayers. Significant effect ( $\alpha = 0.01$ ) of PEG on EIEC 0124 (Expts. 1 - 7) according to the Wilcoxon matched-pairs test. No significant effect ( $\alpha \gg 0.10$ ) of PEG on HB101 (Expts. 8 - 12).
- (c) Arithmetic mean and standard deviation.

Table 2: Effect of PEG treatment of HeLa cells on E. coli yield.

Treatment				
(CFU x 10-3 (a))				
<u>Expt</u>	<u>Organism</u>	<u>PEG</u>	<u>None</u>	<u>Change</u> <sup>(b)</sup>
1	0124	1020	35	+ 21.14
2	0124	1400	495	+ 2.83
3	0124	3530	913	+ 3.90
4	0124	5650	625	+ 9.04
5	0124	6330	1140	+ 5.55
6	0124	10300	9830	+ 1.05
7	0124	12200	2940	+ 4.14
Average <sup>(c)</sup>				+ 6.81 <u>±</u> 6.79
8	HB101	1.80	0.97	+ 1.86
9	HB101	2.80	4.30	- 1.54
10	HB101	2.87	42.80	- 14.91
11	HB101	21.00	3.00	+ 7.00
12	HB101	45.00	44.00	+ 1.02
Average				- 1.31 <u>±</u> 8.21

Figure 2: Correlation of PEG concentration and HeLa cell fusion. HeLa cells were treated with the PEG concentration indicated, and nuclei were scored as to their distribution in mononucleated cells (open), binucleated to tetranucleated cells (hatched) or larger (filled). For PEG concentrations up to 20 %, more than 97 % of the nuclei were distributed in mononucleated cells and less than 3 % were present in multinucleated cells. Multinucleated HeLa cells in untreated monolayers represented less than 3 % of the total nuclei population. For each PEG concentration, a minimum of 500 nuclei were scored.

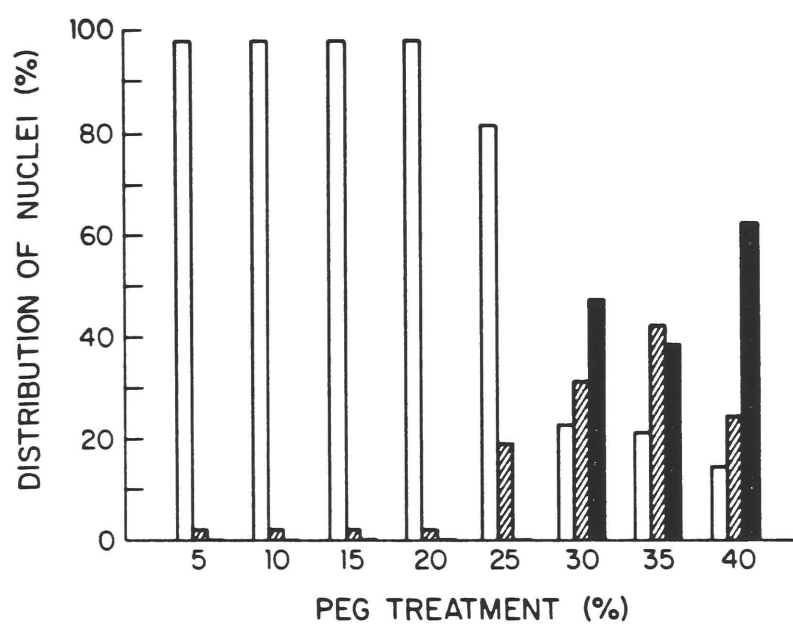


Table 3: Correlation of PEG concentration, HeLa cell fusion,  
and frequency of invasion by EIEC.

(a) HeLa cells were incubated with EIEC as described in Table 1. Cells were grouped as mononucleated, bi- to tetranucleated, and 5 or more nuclei per cell; refer to Figure 2 for the extent of HeLa cell fusion at each PEG concentration. For each PEG concentration, a minimum of 500 nuclei were scored. For multinucleated cells present in cultures treated with 20% PEG or less, a minimum of 15 cells were scored. This is a single representative experiment.

ND, none detected.

Table 3: Correlation of PEG concentration, HeLa cell fusion,  
and frequency of invasion by EIEC.

<u>[PEG]</u>	<u>% of Cells Invaded (a)</u>		
	<u>Mono Nucleated</u>	<u>Bi to Tetra Nucleated</u>	<u>5 or more Nuclei</u>
5 %	16	42	ND
10 %	15	37	ND
15 %	15	52	ND
20 %	19	33	ND
25 %	21	58	ND
30 %	47	67	100
35 %	53	75	91
40 %	63	82	96



Table 4: Effect of cytochalasin B on EIEC yield with  
PEG-treated and untreated HeLa cells.

(a) Monolayers were treated (or untreated) with 40 % PEG, and were exposed (or unexposed) to 5 ug/ml CB. HeLa cells were incubated with EIEC as described in Table 1, in the presence or absence of CB. Colony-forming units were measured after an intracellular growth period of 2 to 3 h.

Table 4: Effect of cytochalasin B on EIEC yield with  
PEG-treated and untreated HeLa cells.

<u>PEG Fusion</u>	<u>CB</u>	CFU x 10 <sup>-3</sup> (a)	
		<u>Expt 1</u>	<u>Expt 2</u>
+	-	3530.0	10300.0
+	+	9.7	20.5
Decrease due to CB		363-fold	502-fold
-	-	913.0	9830.0
-	+	11.6	53.3
Decrease due to CB		79-fold	184-fold

occurred and cells containing 5 or more nuclei appeared. The extent of HeLa cell fusion correlated with the degree of invasion (Table 3). Examination of multinucleated cells revealed that nearly all which possessed 5 or more nuclei per cell were invaded by EIEC. Multinucleated cells which contained 2 to 4 nuclei displayed intermediate levels of invasion; at a given PEG concentration, invasion of these cells was greater than that of mononuclear cells and less than those with 5 or more nuclei. Cells remaining unfused (mononucleated) following PEG pretreatment were invaded by EIEC in direct proportion to increasing PEG concentrations between 30 to 40%; this is likely due to higher levels of reinfection resulting from increased penetration and subsequent intracellular growth associated with multinucleated cells. A direct effect of residual PEG on the bacteria is unlikely since at 20 % PEG HeLa cells resisted fusion and invasion levels were unchanged, while at 30 % PEG both cell fusion and invasion increased dramatically.

The data presented in Table 4 demonstrates that CB diminished EIEC invasion of both PEG-treated and untreated HeLa cells. However, the magnitude of CB inhibition was greater with PEG-treated monolayers. In the presence of CB, infection of PEG-treated cells was equal to (or less than) the level of infection of untreated cells and thus, the enhancing effect of PEG was obviated. This data is consistent with the postulate that the penetration step was augmented in multinucleated cells. In addition, sensitivity of multinucleated cells to CB is consistent with a phagocytic mechanism for EIEC uptake.

Figure 3: Accumulation of EIEC in HeLa cell extensions. Low power view of HeLa cell monolayer treated with 40 % PEG and infected with EIEC. Arrows point to 5 examples of EIEC accumulation in cellular extensions; many additional examples are evident in this field. May-Grunwald and Giemsa stain; magnification x 400.

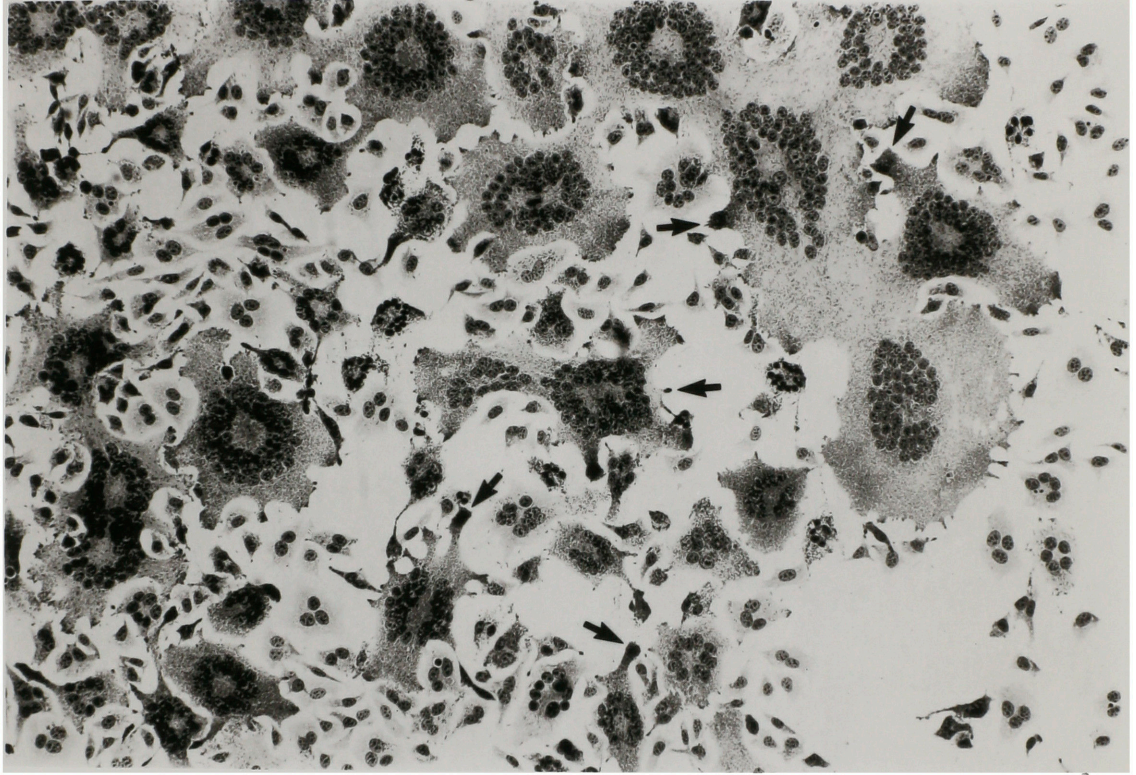
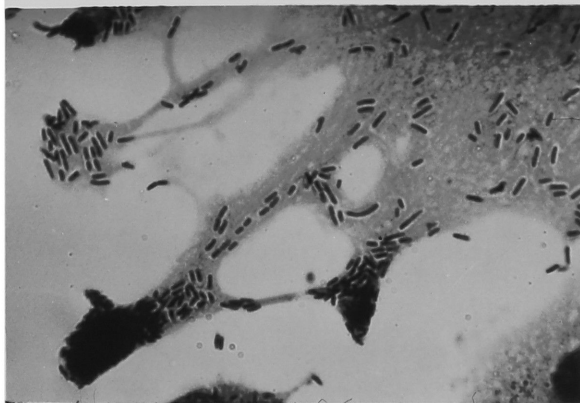
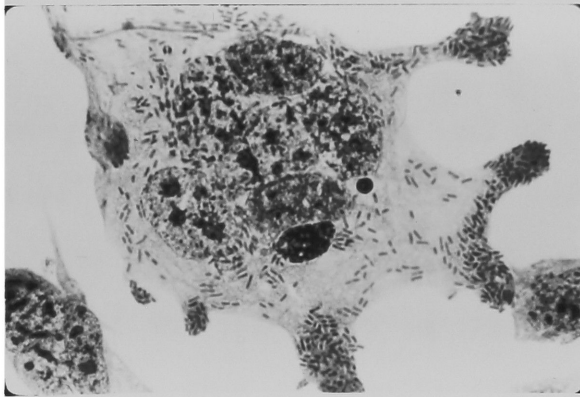
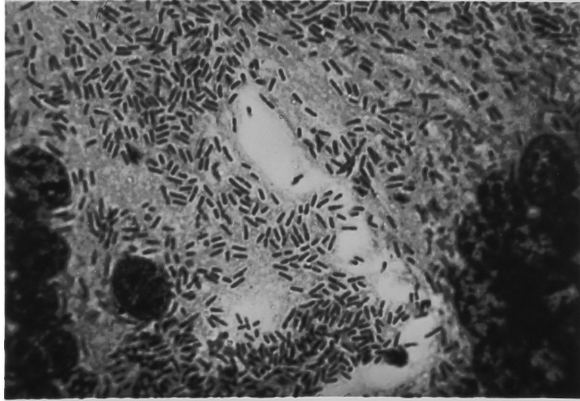


Figure 4: High power view of HeLa cells infected with EIEC. Many cells displayed an even distribution of EIEC (upper panel). Organisms accumulated in cell extensions (middle panel). Heavily infected HeLa cell extensions (lower panel). May-Grunwald and Giemsa stain; magnification x 2100.



### 2.2.2 Morphology of EIEC-Invaded, Multinucleated HeLa Cells

Multinucleated HeLa cells generated by PEG treatment 18 h earlier displayed numerous cell extensions and nuclei occupied a central position in the cytoplasm. In addition, the leading edges spread out in all directions whereas unfused cells tended to align along a single axis. Enteroinvasive E. coli which had survived antibiotic exposure during the intracellular growth period were sometimes found to be evenly distributed throughout the cytoplasm of multinucleated cells (Figures 3 and 4 [upper panel]). However, in a significant proportion of invaded HeLa cells, EIEC displayed a strong predilection for the tips of the cellular extensions (Figures 3 [arrows] and 4 [middle and lower panels]). The accumulation of EIEC in cellular extensions was occasionally observed in unfused HeLa cells as well, but was dramatized in the larger cells. Figure 4 [lower panel] offers a view of heavily infected cellular extensions; note the dense packing of organisms in this structure. The perinuclear region of the multinucleated HeLa cells and the cell periphery aside from the pseudopod-like structures, contained a far lower density of intracellular organisms compared to the cell extensions.

## 2.3 DISCUSSION

A major finding of this study is that alteration of HeLa cell morphology led to increased levels of invasion by EIEC, and stimulation of the penetration step appeared to be a contributing factor. In addition,



PEG can be used as an experimental tool for enhancing invasion by EIEC in vitro. Lastly, the multinucleated HeLa cells generated by fusion showed a striking accumulation of intracellular organisms in pseudopod-like extensions.

Enhanced susceptibility to invasion was demonstrated in multinucleated cells generated both in the presence and absence of PEG. Scoring an individual cell as invaded required 5 intracellular organisms per cell (Mehlman et al., 1982). This represents a measure of the penetration step, albeit a conservative one, since a cell containing 5 or 500 organisms is scored singularly as positive regardless of how many bacteria actually entered. One factor to consider for multinucleated cells is the larger surface area which increases the probability of being penetrated. The magnitude of increased surface area of a multinucleated cell is not known for certain, but it seems reasonable to estimate that a tetranucleated cell has a nuclear region twice the diameter of a mononuclear cell, and four times the volume. Assuming the ratio of the nuclear region to cytoplasm remains constant upon fusion, then a tetranucleated cell would have twice the surface area and a binucleated cell would have 1.41 times (square root of 2) the surface area of a mononuclear cell. According to Figure 3, these estimates seem to be reasonable. At low invasion levels (Table 1, Expts. 1 to 5), it appears that increased invasion of multinucleated cells was due to a direct enhancement of the penetration step since the proportion of cells invaded exceeded that of mononucleated cells by 7- to 18-fold; it is unlikely that surface area alone could account for this difference.

A second measurement was employed to demonstrate increased survival of EIEC with multinucleated HeLa cells. Colony-forming units represent total viable organisms: those which penetrated, their progeny from intracellular multiplication, and the few extracellular organisms which escaped gentamicin/lysozyme killing. Enteroinvasive E. coli survival typically exceeded that of HB101 by 1000-fold. The overall net yield of EIEC cultivated on PEG-treated HeLa cells was significantly greater than for untreated monolayers, averaging a 7-fold enhancement. By this measurement alone, it is difficult to assess the actual contribution of PEG fusion to penetration. Increased invasion resulting from enhancement of the penetration step could be further amplified at the intracellular stage, where organisms multiply freely in the protective environment of the cytoplasm. In conclusion, artificially fused HeLa cells supported higher levels of overall EIEC invasion.

Several features of HeLa cell extensions are well-characterized, and may help to explain why EIEC selectively concentrate in this cell structure. For example, plasma membrane ruffling is associated with the leading lamellae and cell perimeter of normal, spreading HeLa cells and actin is enriched at these regions (Herman et al., 1981); Shigella stimulate plasma membrane ruffling at the site of attachment, leading to CB-sensitive endocytosis (Hale et al., 1979; Ogawa et al., 1968). The study of giant, mononucleated HeLa cells demonstrates a second important feature of cellular extensions. Giant HeLa cells which arise by growth in the absence of division (due to lethal irradiation), are well-spread and flattened, and their receptors for low density lipoprotein, transferrin, and ferritin (but not for concanavalin A) are concentrated toward the tips of the leading edge (Bretscher and Thomson, 1983;

Bretscher, 1983). These receptors are associated with coated pits in a random distribution over the dorsal cell surface, endocytosed upon ligand binding, returned to the plasma membrane free of bound ligand by exocytosis at the leading edge, and accumulate in this region. Given that select HeLa cell membrane constituents concentrate at cell extensions, this might be a preferred site for invasion if the receptor for EIEC attachment were enriched at this site as well.

It is unclear what differentiates mononucleated and multinucleated HeLa cells in their ability to (i) take up invasive bacteria by what appears to be phagocytosis, and (ii) accumulate organisms in cellular extensions. Elucidation of the bacterial elements responsible for induced phagocytosis, and their mechanism of interaction with epithelial cells, should shed light on the basis for the distinct behavior of mono- and multinucleated cells. In addition, a deeper understanding the differences between mono- and multinucleated cells may provide insight as to the mechanisms by which EIEC induce phagocytosis by nonprofessional phagocytes.

## CHAPTER 3

### INTERACTIONS OF GONOCOCCI WITH EPITHELIAL CELLS

Tissue culture cells provide a model for some of the events which are likely to occur during infection in vivo. In this chapter, several events which take place during the first 5 h of cultivation of gonococci on HeLa cell monolayers are described. Gonococci of the opaque phenotype predominate on the monolayer, and mechanisms which contribute to their dominance are defined. In addition, the nature and extent of contact between adherent gonococci and the host cell is explored.

### 3.1 MATERIALS AND METHODS

Bacteria: Strain R10 of Neisseria gonorrhoeae (a cervical isolate) and Neisseria sicca were grown for 21 to 24 h on GC agar plates prepared as specified (Swanson, 1978a). Colonies were viewed by transmitted illumination from a substage mirror or a substage diffusing surface of a Bausch and Lomb microscope.

Gonococcal-HeLa cell co-cultures: Monolayers of HeLa cells were grown to confluence in 35 mm polystyrene wells as described in Section 2.1. Growth medium was replaced with antibiotic-free zeta-DME 18 h prior to inoculation with gonococci. Gonococcal colonies were swabbed off the agar plates and suspended in zeta-DME, vortexed vigorously to dissociate aggregates, and added to HeLa monolayers in a 1.0 ml volume at bacterium to HeLa cell ratios ranging from approximately 10:1 to 1:10 (multiplicity of infection, MOI). When CB was used, it was added to the HeLa monolayers 30 minutes prior to inoculation, and maintained throughout co-cultivation with gonococci at 5 ug/ml. Co-cultures were

incubated at 37°C with 6% CO<sub>2</sub> for the times indicated. Fluid-phase organisms were removed, monolayers washed twice, and the washes pooled to constitute the nonadherent (nonAd) fraction. Gonococci attached to the HeLa monolayer were collected by addition of 0.25% trypsin (Type II crude, Sigma) in PBS. HeLa cells and adherent (Ad) gonococci detached as a result of trypsinization, and were mixed with an equal volume of zeta-DME. Pellets were collected following 10 min at 15,000 x g, resuspended in zeta-DME, and vigorously vortexed for even dispersal. Dilutions of Ad and nonAd organisms were plated on GC agar and CFUs were enumerated. Colony phenotypes were identified by light microscopy as described above, and CFU measurements are presented for the colony phenotype specified.

Bactericidal agents: Serum was collected by cardiac puncture of Sprague-Dawley rats, blood was pooled and allowed to clot for 2 h at room temperature, erythrocytes were removed by centrifugation, and serum aliquots were immediately frozen at -70°C. Following removal of nonAd gonococci from the HeLa cell monolayer, Ad organisms were subjected to 10% fresh rat serum in zeta-DME for 30 min at 37°C. For gentamicin treatment, Ad organisms were incubated for 3 h in 5 to 20 ug/ml gentamicin sulfate in zeta-DME. After bactericidal treatments, monolayers plus Ad organisms were detached by trypsin and processed as usual.

Immunofluorescent staining: Gonococci were cultivated on HeLa cell monolayers grown on Lab-Tek chamber slides and subjected to FRS as described above, except that the multiplicity of infection was 500:1 (gonococci per HeLa cell nucleus). Following FRS treatment, monolayers were washed with PBS, fixed 10 min in 3.7% formaldehyde-PBS, washed 3 x

5 min in PBS, and permeabilized at  $-20^{\circ}\text{C}$  in methanol (10 min), acetone (5 min), and methanol (10 min). Anti-whole gonococci sera raised in rabbits was diluted 1:10 in PBS and incubated on the air-dried slides for 1 h. Monolayers were washed in PBS and incubated 1 h with GARIg conjugated to fluorescein (FITC-GARIg) (Cappel). Following extensive washing, slides were mounted with glycerol containing phenylenediamine to prevent quenching (Johnson and Nogueria Araujo, 1981), and viewed with a Nikon microscope equipped with epifluorescent illumination.

Immunoblots: Gonococci were lysed in 1.0% SDS, boiled with beta-mercaptoethanol, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Whole gonococcal lysates were transferred electrophoretically from gels to nitrocellulose (Bio-Rad Laboratories, Richmond, CA) (Towbin et al., 1979), blocked with 0.5% Tween 20, and incubated with monoclonal antibody raised against P.IIa (see below) of R10 (kindly provided by Dr. M. Blake), in 0.1 M Tris-HCl (pH 8.0), 0.4 M NaCl, and 0.5% Tween 20 for 2 h with shaking at room temperature. Blots were washed free of unbound antibody and stained with goat anti-mouse Ig (GAMIg) conjugated to alkaline phosphatase (Blake et al., 1984).

## 3.2 RESULTS

### 3.2.1 P.II Content of Gonococcal Colony Types

The colonial morphology and P.II content of the R10 variants under study is presented in Table 5 and Figure 5. The heat-denatured forms of 5 distinct P.IIs from strain R10 have  $M_r$  of 29.0, 30.5, 31.5, 31.8, and 33.9 and are designated P.IIa, P.IIb, P.IIc, P.IId, and P.IIe, respectively (Figure 5, upper panel). The isoelectric points of P.IIa and P.IIc are approximately 9.5 and 7.5 to 8.0, respectively; P.IIe appears to be that previously described (Blake and Gotschlich, 1983) with an isoelectric point of 8.0. Colonies viewed with the mirror substage are highly opaque ( $0^{++}$ ), moderately opaque ( $0^+$ ), or transparent ( $0^-$ ), and variation in darkness (viewed with the diffuser substage) correlated well with opacity. Some  $0^{++}$  colonies were slightly more opaque and darker than others; these had distinct rough edges and appeared to be more compact (P.IIac $0^{++}$ , P.IIae $0^{++}$ , and P.IIade $0^{++}$ ). Two colony types were observed which differed in their degree of opacity, and both types expressed a single P.II molecule indistinguishable in molecular weight and temperature of conversion to the slower migrating form (Figure 5, lower panel). The P.I bands serve as an internal control for the quantity of organisms loaded per lane. The data suggests that P.IIa $0^{++}$  expresses higher quantities of P.IIa than does P.IIa $0^+$ . Piliated organisms were  $P^+$  as described (Swanson, 1978a).



Table 5: Colony variants of strain R10 of Neisseria gonorrhoeae(a)

(a) Opacity was determined using the mirror substage and darkness using the diffuser substage. Variant P<sup>-</sup>P.IIe(a)O<sup>++</sup> contained P.IIe as its major P.II band, and only small quantities of P.IIa.

Table 5: Colony variants of strain R10 of Neisseria gonorrhoeae(a)

<u>Nomen- Clature</u>	<u>Opacity</u>	<u>Darkness</u>	<u>P.II Content</u>	<u>Piliation</u>
P <sup>-</sup> O <sup>-</sup>	0 blue-clear	0	0	P <sup>-</sup>
P <sup>-</sup> P.IIaO <sup>+</sup>	+	+	a	P <sup>-</sup>
	yellow-blue			
P <sup>-</sup> P.IIcO <sup>+</sup>	+	+	c	P <sup>-</sup>
	yellow-grey			
P <sup>-</sup> P.IIaO <sup>++</sup>	++	++	a	P <sup>-</sup>
	yellow			
P <sup>-</sup> P.IIe(a)O <sup>++</sup>	++	++	e(a)	P <sup>-</sup>
	yellow			
P <sup>-</sup> P.IIadO <sup>++</sup>	++	++	ad	P <sup>-</sup>
	yellow			
P <sup>-</sup> P.IIacO <sup>++</sup>	+++	+++	ac	P <sup>-</sup>
	yellow			
P <sup>-</sup> P.IIaeO <sup>++</sup>	+++	+++	ae	P <sup>-</sup>
	yellow			
P <sup>-</sup> P.IIadeO <sup>++</sup>	+++	+++	ade	P <sup>-</sup>
	yellow			
P <sup>+</sup> P.IIbO <sup>+</sup>	+	+	b	P <sup>+</sup>
	yellow-white			
P <sup>+</sup> O <sup>-</sup>	0	0	0	P <sup>+</sup>
	blue-clear			

Figure 5:

Upper panel: Western blot of whole gonococcal lysates stained with monoclonal antibody against P.II. Lysates of colony variants of strain R10 gonococci were boiled in sample buffer, subjected to SDS-PAGE, electrotransferred to nitrocellulose, and stained with monoclonal antibody against P.II using GAMIg conjugated to alkaline phosphatase. Lanes: (1) P.IIcO<sup>+</sup>; (2) P.IIacO<sup>++</sup>; (3) P.IIadO<sup>++</sup>; (4) P.IIaO<sup>++</sup>; (5) P.IIadeO<sup>++</sup>; and (6) P.IIe(a)O<sup>++</sup>.

Lower panel: Autoradiogram of Western blot of gonococcal lysates P.IIaO<sup>++</sup> (Lanes 1,3,5) and P.IIaO<sup>+</sup> (Lanes 2,4,6). Lysates were solubilized in sample buffer at 37 (1,2), 68 (3,4), and 100°C (5,6) for 15, 15, and 5 min, respectively. Samples were subjected to SDS-PAGE (15 % polyacrylamide), electrotransferred to a polyvinylidene difluoride membrane (Millipore), incubated with a combination of monoclonal antibodies to P.I and P.II, and reacted with <sup>125</sup>I-Protein A. Arrows indicate P.I (a), heat-modified form of P.IIa (b), and unmodified form of P.IIa (c).

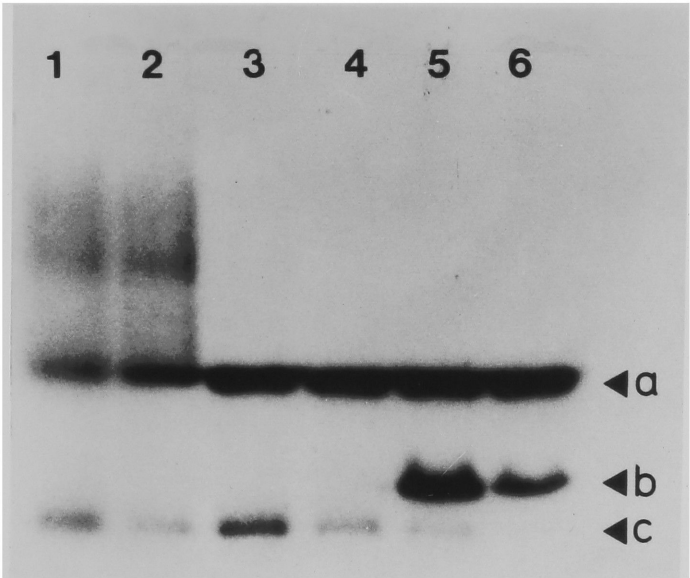
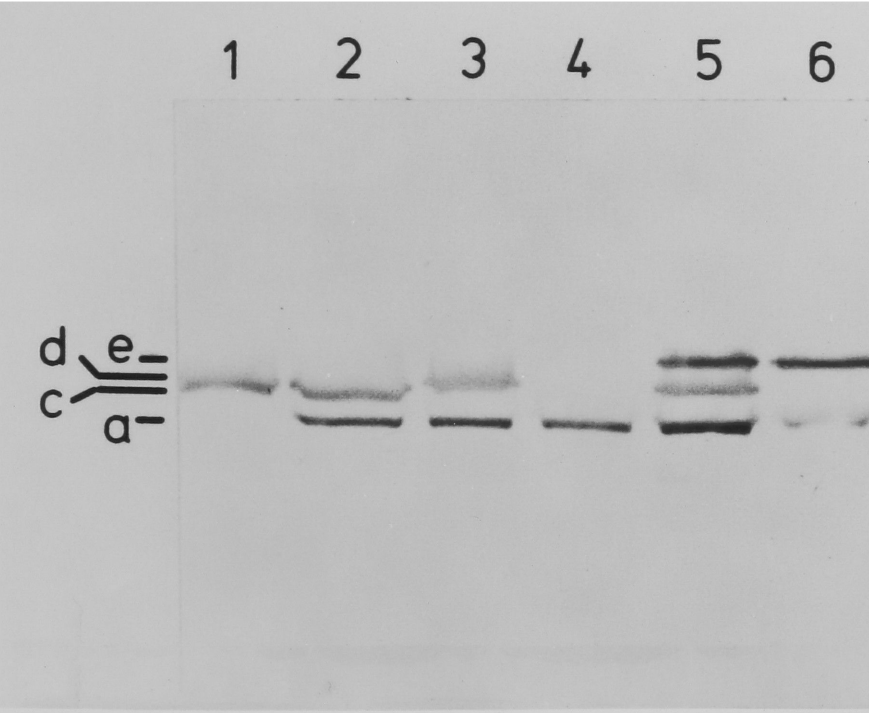
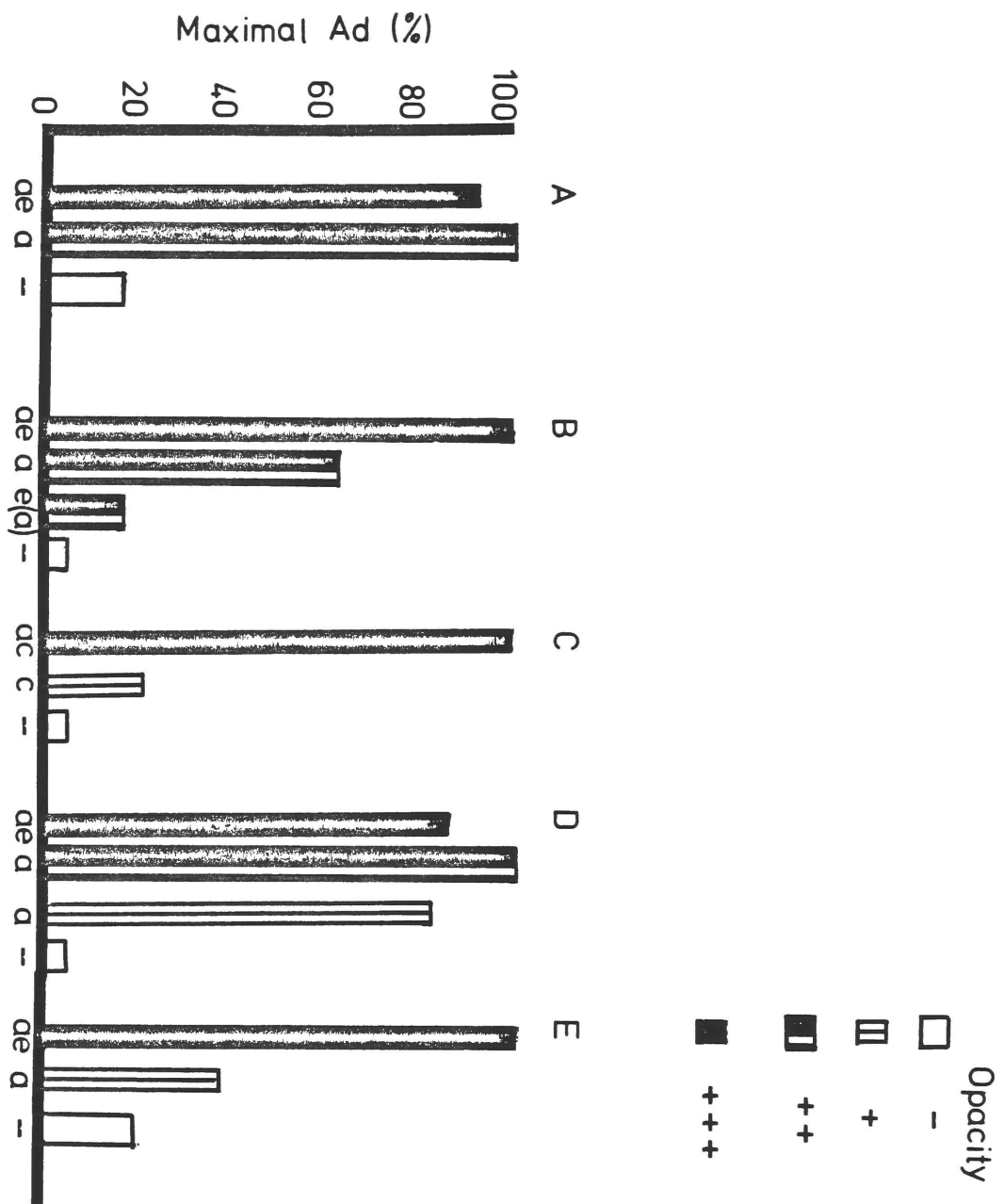


Figure 6: Effect of P.II and opacity on relative distribution in Ad fraction. Panels A through E represent 5 different experiments whereby gonococci were incubated on HeLa cell monolayers for 2 to 5 h, and CFUs in the Ad and nonAd fractions were measured. The fraction in the Ad pool was calculated (Ad CFUs divided by the sum of Ad and nonAd CFUs); the variant having the highest fraction of CFUs in the Ad pool was normalized to 100 % (maximal Ad) and the remaining variants were adjusted accordingly. The % of maximal Ad is plotted for each variant. The degree of opacity and P.II content of each variant is given, and is further defined in Table 5. The following MOIs (gonococci to HeLa cell ratio) were used: panel A, 2.67 (P.IIa0<sup>++</sup>), 3.84 (0<sup>-</sup>); panel B, 0.36 (P.IIae0<sup>++</sup>) 0.33 (P.IIa0<sup>++</sup>), 0.17 (P.IIe(a)0<sup>++</sup>), 0.43 (0<sup>-</sup>); panel C, 3.67 (P.IIac0<sup>++</sup>), 0.83 (P.IIc0<sup>+</sup>), 28.8 (0<sup>-</sup>), panel D, 0.158 (P.IIae0<sup>++</sup>), 0.263 (P.IIa0<sup>++</sup>), 0.028 (P.IIa0<sup>+</sup>), 0.255 (0<sup>-</sup>); panel E, 0.27 (P.IIae0<sup>++</sup>), 0.16 (P.IIa0<sup>+</sup>), 0.41 (0<sup>-</sup>).

# PII CONTENT



### 3.2.2 Net Distribution of Gonococcal Colony Types

Gonococci cultivated with HeLa cell monolayers for a given period of time distributed into adherent (Ad) and nonadherent (nonAd) fractions. The percentage of a population distributing in the Ad phase (% Ad) can be calculated by dividing the number of CFUs measured in the Ad pool by the sum of the Ad and nonAd pool CFUs (total population). The Ad-nonAd distribution of isogenic variants varying in P.II content and opacity was compared (Figure 6A-E); for each group the variant displaying the highest proportion of CFUs in the Ad pool was defined as having maximal distribution in the Ad fraction, and was normalized to 100%. Transparent colony types lacked P.II and displayed low levels of distribution in the Ad fraction relative to those variants designated  $O^{++}$  (solid and solid/bar) which generally exhibited the maximal distribution in the Ad pool. An exception is illustrated in Figure 6B with variant P.IIe(a) $O^{++}$ , which displayed a significantly lower proportion in the Ad fraction despite the presence of P.II and a highly opaque phenotype. Moderately opaque ( $O^+$ ) colonies were often intermediate in their distribution relative to  $O^-$  and  $O^{++}$  (Figure 6C and 6E). However, a variant was identified which displayed high levels of adherence despite a moderate degree of opacity (P.IIa $O^+$ , Figure 6D). Other gonococci of the P.IIa $O^+$  phenotype (Figure 6E) displayed intermediate levels of adherence similar to that of P.IIc $O^+$  as in Figure 6C; the basis for variability of P.IIa $O^+$  adherence is not understood. Nevertheless, colonial opacity does not strictly correlate with distribution of organisms in the Ad fraction. Equivalent amounts of P.II were produced by P.IIc $O^+$  and P.IIa $O^{++}$  (Figure 5) despite differences in the degree of opacity and

adherence. Gonococci bearing P.IIa and/or P.IIc are the focus of most of the studies presented below and in Chapters 4 and 5.

The distribution of  $P^-P.IIaO^{++}$  gonococci in Ad and nonAd pools over 4.3 h of co-cultivation with HeLa cell monolayers was measured (Figure 7A). The proportion of gonococci in the Ad fraction (% Ad) was 9%, 18%, and 88%, at 1, 2, and 4.3 h, respectively. The extent of binding by  $P^-O^-$  organisms rose steadily over time, but remained below 10% of the total population (Figure 7B). Using a single inoculum consisting of a mixture of 98.5%  $P^-O^-$  and 1.5%  $P^-P.IIaO^{++}$  gonococci, the time course of distribution of transparent and opaque phenotypes in the Ad and nonAd fractions was measured (Figure 8). Examination of colony phenotypes in each pool reveals that opaque gonococci account for a disproportionate share of the attached bacteria. Whereas opaque colonies comprised only 1.5% of the initial inoculum, they constituted 80% of the CFUs in the Ad fraction by 4 h. These results provide further indication that opacity correlates with significant enhancement in the association between gonococci and HeLa cells.

### 3.2.3 Detachment of Gonococci

Several factors can contribute to the overall distribution of a gonococcal phenotype in the Ad and nonAd subpopulations. Those events which follow the initial attachment, namely detachment and bacterial multiplication, were characterized. Net flux of gonococci from the Ad to the nonAd fraction was measured. Bacteria were allowed to attach to HeLa cell monolayers for 60 to 150 min, unbound organisms were removed,



Figure 7: Distribution of P-P.IIaO<sup>++</sup> (A) and P<sup>-</sup>O<sup>-</sup> (B) gonococci in Ad (circle) and nonAd (triangle) fractions. Gonococci were incubated on HeLa cell monolayers for the times indicated, and CFUs of each fraction were measured. Control (square) represents gonococci in zeta-DME growth medium without HeLa cells. The MOI for P-P.IIaO<sup>++</sup> and P<sup>-</sup>O<sup>-</sup> was 4.98 and 5.78, respectively.

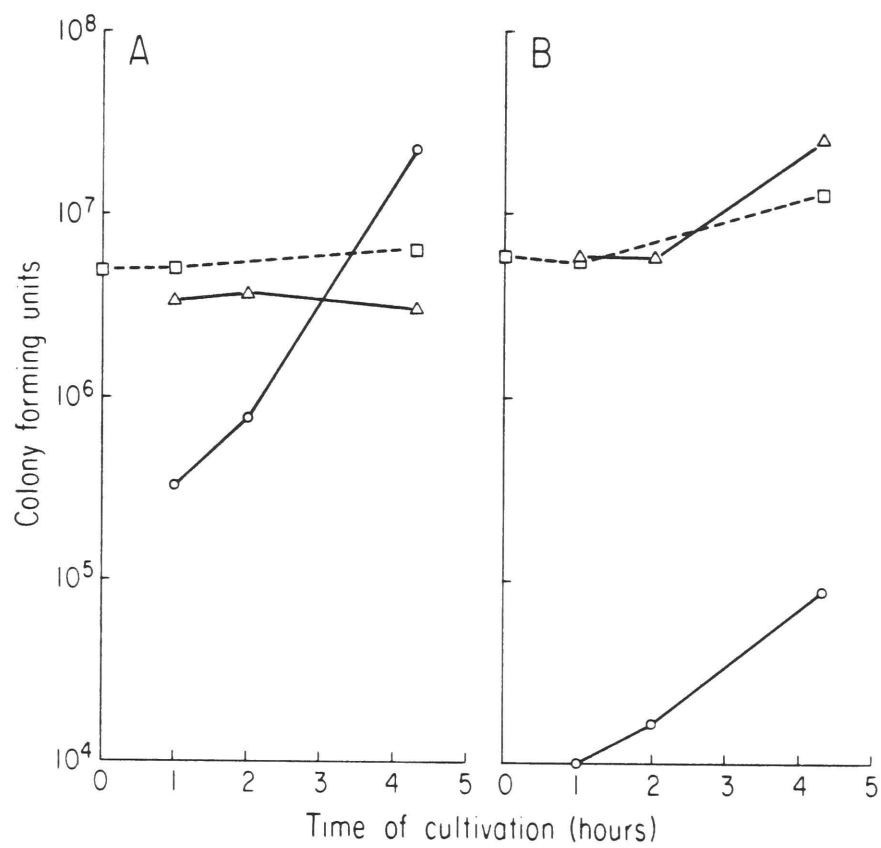


Figure 8: Distribution of  $P^-P.IIaO^{++}$  and  $P^-O^-$  gonococci in Ad and nonAd fractions. A single inoculum containing a mixture of  $P^-O^-$  and  $P^-P.IIaO^{++}$  gonococci was incubated on HeLa cell monolayers for the times indicated, and the CFUs and colony phenotype of each fraction was measured. The proportion  $O^{++}$  and  $O^-$  organisms constituting the inoculum is indicated at 0 h. The proportion of  $O^{++}$  (solid line) and  $O^-$  (broken line) constituting the Ad fraction (circle) or nonAd fraction (triangle) is plotted. The MOI for  $P^-P.IIaO^{++}$  and  $P^-O^-$  was 0.087 and 5.69, respectively.

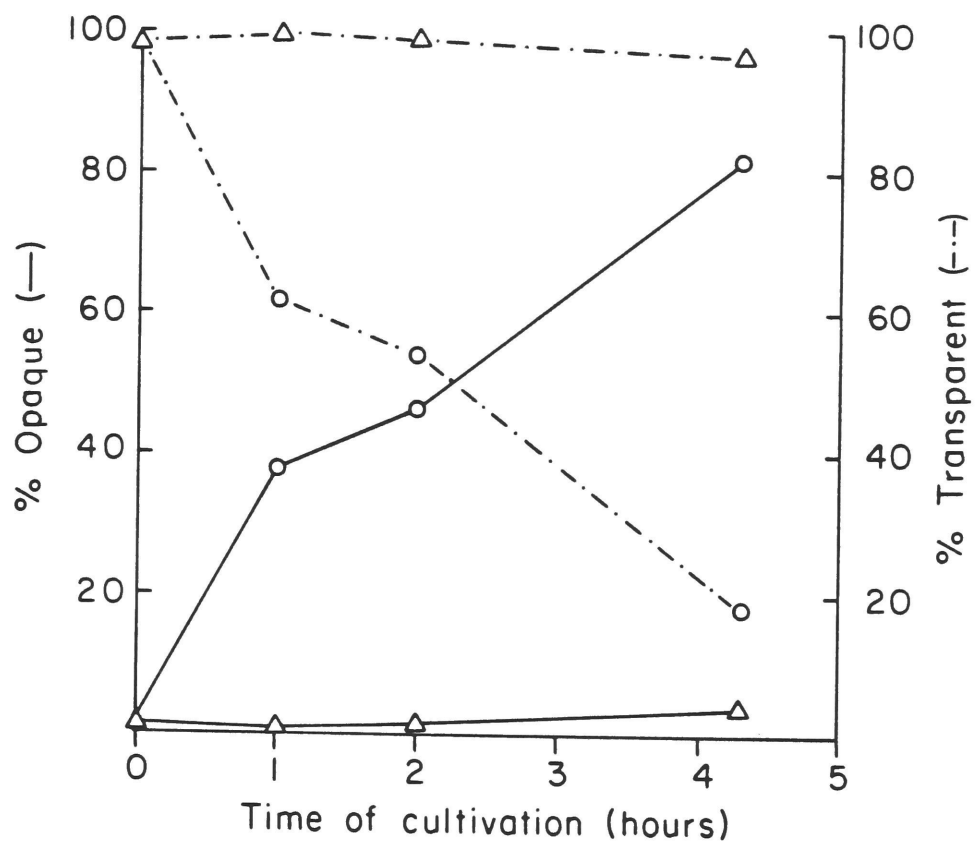
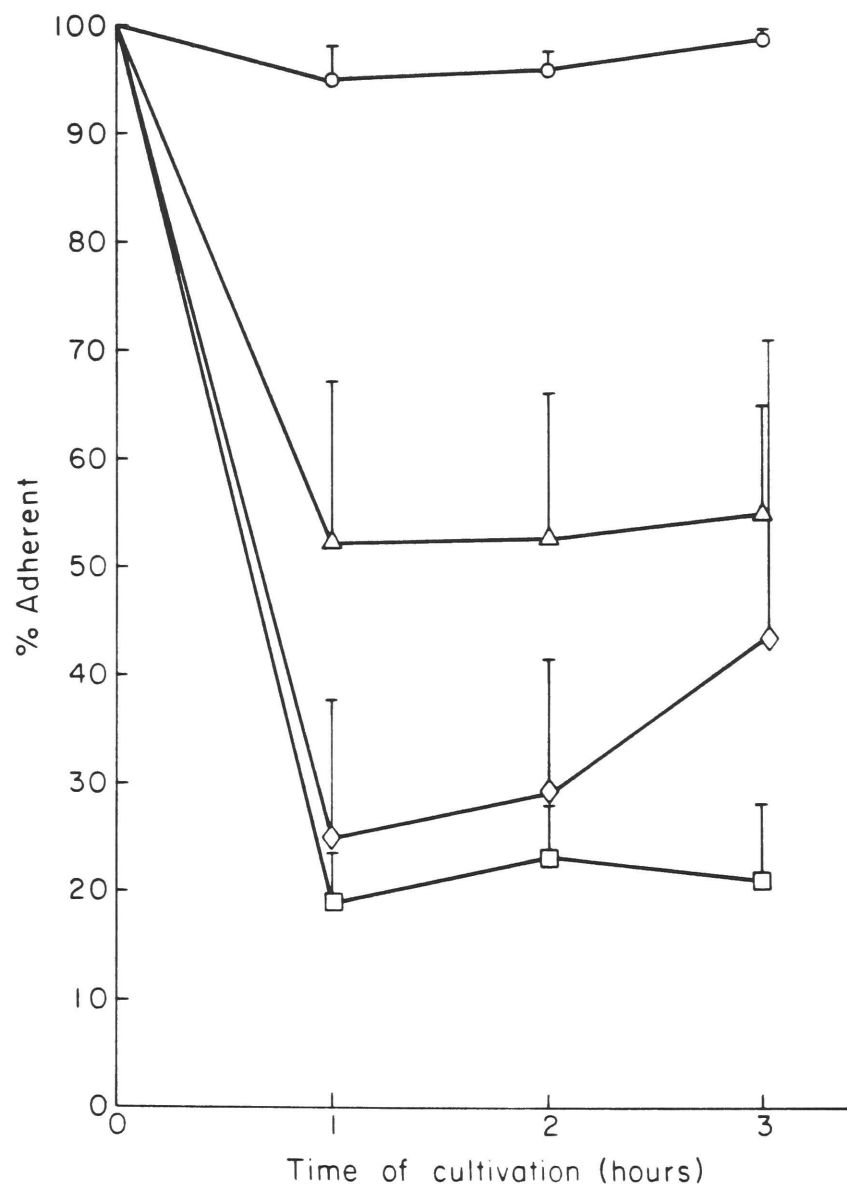


Figure 9: Proportion of organisms in the Ad fraction following removal of unbound organisms at 0 h. At 0 h, 100% of gonococci were Ad; cultivation continued and the percentage of the total population which were Ad at various times is indicated. The colony variants represented are:  $P^-P.IIacO^{++}$  (circle),  $P^-P.IIcO^+$  (triangle),  $P^-O^-$  (square), and  $P^+O^-$  (diamond). Each point (except  $P^+O^-$ ) represents the mean of 4 experiments, and the standard deviation is indicated by bars. The following MOIs were used:  $P^-P.IIacO^{++}$  (5.17, 3.37, 0.30, 0.25);  $P^-P.IIcO^+$  (1.83, 1.37, 0.07, 0.05);  $P^-O^-$  (13.8, 3.70, 3.15, 1.85);  $P^+O^-$  (5.63, 2.13).



fresh zeta-DME was added to the monolayer, and incubation continued for the times indicated (Figure 9). The proportion of gonococci in the Ad phase decreased within 1 h following removal of the unbound inoculum. The exception was the  $O^{++}$  variant,  $P^-P.IIacO^{++}$ , which remained almost exclusively in the Ad fraction. A steady-state was established for each phenotype, with the possible exception of  $P^+O^-$ , which showed a slight rise in the Ad pool following the initial drop. The data supports the postulate that redistribution of the Ad subpopulation is a consequence of detachment of gonococci from the HeLa cell monolayer. The  $P.IIcO^+$  variant displays intermediate levels of detachment, suggesting a direct correlation between detachment and P.II content and/or opacity.

A comparison of  $P^-O^-$  and  $P^+O^-$  gonococci indicated that piliation does not confer enhanced attachment to HeLa cells, and the percentage of organisms which adhered was less than 10% of the total population (data not shown). Although  $P^+O^-$  exhibits significant detachment in this study, it should not be inferred that detachment is a general property of pili-mediated adherence. It is possible that a cell abundant in pilus receptors and/or a gonococcus rich in pili would lead to a more stable interaction.

### 3.2.4 Multiplication of Gonococci

The Ad pool has the potential to increase in CFUs either by attachment of organisms originating in the nonAd fraction, or by replication (and retention) of organisms already bound to the HeLa cell surface. Both transparent and opaque gonococci cultured with HeLa cells divide

more rapidly than control cultures which lack HeLa cells (see Figure 7). This finding implies that gonococcal replication is stimulated in the presence of HeLa cells. To accurately measure the multiplication of organisms bound to the HeLa cell surface, exchange of organisms between the Ad and nonAd subpopulations should be minimal. According to Figure 9, the P.IIacO<sup>++</sup> variant appears to best fit this condition, because bound P.IIacO<sup>++</sup> organisms do not readily detach. Bacteria were allowed to adhere to HeLa cell monolayers for 30 min, unbound organisms were subsequently removed, replaced with fresh zeta-DME, and co-cultivation continued (Table 6). Attached gonococci underwent one division between 30 min and 2.5 h. From 2.5 to 5.0 h, the average generation time dropped to 35 min. Control organisms, which were incubated in zeta-DME without HeLa cell monolayers, displayed an extended lag phase and showed net growth only after 2.5 h. Between 2.5 and 5.0 h the doubling rate of the control culture was significantly slower (by 3-fold) than the generation time of Ad gonococci during a parallel time period. The data indicates that for the P.IIacO<sup>++</sup> variant attachment to HeLa cells is coupled to a significant stimulation of gonococcal division. Furthermore, daughter cells are retained in the Ad population.

The gonococcal colonial forms each attain a different level of distribution between the Ad and nonAd subpopulations (see Figure 9). There is a correlation between the proportion of the population in the Ad phase, and the growth rate of the total (Ad plus nonAd) population (Table 7). Opaque organisms multiply faster than transparent in the presence of HeLa cells.



Table 6: Multiplication of gonococci on HeLa cell monolayers.

- (a) Colony variant used was P-P.IIaO<sup>++</sup>, and is expressed as CFUs/ml. HeLa cells were inoculated at 0 min (MOI = 0.18), and unbound gonococci were removed at 30 min (nonAd is 0). This is a representative experiment.
- (b) Control represents gonococci in zeta-DME growth medium lacking HeLa cells.

Table 6: Multiplication of gonococci on HeLa cell monolayers.

Infection <u>Time</u>	<u>HeLa Culture Fractions</u> (a)		<u>Control</u> (b)
	<u>Adherent</u>	<u>Nonadherent</u>	
30 min	7,682	0	179,000
2.5 h	15,531	1,400	184,000
5.0 h	295,000	7,400	471,000

Table 7: Correlation of adherence and replication.

(a) Gonococci were allowed to attach to HeLa cells for 150 or 60 min, unbound organisms removed, and cultivation continued. Measurements were made beginning 1 h after removal of unbound organisms, and continued for 1 or 2 h, respectively (150 or 60 min initial attachment period). The percentage growth increase per hour and generation times are calculated for the total population (Ad plus nonAd fractions). Mean and standard deviations of 4 experiments are presented.

(b) Differences between  $P^-P.IIacO^{++}$  and  $P^-O^-$ , and between  $P^-P.IIcO^+$  and  $P^-O^-$  were significant ( $P < 0.001$ ) according to the t-test.

(c) Difference between  $P^-P.IIacO^{++}$  and  $P^-O^-$  was significant ( $\alpha = 0.10$ ) according to the Mann-Whitney U test.

Table 7: Correlation of adherence and replication.

<u>Colony Phenotype</u>	<u>Percentage Adherent<sup>(a)</sup></u>	<u>% Growth Increase Per Hour</u>	<u>Generation Time (min)</u>
P <sup>-</sup> P.IIacO <sup>++</sup>	96.2 $\pm$ 2.4 <sup>(b)</sup>	196.3 $\pm$ 83.1 <sup>(c)</sup>	61.5
P <sup>-</sup> P.IIcO <sup>+</sup>	52.5 $\pm$ 12.3	170.9 $\pm$ 93.4	77.6
P <sup>-</sup> O <sup>-</sup>	19.6 $\pm$ 5.1	163.1 $\pm$ 85.3	85.0

### 3.2.5 Nature of the Association with HeLa Cells

Gonococci invade host epithelium in vivo (Harkness, 1948), and tissue culture monolayers have served as valuable in vitro models for the study of invasion by other micro-organisms, such as Shigella, Salmonella, and Chlamydia spp. (Gianella et al., 1973; LaBrec et al., 1964; Friis, 1972). Bacteria which completely penetrate the epithelial cell are no longer accessible to microbicidal agents which do not permeate across the eucaryotic plasma membrane. Fresh rat serum (FRS) is extremely cytolytic for Neisseriae spp., and evidence indicates that its action on the cell surface is mediated by complement (Swanson and Goldschneider, 1969). Experiments were performed to determine whether Ad gonococci are protected from the bactericidal action of FRS (Table 8). A small percentage of Ad gonococci retained their viability following treatment with FRS. Neither piliation or opacity conferred a survival advantage (or disadvantage) on the organism. The piliated gonococci differed from those described previously (Figure 9) in that these piliated organisms adhered strongly to HeLa cells.

If HeLa cells ingest gonococci and thereby render them inaccessible to FRS action, one would expect that disruption of the endocytic process would lead to fewer survivors of serum action. Cytochalasin B disrupts microfilament function and impairs endocytosis by professional phagocytes, and by epithelial cells presented with invasive bacteria (Hale et al., 1979; Kihlstrom and Nilsson, 1977). HeLa cells subjected to CB round up and cell extensions retract (data not shown). Resistance to serum-mediated killing of gonococci bound to CB-treated and untreated HeLa monolayers is given in Table 9. The percentage of survivors was

Table 8: Survival of gonococci in FRS.

- (a) Gonococci were cultivated with HeLa cell monolayers for 4 h before unbound organisms were removed and monolayers treated with FRS. The percentage of organisms which survived FRS treatment was calculated from the number of adherent organisms before and after FRS treatment. The following MOIs were used:  $P^-P.IIa0^{++}$  (1.0),  $P^-0^-$  (2.1),  $P^+0^-$  (0.8),  $P^+P.IIb0^+$  (0.6).
- (b) Significant difference ( $P < 0.001$ ) between mean of adherent serum survivors and controls by the t-test.
- (c) In controls, gonococci were suspended in growth medium at a concentration of  $10^6$  CFU per ml, FRS was added to a concentration of 10 %, incubation proceeded for 30 min at  $37^\circ\text{C}$ , and number of surviving organisms was determined. Addition of HeLa cells at the time of FRS addition to control mixtures had no effect on the viability of gonococci (data not shown).

Table 8: Survival of gonococci in FRS.

<u>Colony Phenotype</u>	<u>% Serum Survivors</u>	
	<u>Adherent<sup>(b)</sup> to HeLa Cells</u>	<u>Suspension<sup>(c)</sup> Control</u>
P <sup>-</sup> P.IIaO <sup>++</sup>	1.22%	< 0.0016%
P <sup>-</sup> O <sup>-</sup>	1.37%	< 0.0016%
P <sup>+</sup> O <sup>-</sup>	0.48%	< 0.0016%
P <sup>+</sup> P.IIbO <sup>+</sup>	1.89%	< 0.0016%

Table 9: Effect of cytochalasin B on survival of  
adherent Neisseria following FRS treatment.

(a) Neisseria gonorrhoeae or Neisseria sicca were allowed to adhere to CB-treated or untreated monolayers for 4 to 5 h, unbound bacteria were removed, and Ad organisms were subjected to FRS as described previously. Initial inoculums ranged from bacteria to HeLa cell ratios of 1:10 to 10:1. The colonial variants of N. gonorrhoeae are indicated.

(b) "- CB/ + CB" represents the ratio of the percentage of Ad organisms surviving serum treatment, on HeLa cells without (-) or with (+) CB treatment. The percentage of Ad gonococci surviving FRS treatment was significantly greater in the absence of CB compared to the presence of CB, according to the Wilcoxon matched-pairs test ( $\alpha = 0.01$ ).



Table 9: Effect of cytochalasin B on survival of  
adherent Neisseria following FRS treatment.

<u>ORGANISM</u>	<u>MOI</u>	<u>CB</u>	CFUs x 10 <sup>3</sup>				RATIO (b) - CB/+ CB
			<u>Ad (a)</u>	<u>SERUM SURVIVAL</u>	<u>PERCENTAGE SURVIVAL</u>		
P <sup>-</sup> P.IIa0 <sup>++</sup>	0.32	-	150	5.5	3.67		5.02
		+	350	2.6	0.73		
P <sup>-</sup> P.IIa0 <sup>++</sup>	0.13	-	950	6.2	0.65		1.10
		+	1600	9.5	0.59		
P <sup>-</sup> P.IIa0 <sup>++</sup>	1.0	-	1470	17.9	1.22		7.63
		+	3290	5.3	0.16		
P <sup>-</sup> P.IIa0 <sup>++</sup>	4.8	-	21000	456.0	2.17		4.43
		+	28600	139.0	0.49		
P <sup>-</sup> P.IIa0 <sup>++</sup>	5.0	-	22700	382.0	1.68		1.89
		+	37300	332.0	0.89		
P <sup>-</sup> O <sup>-</sup>	2.1	-	195	2.6	1.37		1.62
		+	379	3.2	0.85		
P <sup>+</sup> O <sup>-</sup>	0.8	-	35100	169.0	0.48		3.43
		+	17500	24.5	0.14		
P <sup>+</sup> P.IIb0 <sup>+</sup>	0.6	-	5560	105.0	1.89		2.28
		+	10200	85.0	0.83		
N. <u>sicca</u>	0.15	-	1870	6.2	0.33		0.38
		+	2250	19.8	0.88		
N. <u>sicca</u>	0.02	-	117	0.07	0.06		0.38
		+	117	0.18	0.16		

consistently lower if monolayers were pretreated with CB. Cytochalasin B did not hamper overall levels of adherence, and had no effect on gonococcal viability. The ratio of percent survivors without CB (- CB) to survivors with CB (+ CB) exceeds one, and typically falls in a range between 2- and 8-fold. In contrast, a ratio of less than one (about 0.4) is observed with the nonpathogenic commensal, Neisseria sicca (Table 9). Adherent gonococci were treated for 3 h with microbicidal concentrations of gentamicin, and less than 0.1% of the organisms survived (Table 10). This represents 10- to 50-fold less survival than that following serum exposure. Treatment of monolayers with CB caused a 5-fold decrease in gentamicin resistance compared to cultures lacking CB. The results indicate that resistance to both serum and gentamicin is depressed when HeLa cell cytoskeleton function is impaired. However, the action of serum and gentamicin is distinct since the level of survival is significantly lower following gentamicin treatment.

The co-cultivation period was extended to determine whether protection of Ad gonococci could be enhanced. Gonococci were allowed to bind to monolayers for 2 to 3 h, and unbound organisms were removed and replaced with fresh zeta-DME. Cultivation continued for 1 or 2 days, after which time monolayers were subjected to FRS or gentamicin. The results presented in Table 11 indicate that survival following gentamicin treatment remains unchanged from short term cultures, at about 0.1 to 0.01% of Ad gonococci. However, an increase in the proportion of organisms surviving bactericidal FRS is apparent. Mean averages for percent serum survival of  $P^{-}O^{++}$  gonococci in short term (Table 9) and long term (Table 11) cultures are 1.88% (S.D. 1.15%) and 4.67% (S.D. 1.45%), respectively.

Table 10: Effect of cytochalasin B on survival of Ad gonococci following gentamicin treatment.

(a) Gonococci (colony variant P<sup>-</sup>P.IIa0<sup>++</sup>) were allowed to adhere to CB-treated or untreated monolayers for 4 to 5 h, unbound bacteria were removed, and Ad organisms were subjected to gentamicin for 3 h (MOI = 0.125). The percentage of organisms which survived gentamicin was calculated from the number of adherent organisms before and after treatment. Controls ( $3 \times 10^6$  CFU/ml) for survival of gonococcal suspensions in gentamicin are indicated.

(b) "- CB/ + CB" represents the ratio of the percentage of Ad organisms surviving gentamicin treatment, on HeLa cells without (-) or with (+) CB treatment.

Table 10: Effect of cytochalasin B on survival of Ad gonococci  
following gentamicin treatment.

<u>Gentamicin</u> <u>ug/ml</u>	<u>CB</u>	<u>% Gentamicin Survivors</u>		Ratio (b) <u>+ CB/- CB</u>
		<u>Adherent<sup>(a)</sup></u>	<u>Control</u>	
5	-	0.0447	< 0.0005	5.45
	+	0.0082		
20	-	0.0571	< 0.0005	6.96
	+	0.0082		

Table 11: Effect of FRS and gentamicin on survival of Ad gonococci  
in long-term cultures.

(a) Gonococci (P<sup>-</sup>P.IIaO<sup>++</sup>) were allowed to attach to HeLa cells for 2 to 3 h, unbound organisms were removed, and co-cultivation continued for an additional 19 h (Expts. 3 and 4), or unbound organisms were again removed after 25 h, and co-cultivation continued for an additional 19 h (Expts. 1 and 2). The following MOIs were used: Expt 1 (0.0017); Expt 2 (0.0002); Expt 3 (0.048); Expt 4 (0.433). Treatment with bactericidal agents was as previously described. Gentamicin was used at 5 ug/ml.

(b) Comparison of % of gonococci surviving FRS in short term (Table 9) and long term cultures (Table 11) of Ad P<sup>-</sup>P.IIaO<sup>++</sup> were significantly different ( $\alpha = 0.05$ ) according to the Mann-Whitney U test.

N.D., not determined.

Table 11: Effect of FRS and gentamicin on survival of Ad gonococci  
in long-term cultures.

<u>Expt</u>	<u>% Survival (a)</u>	
	<u>Serum<sup>(b)</sup></u>	<u>Gentamicin</u>
1	5.73	0.078
2	2.94	0.034
3	4.02	N.D.
4	6.00	N.D.

The results presented in Chapter 2 indicated that treatment of HeLa cells with PEG led to increased levels of invasion by EIEC. Gonococci were cultivated on PEG-treated and untreated HeLa cell monolayers and survival following FRS treatment was measured (Table 12). Treatment of monolayers with PEG was without a significant effect on the number of gonococci surviving FRS treatment. Immunofluorescent staining of Ad gonococci following FRS treatment showed an accumulation of organisms above the perinuclear region of unfused HeLa cells (Figure 10, left lower panel), and the perinuclear rimming pattern is more dramatic with multinucleated HeLa cells (Figure 10, upper panel and right lower panel). The distribution of Ad gonococci on multinucleated HeLa cells contrasts sharply with that observed with EIEC (Figure 4).

### 3.3 DISCUSSION

Monolayers of the epithelial-like, HeLa cell line were employed as a model for the study of interactions between gonococci and epithelial cells. Several factors contributed to the enrichment of opaque gonococci in the Ad phase: high avidity for HeLa cells, low levels of detachment, HeLa cell-stimulated replication, and retention of daughter cells. Once organisms were attached, HeLa cell-mediated protection from microbicidal agents occurred independent of opacity and piliation.

Enumeration of gonococci by measuring CFUs was chosen because it is a highly sensitive analysis (as few as 50 CFUs can be detected), and it allows one to readily discern colonial phenotypes. Since opaque

Table 12: Effect of PEG treatment of HeLa cells  
on survival of gonococci in FRS.

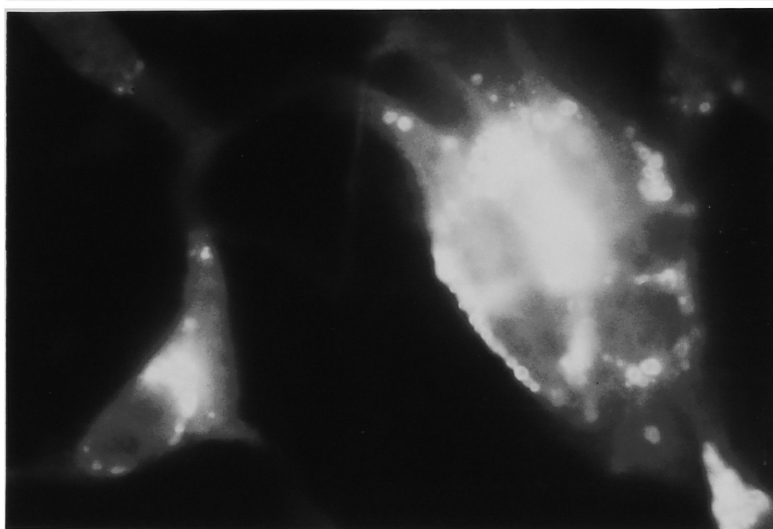
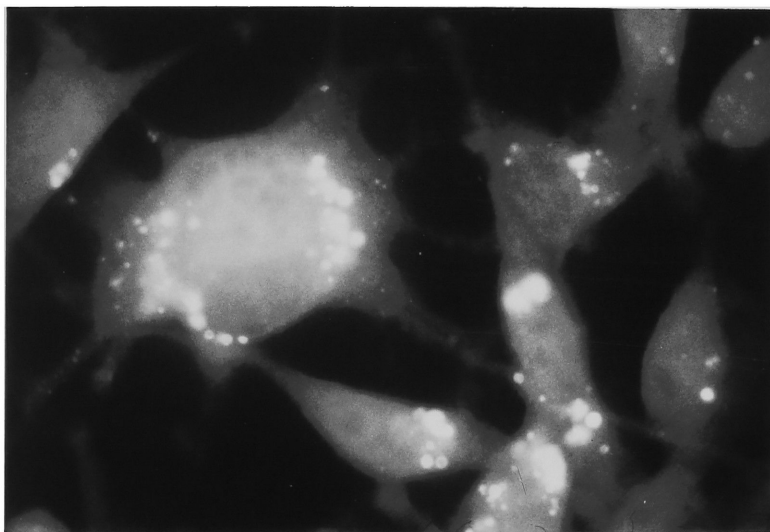
- (a) HeLa cells were treated or untreated with 40 % PEG as described in Section 2.1. Gonococci ( $P^{-}P.IIa0^{++}$ ) were cultivated on HeLa cell monolayers and subjected to FRS as described in Table 8. The MOI for Expt 1 and 3 was 0.32 and 0.27, respectively.
- (b) The increase (+) or decrease (-) in yield (CFUs) as a consequence of PEG-treatment of HeLa monolayers.
- (c) Arithmetic mean and standard deviation.



Table 12: Effect of PEG treatment of HeLa cells  
on survival of gonococci in FRS.

Treatment <sup>(a)</sup> (CFU x 10 <sup>-2</sup> )			
<hr/>			
<u>EXPT</u>	<u>PEG</u>	<u>NONE</u>	<u>CHANGE</u> <sup>(b)</sup>
1	95	70	+ 1.36
2	355	98.5	+ 3.60
3	300	1000	- 3.33
Average (c)			+ 0.54 $\pm$ 3.54

Figure 10: Immunofluorescent staining of gonococci bound to HeLa cells. HeLa cell monolayers treated with PEG (as in Section 2.1) were cultivated with P-P.IIa0<sup>++</sup> gonococci (500:1 gonococci per HeLa cell nucleus), subjected to FRS, and stained with anti-gonococcal sera and FITC-GARig. Multinucleated and mononucleated (lower left of lower panel) HeLa cells are observed with Ad gonococci. Magnification 2800x.



gonococci clump (Swanson, 1978b) and aggregation could influence CFU measurements, several controls were performed to ensure that single organisms were being quantified. The pattern of interaction of opaque and transparent gonococci with HeLa cells was unaltered by filtration of the inocula through 1.0  $\mu$ m pores. Neither trypsinization of organisms nor passage through a 30 gauge needle had an effect on the CFU yield. In addition, uptake of radiolabel by Ad gonococci following serum treatment displayed the same pattern of survival on CB-treated and untreated monolayers as revealed by the CFU measurements reported.

The colonial variants under study exhibited strong association with HeLa cells when the basic P.IIa was present. The role of P.IIc and pili in mediating HeLa cell association was less well-defined, but neither provided the adherence level attributable to P.IIa. Inspection of P.IIacO<sup>++</sup> and P.IIcO<sup>+</sup> colonies by light microscopy revealed differences in morphology, with the P.IIcO<sup>+</sup> type being lighter and less intense in color. Although this analysis is subjective, it appears that P.IIcO<sup>+</sup> is less opaque than P.IIacO<sup>++</sup>. Detachment occurred most readily when P.II was absent, and P.IIc allowed for greater detachment than P.IIac. Therefore, as the opacity of the gonococcus decreased, detachment from HeLa cells increased.

The opaque phenotype exhibited an altered growth rate in this tissue culture model. Growth of both opaque and transparent gonococci was enhanced in the presence of HeLa cells, however, replication of opaque organisms exceeded that of the transparent type. There was an inverse correlation between the proportion of the population in the Ad phase, and the net generation time of the total (Ad plus nonAd) population.

Opacity and adherence were inseparable in this study and therefore, it is difficult to distinguish between two reasonable interpretations for the enhanced growth of the opaque gonococcus: a microenvironment favorable for reproduction of all gonococci adherent to the HeLa cell surface, or an opacity-specific stimulation arising from the HeLa cell. The progeny of the Ad and replicating opaque gonococcus remained bound to the monolayer. This could be the consequence of intergonococcal adhesions. The daughter cell has the option of attaching directly to the HeLa cell surface, or adhering indirectly by binding to another gonococcus, which in turn makes direct contact with the monolayer. Although this concept is not new (Swanson, 1980b), these experiments provide a working model. In conclusion, low levels of detachment, combined with enhanced multiplication and retention of daughter cells, contribute to the ability of opaque gonococci to extensively colonize the HeLa cell surface.

Tissue culture cells have served as valuable tools for studying the invasive capacity of several other micro-organisms (Friis, 1972; Hale et al., 1979; Kihlstrom and Nilsson, 1977; Gianella et al., 1973), and for this reason, I sought to determine whether HeLa cells provide a useful model for gonococcal invasion. The data indicates that a small proportion (0.5 to 2.5% or more) of Ad gonococci survive the bactericidal action of FRS. Although the various colony phenotypes attached to HeLa cells with different levels of efficiency, once attached, serum survival was approximately the same for all types. In FTOC, both  $P^+O^-$  and  $P^-O^-$  colony types penetrate at comparable levels once the organism is firmly attached to the surface (Ward et al., 1975; McGee et al., 1983), and in this way, our findings parallel the FTOC invasive model. However, less

than 0.1% of organisms adhering to HeLa cells survived gentamicin treatment, which is substantially lower than the 1.0% survival rate reported for Ad gonococci cultivated in FTOC and subjected to spectinomycin (Ward et al., 1975). In addition, long term cultivation of gonococci with HeLa cells failed to increase the proportion of Ad organisms which were resistant to gentamicin. If survivors of gentamicin represent organisms which have completely penetrated HeLa cells, then the level of invasion is significantly less than that observed with columnar epithelium of the FTOC. Our data supports some degree of penetration of HeLa cells by gonococci. However, the level of invasion is low and therefore limits the usefulness of HeLa cells as an experimental model for gonococcal invasion. One distinction between the two microbicidal agents employed in this study is that long term co-cultivation of gonococci on HeLa cells led to a small but significant increase in the extent of survival in serum but not in gentamicin. Another difference between gentamicin and the complement components of FRS is molecular size, and this may have bearing on their accessibility to a partially engulfed or deeply embedded gonococcus. The results obtained with CB support a key role for the HeLa cell cytoskeleton in protection. Gentamicin-resistant organisms may represent those few which have completely invaded the HeLa cell, whereas those surviving serum are more likely to be present on the cell surface in a partially engulfed state.

The action of CB on professional phagocytes is well-documented. By disrupting microfilament function, pseudopod extension is blocked and phagocytosis diminished (Zigmond and Hirsch, 1972; Davies et al., 1973; Hartwig and Stossel, 1979). Uptake of several invasive bacteria by epithelial cell monolayers is retarded in the presence of CB, and

treatment of FTOC with CB reduces gonococcal penetration by 5-fold (Ward et al., 1975). This drug had profound effects on HeLa cell morphology, causing cells to round up and edges to retract. The survival of Ad gonococci following either serum or gentamicin treatment was reduced on CB-treated monolayers by up to 8-fold. Cytochalasin B treatment did not increase the susceptibility to FRS of the commensal Neisseria sicca. Our data indicates that the function of the HeLa cell cytoskeleton enhances the survival of the attached gonococcus. The distinctive behavior of nonpathogenic Neisseria provides further support that the association between the gonococcus and HeLa cell is highly specific.

A speculative scenario for uncomplicated gonorrhea has been proposed, whole or in part, by several investigators. The sites of infection under consideration are the male urethra (Ward et al., 1975; Ward and Watt, 1972; Swanson and Mayer, 1984) and the ectocervix or squamocolumnar junction (Swanson, 1980b; Swanson and Mayer, 1984; Evans, 1977). Gonococci are embedded and partially engulfed on the epithelial cell surface (Evans, 1977; Ward et al., 1975; Ward and Watt, 1972; Swanson, 1980b; Swanson and Mayer, 1984; Novotny and Short, 1977). The attached organisms multiply (Evans, 1977; Ward et al., 1975; Swanson, 1980b), forming microcolonies on urethral cells (Ward et al., 1975; Novotny and Short, 1977). Daughter cells are retained by those organisms possessing opacity-associated P.II (Swanson, 1980b). An effective mode for transmission to a new host may be desquamated epithelial cells studded with gonococci (Evans, 1977), which are replicating (Swanson and Mayer, 1984; Novotny and Short, 1977). There is controversy as to whether squamous cells of the cervix are truly invaded by gonococci (Ward et al., 1975; Evans, 1977; Harkness, 1948).

Cultivation of gonococci on HeLa cell monolayers appears to best resemble those events which histopathological studies have suggested to be the hallmarks of urethral and cervical cell infection. Adherent gonococci displayed increased multiplication and the daughter cells of the opaque variant were retained in the adherent fraction. The data suggests that some degree of partial engulfment occurs on the HeLa cell surface. The preponderance of opaque colony types in clinical isolates taken from the urethra or cervix at midcycle (Draper et al., 1980; James and Swanson, 1978b), further supports the parallels between the gonococcal-HeLa cell model and events in vivo. The value of an in vitro model for the interaction of gonococci with epithelium is that it can potentially lead to a better understanding of molecular mechanisms which underlie gonococcal infection.

### 3.4 GENERAL DISCUSSION OF CHAPTERS 2 AND 3

Neisseria gonorrhoeae and enteroinvasive E. coli share the general property of invasion. During the course of infection, EIEC invade epithelial cells lining the gut in order to elicit the clinical symptoms of dysentery. Gonococci penetrate the epithelial barrier during DGI, and become embedded in, and engulfed by epithelial cells in uncomplicated infections. However, the interactions of these two organisms with cultured epithelial cells are quite distinct in several respects.

Invasion of HeLa cells by EIEC occurred at a faster rate and attained higher intracellular levels than invasion by gonococci. The HeLa



cell may not be a suitable target for gonococcal invasion, despite the fact that (i) gonococci adhere strongly to the cell surface; and (ii) HeLa cells are competent for invasion of not only EIEC and Shigella, but many other microorganisms including Salmonella, Yersinia, and Chlamydia. Other cultured epithelial cells of human origin (HEp-2, Henle 407) which sustained EIEC invasion were tested with gonococci and invasion levels remained low (data not shown). In the FTOC model, gonococci invade 20 h post-attachment (McGee et al., 1983; Ward et al., 1974), yet long-term cultivation of gonococci on HeLa cells failed to increase the yield of gentamicin-resistant organisms. The mechanism(s) by which invasive bacteria induce their phagocytosis by nonprofessional cells has not been defined. It may be that induction mechanisms employed by EIEC and gonococci are quite distinct. The interactions of both gonococci and EIEC with cultured epithelial cells was influenced by CB. Although CB has a multitude of physiological effects on eucaryotic cells, the most striking is on those functions requiring actin which include maintenance of cell shape, motility, and phagocytosis. Inhibition of EIEC invasion and further diminution of gonococcal viability by bactericidal agents in the presence of CB strongly suggests that both organisms rely on cytoskeletal elements for their interactions with HeLa cells.

Multinucleated HeLa cells generated by PEG treatment supported higher levels of EIEC invasion but had no obvious effect on uptake of HB101 or gonococci. It is unclear as to the actual contribution of PEG treatment to promoting phagocytosis and perhaps to enhancing intracellular growth as well. If in fact PEG acts largely on the phagocytic step, the lack of enhancement of gonococcal invasion by PEG may be indicative of distinct inductive mechanisms for invasion. The increased

cell size of multinucleated HeLa cells amplified the distances between morphological features, and the subtle patterns of bacterial association occasionally observed in unfused cells became obvious in multinucleated cells. Gonococci which remained attached following FRS treatment were concentrated close to the perinuclear region of multinucleated HeLa cells. Interestingly, a perinuclear distribution is observed with piliated gonococci attached to human amnion cells; furthermore, electron microscopy of amnion cells shows several gonococci at an intracellular location, some lying adjacent to the nuclear region (Swanson, 1973). In contrast, EIEC displayed a strong predilection for the extensions of fused cells. It has not been resolved whether EIEC entered the cell at this site, or followed an intracellular route in getting there. Nevertheless, it is clear that EIEC and gonococci accumulate at distinct morphological sites during cultivation with HeLa cells.

## CHAPTER 4

### BINDING PROPERTIES OF GONOCOCCAL PROTEIN II

A gonococcus may express one, several, or none of the outer membrane proteins classified as Protein II (P.II). Proteins II are chemically defined by a shift in apparent molecular weight upon heating, and are structurally related (Heckels, 1977; Swanson, 1978b; Swanson and Barrera, 1983). In some gonococcal variants P.II is abundant, equaling the major outer membrane protein in content. One consequence of P.II expression is an alteration in the capacity of the gonococcus to engage in cell-cell interactions, with other gonococci and with a variety of eucaryotic cell types. Whether P.IIs impart nonspecific adhesive properties to the gonococcal cell surface, or whether P.IIs act as specific adhesins, combining stereochemically by a lock-and-key mechanism, is unknown. A role for surface carbohydrate in P.II-mediated adhesion has been addressed by several investigators. It has been proposed that a receptor-ligand relationship between P.II<sub>op</sub> and LPS underlies the basis for the intergonococcal adhesions which give rise to the opaque colony phenotype (Blake, 1985). The objective of this study is to better understand the chemical nature of the interaction between P.II<sub>op</sub> and eucaryotic cells, and of P.II-carbohydrate interactions in particular. The binding activity of purified P.IIs to HeLa cell components and to other eucaryotic-derived macromolecules is presented.

## 4.1 MATERIALS AND METHODS

Reagents: Carrier-free Na  $^{125}\text{I}$  (17 Ci/mg), and D-[6- $^3\text{H}(\text{N})$ ]-glucosamine hydrochloride (28 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). The lectins wheat germ agglutinin (WGA) and concanavalin A (ConA), rabbit anti-WGA and anti-ConA sera, N,N'-diacetylchitobiose, and N,N,N''-triacetylchitotriose were purchased from E-Y Laboratories, Inc. (San Mateo, CA). Goat anti-rabbit Ig (GARIG) and goat anti-mouse Ig (GAMIG) antibody was purchased from Tago, Inc. (Burlingame, CA). The following materials were obtained from Sigma Chemical Corp. (St. Louis, MO): human serum albumin (HSA) (Fraction V), bovine submaxillary gland mucin (Type I), fetuin (Type III), ovomucoid (Type III-O), ovalbumin (Grade V), lactic dehydrogenase (Type III), cytochrome c (Type IV). Zwittergent 3-14 and Pronase B were obtained from Calbiochem-Behring (La Jolla, CA). Crystalline bovine serum albumin (BSA) was purchased from Armour Pharmaceutical Company (Kankanee, IL).

Purification of P.IIa: Strain R10 of Neisseria gonorrhoeae was grown overnight in Proteose Peptone broth, and organisms were extracted with Zwittergent 3-14 in the presence of  $\text{CaCl}_2$  at pH 4.0. Protein IIa (a P.IIop, described in Chapter 3) was purified by salt elution from a CM-Sepharose ion exchange column (pH 8.1), followed by molecular sieve chromatography (Blake and Gotschlich, 1984). Purified P.IIa had a  $M_r$  of approximately 29 kDa (heat-modified form) and probably is identical to the R10 P.IIop previously described (Blake and Gotschlich, 1984), with a pI of 9.5. Purification of P.IIc, which is also a P.IIop, is presented in the Results section (4.2.5.)

Iodination of P.II: Purified P.II (20 ug) in 0.2 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.2 M NaCl, 0.01 M EDTA, and 0.05% Zwittergent was iodinated for 15 min at room temperature by addition of 0.1 mM carrier KI, 0.5 mCi Na<sup>125</sup>I, and Iodo-Beads (Pierce Chemical Company, Rockford, IL) coated with chloroglycoluril (Markwell and Fox, 1978). The reaction was terminated by addition of sodium metabisulfite. Iodinated P.II was separated from unreacted iodine by passage over a column of Sephadex G-50 Fine, which had been equilibrated in the iodination buffer and precoated with BSA. Typically 70% of the radiolabel was incorporated into trichloroacetic acid (TCA) precipitable counts.

Gel electrophoresis: Samples were solubilized in 1.0 % SDS at 100°C with beta-mercaptoethanol unless indicated otherwise, and separated by SDS-PAGE (Laemmli, 1970), on gels composed of 12.5% (30:0.8) acrylamide:bis-acrylamide. Gels were either stained with Coomassie blue, stained with silver following periodate oxidation (Tsai and Frasch, 1982), dried for autoradiography, or electroblotted to nitrocellulose membranes (details below).

Ligand immobilization on blots: HeLa cell protein and glycoprotein was obtained from confluent monolayers which were lysed in 1.0 % SDS, and separated by SDS-PAGE; each lane contained an equivalent of 10<sup>5</sup> HeLa cells, representing 10 ug or less of total protein. HeLa cell protein was transferred electrophoretically from gels to nitrocellulose (Bio-Rad Laboratories, Richmond, CA) (Towbin et al., 1979). Western blots of proteins and glycoproteins of defined composition were prepared in a similar manner. For dot blots, proteins and glycoproteins were

immobilized on nitrocellulose membranes using a BioDot apparatus (Bio-Rad).

Probe-ligand binding assays and immunoblots: Ligands immobilized on nitrocellulose were incubated with probes which could be detected by autoradiography or by immunochemical means. Membranes bearing immobilized ligand were blocked with 0.5% Tween 20 (Batteiger et al., 1982). All incubations were carried out at room temperature in 0.1 M Tris-HCl (pH 8.0), 0.4 M NaCl, and 0.5% Tween 20 for 1 to 3 h with shaking, unless indicated otherwise. Extensive washing in buffer followed the removal of each probe. For inhibition studies, probes were preincubated with inhibitors in 0.1 M Tris-HCl and 0.5% Tween 20 for 1 h, after which time the NaCl concentration was adjusted to 0.4 M, and the probe-inhibitor mixture added to immobilized ligand on membranes. Probes were used at the following concentrations: iodinated P.II (from 20 ng/ml or approximately 1 nM when fresh, to 200 ng/ml for up to 6 months post-labeling), noniodinated P.II (20 to 50 ug/ml), WGA (1 ug/ml), and ConA (100 ng/ml). To ensure optimal binding of ConA, the pH of Tris-HCl was 7.2 and 1 mM of  $\text{CaCl}_2$  and  $\text{MnCl}_2$  were included. Iodinated P.II was detected by autoradiography. Noniodinated P.II, WGA, and ConA were localized using polyclonal rabbit serum against P.IIa (kindly provided by Dr. M. Blake), or rabbit anti-lectin sera, and stained with GAR Ig conjugated to alkaline phosphatase (Blake et al., 1984).

Densitometric measurements: Autoradiograms of dot blots were analyzed on a Gilford densitometer equipped with a Hewlett-Packard integrator, and peak heights were measured.

HeLa cell maintenance and radiolabeling: HeLa cells which were used for Western blotting were grown as monolayers to confluence in Dulbecco's MEM (Flow Laboratories, Inc., McLean, VA) containing 10% ZetaSera D (AMF Biological, Seguin, TX) and antibiotics. For Bligh-Dyer extraction (Bligh and Dyer, 1959), HeLa S3 cells (a variant adapted for growth in suspension culture) were grown to  $5 \times 10^5$  cells per ml in MEM/Spinner medium (KC Biological, Inc., Lenexa, KS) containing 10% serum and antibiotics. For intrinsic labeling of HeLa S3 carbohydrate (Yurchenco et al., 1978), 50 uCi of tritiated glucosamine was added to 50 ml of actively growing culture, and cells harvested after 18 h.

HeLa cell extraction: HeLa S3 cells were washed by centrifugation, and extracted in chloroform:methanol by the Bligh-Dyer procedure (Finne and Krusuis, 1982; Bligh and Dyer, 1959). For compositional analysis, HeLa cells radiolabeled with ( $^3\text{H}$ )-glucosamine were included as 5% of the total cell population. In brief, cells were subjected to Dounce homogenization (Type A pestle) in 1:2:1 chloroform:methanol:H<sub>2</sub>O, KCl added, and the lower phase separated from the upper phase and interfacial precipitate. The lower phase was evaporated under a stream of N<sub>2</sub> and stored dessicated. The upper phase and interface were pooled, precipitated in 90% methanol, washed 2x in methanol, washed 1x in acetone, and dried overnight in vacuo.

Glycopeptide preparation (Finne and Krusuis, 1982): Bovine submaxillary (BSM) gland mucin or the dried acetone powder of HeLa cell extract was brought to 20 mg protein per ml in 0.05 M Tris-HCl (pH 8.0) containing 0.01 M MgCl<sub>2</sub>, and nucleic acids were digested by 1 h incubation with 50 ug/ml each of RNase and DNase. Pronase B suspended



in 0.05 M Tris-HCl (pH 8.0) with 0.01 M  $\text{CaCl}_2$  was preincubated at  $56^\circ\text{C}$  for 30 min to inactivate residual glycosidase activity. Nuclease-digested HeLa upper phase/interface material was incubated at pH 8.0 with 1 mg/ml of pronase at  $37^\circ\text{C}$  or  $56^\circ\text{C}$  for 48 to 72 h. Fresh pronase (1 mg/ml) was added daily, and a toluene layer maintained to prevent microbial growth. At the completion of digestion, pronase was inactivated by boiling for 5 min, and the digest centrifuged at  $15,000 \times g$  for 10 min. The supernatant containing HeLa cell glycopeptide was dialyzed overnight in SpectraPor 6 tubing (molecular weight cutoff 1000) (Spectrum Medical Industries, Inc., Los Angeles, CA) to eliminate free amino acids, sugars, and nucleotides. Mucin glycopeptide was not dialyzed.

Methylation of BSA: Esterification of free carboxyl groups of BSA in methanolic HCl was performed as described (Sueoka and Cheng, 1962). The final precipitate was washed free of residual acid using methanol, and stored as a dry powder.

Analytical determinations: Protein content was measured with the bicinchoninic acid (BCA) reagent (Pierce) (Smith et al., 1985).

Gonococcal Binding Studies: Viable gonococci were tested for binding to ligands immobilized on nitrocellulose disks, or to HeLa cell monolayers (grown in 35-mm wells) in the presence of ligand inhibitors. Gonococci grown 21 h on GC agar were starved 30 min in methionine-free RPMI (Flow Labs) containing 5% zeta sera;  $^{35}\text{S}$ -methionine (New England Nuclear) was added at 1  $\mu\text{Ci}/\text{ml}$ , organisms were incubated for 60 min at  $37^\circ\text{C}$ , washed, and resuspended in either DME containing 0.1 % BSA or

chemically-defined Catlin medium (Gibco Diagnostics, Madison, WI). Radiolabeled gonococci were tested for binding to nitrocellulose disks or in some cases, to HeLa cell monolayers. Nitrocellulose disks (BA85, 13 mm; Schleicher and Scheull, Keene, NH) were boiled in  $\text{dH}_2\text{O}$  to remove the wetting agent, mounted in a Millex filter holder, and ligands were loaded into a syringe and pushed onto disks. Disks contained the following ligands: HeLa S3 cells ( $10^5$  cells/disk),  $\text{O}^{++}$  gonococci ( $10^8$  CFU/disk), albumin (1 mg/disk). Following ligand immobilization, disks were blocked 2 x 30 min with a 1 % solution of blocking agent as indicated; radiolabeled gonococci added to disks were incubated 90 min at  $37^\circ\text{C}$  with end-over-end rotation. For inhibition studies, both gonococci and HeLa cell monolayers were preincubated 30 min with the inhibitor under study in 0.1 % BSA-DME, organisms were allowed to bind for 90 to 120 min, and Ad and nonAd gonococcal fractions were assessed by CFUs or by radioactivity. For CFU measurements, Ad and nonAd fractions were collected as described in Section 3.1. For radiolabeled organisms, nonAd fractions were removed, disks or monolayers washed, and SDS added to each fraction at a final concentration of 1 %. A portion of the detergent lysate was added to scintillation fluid and counted.

## 4.2 RESULTS

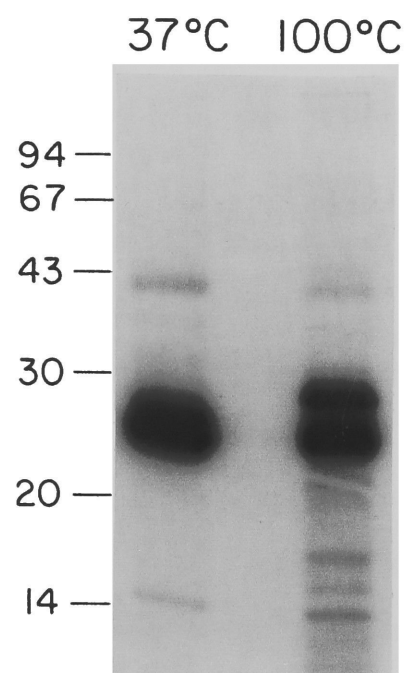
### 4.2.1 Purity of P.IIa

The radioiodinated P.IIa probe gave a single major band on SDS-PAGE and autoradiography, and displayed heat-modifiability (Figure 11). Shorter exposure times failed to reveal additional bands in the 20 kDa to 35 kDa region. Coomassie blue staining of purified P.IIa on SDS-PAGE exhibited the same pattern observed with radioiodinated P.IIa by autoradiography (data not shown). The gonococcal outer membrane contains abundant quantities of LPS and H.8 antigen, neither of which binds Coomassie blue or incorporates  $I^-$  by the chloroglycoluril method. Purified P.IIa was free of LPS as determined by silver staining following periodate oxidation of the purified protein on SDS-PAGE, and free of H.8 antigen as determined by Western immunoblot analysis (data not shown). Polyclonal antisera raised against whole gonococci and purified P.II each failed to detect major contaminants in the purified P.IIa preparation (data not shown).

### 4.2.2 Binding to HeLa Cell Protein/Glycoprotein: Role of Carbohydrate

A variety of substances (eg., antibodies, lectins, calmodulin, DNA, epidermal growth factor, bungarotoxin) have been employed as probes to demonstrate biologically meaningful interactions with proteins immobilized on Western blots (Gershoni and Palade, 1983). The binding of radioiodinated P.IIa to total HeLa cell protein and glycoprotein is

Figure 11: SDS-PAGE and autoradiography of the radioiodinated P.IIa probe. Radioiodinated P.IIa probe was solubilized in buffer containing SDS and beta-2-mercaptoethanol, and was incubated at either 37°C or 100°C for 30 or 5 min, respectively. Molecular weight standards are indicated.



presented in Figure 12 (Lane 1). About 30 to 50 distinct molecular species were recognized by P.IIa. The iodinated material which bound to HeLa cell components immobilized on nitrocellulose could be eluted by SDS; when subjected to SDS-PAGE and autoradiography, a single major band corresponding in position to P.IIa was observed (data not shown). Measurements of the percentage of a nM solution (1.0 ml) of  $^{125}\text{I}$ -P.IIa which bound to 10 ug or less of immobilized HeLa cell macromolecules ranged from 5 to 30%. The binding of wheat germ agglutinin (WGA), a model carbohydrate-binding protein, to HeLa cell components was compared to P.IIa binding (Figure 12). Many molecular species were recognized by WGA, some of which appear to be shared with P.IIa, and others unique.

The lectins WGA and ConA display high affinity for N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid (Bhavanandan and Katlic, 1979), or D-mannose and D-glucose, respectively. The lectins maintained their sugar specificities in this probe-ligand binding assay (Figure 13). WGA (present at 50 nM) was completely blocked in the presence of 10 mg/ml (45 mM) N-acetyl-D-glucosamine, 1.0 mg/ml (2.4 mM) N,N'-diacetylchitobiose, or 0.1 mg/ml (160 uM) N,N,N''-triacetylchitotriose. Similarly, ConA required 0.2 M alpha-methyl-D-mannoside for complete inhibition of binding. In the presence of inhibitory carbohydrate, band intensity of all lectin-ligand complexes appeared to diminish stoichiometrically; the exception was a series of low molecular weight bands which were often bound by GARIG and were unaffected by inhibitors.

To determine whether P.IIa is lectin-like and interacts with carbohydrate at its binding site, the monosaccharides most common to the

Figure 12: Western blot of HeLa cell protein and glycoprotein, and binding by P.IIa and WGA. Lanes: 1, iodinated P.IIa detected by autoradiography; 2, WGA detected with rabbit anti-WGA sera and GAR Ig conjugated to alkaline phosphatase.

1 2

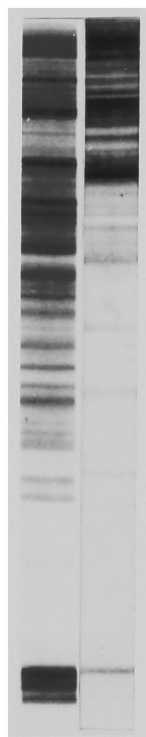




Figure 13: Western blot of HeLa cell protein and glycoprotein, and carbohydrate-specific binding by lectins. Lectins WGA and ConA were detected with rabbit anti-lectin sera and GAR Ig conjugated to alkaline phosphatase. Prior to incubation with nitrocellulose, WGA was preincubated with the following carbohydrates: 50 mg/ml D-glucosamine hydrochloride (lane 1); N,N,N''-triacetylchitotriose at 0.1 (lane 2) and 1 mg/ml (lane 3); N,N'-diacetylchitobiose at 0.1 (lane 4) and 1 mg/ml (lane 5); N-acetyl-D-glucosamine at 0.1 (lane 6), 1 (lane 7), and 10 mg/ml (lane 8); and no inhibitor (lane 9). For ConA, preincubation with 0.2 M alpha-methyl-D-mannoside (lane 11) or no inhibitor (lane 10).

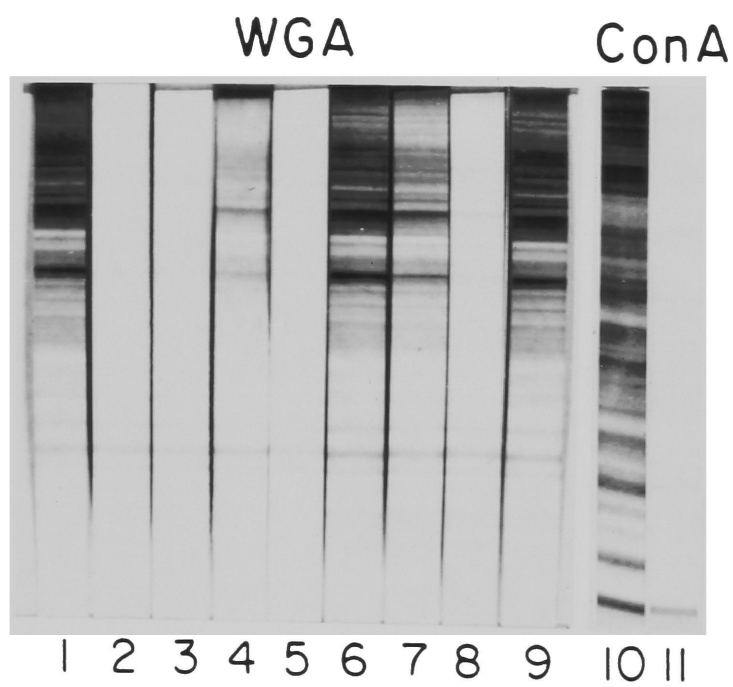


Table 13: Inhibition of P.IIa Binding to HeLa Cell (Glyco)protein.

(a) Radioiodinated P.IIa was used at 50 ng/ml (about 2.5 nM). The following abbreviations are used: glucose (Glu), glucosamine ( $\text{Glu-NH}_2$ ), N-acetyl-D-glucosamine (NAcGlu), galactose (Gal), galactosamine ( $\text{Gal-NH}_2$ ), N-acetyl-D-galactosamine (NAcGal) mannose (Man), fucose (Fuc), N-acetyl-D-neuraminic acid (NAcNeu).

Table 13: Inhibition of P.IIa Binding to HeLa Cell (Glyco)protein.

<u>Carbohydrates</u> <sup>(a)</sup>	<u>Concentration</u>	<u>Inhibition</u>
Glu, Glu-NH <sub>2</sub> , NAcGlu	0.25 M	0
Gal, Gal-NH <sub>2</sub> , NAcGal	0.25 M	0
Man	0.25 M	0
Fuc	0.25 M	0
Lactose	0.25 M	0
$\beta$ -methyl-Glu	0.25 M	0
$\beta$ -methyl-Gal	0.25 M	0
$\alpha$ -methyl-Man	0.25 M	0
NAcNeu	0.05 M	0
Colominic acid	1 mg/ml	0
<u>N. meningitidis</u> (C,Y) capsule	1 mg/ml	0

eucaryotic cell surface were tested at concentrations of 0.25 M or higher (at pH 8.0), and each failed to diminish binding of P.IIa to HeLa cell components immobilized on nitrocellulose (Table 13). In addition, neither 0.05 M N-acetyl-D-neuraminic acid nor polysialyl capsular material (1 mg/ml) from Escherichia coli K1 (colominic acid) or Neisseria meningitidis (Groups C and Y) were capable of blocking P.IIa binding activity. In further attempts to test the role of carbohydrate in P.IIa binding, four eucaryotic glycoproteins were chosen for study: BSM mucin, fetuin, ovomucoid, and ovalbumin. This particular combination of glycoproteins provides an array of many important features of oligosaccharides which could influence recognition by lectin-like molecules. Figure 14 shows the inhibitory capacities of glycoproteins in blocking WGA and P.IIa binding to nitrocellulose-bound HeLa cell components. Inhibitor glycoproteins were tested at 200 ug/ml, which represents approximately 5 uM for fetuin, ovomucoid, and ovalbumin. WGA binding was significantly diminished in the presence of all four glycoproteins. Blockage of P.IIa binding was complete with mucin, near complete with fetuin, and partial with ovalbumin. ConA binding was inhibited by mucin and ovalbumin (data not shown). In addition, intact HeLa cells were gently trypsinized and the remaining cell-associated protein and glycoprotein was tested for binding by P.IIa and lectin. A multitude of high molecular weight HeLa cell components bound by P.IIa and WGA were absent from surface-trypsinized HeLa cells (data not shown).

In order to better ascertain the chemical nature of the HeLa cell substance engaged in P.IIa interactions, HeLa cells were fractionated and tested for P.IIa inhibitory activity (Figure 15). Homogenized HeLa S3 cells were extracted with a chloroform:methanol:water mixture;

Figure 14: Western blot of HeLa cell protein and glycoprotein, and inhibition of P.IIa and WGA binding by model glycoproteins. Inhibitors were present at 200 ug/ml: mucin (Lane 1); ovalbumin (Lane 2); fetuin (Lane 3); ovomucoid (Lane 4); and no inhibitor (Lane 5). P.IIa was used at approximately 200 ng/ml.

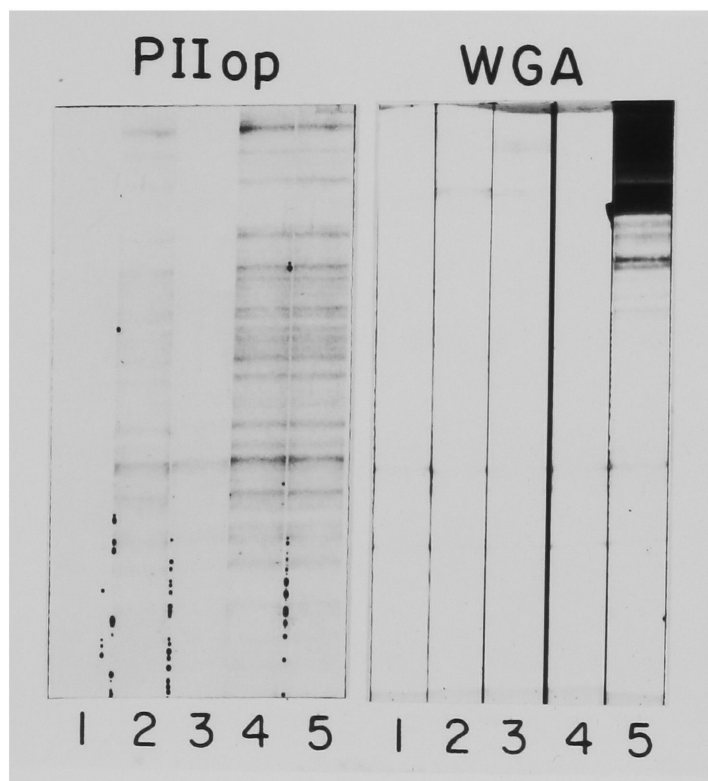


Figure 15: Flow chart of HeLa cell glycopeptide preparation.



HELA CELL  
FRACTIONATION

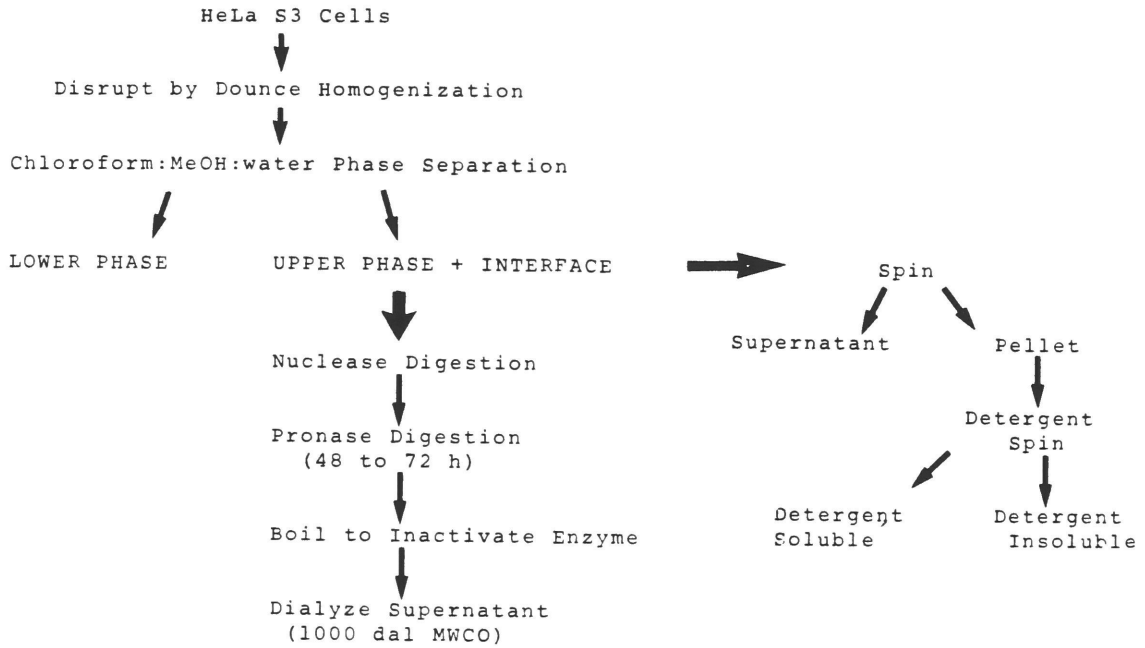


Table 14: Composition of HeLa Cell Fractions

- (a) Supernatant and pellet subfractions were prepared by centrifugation at 15,000 x g for 10 min.
- (b) Glucosamine measurements represent counts per minute (cpm) of tritiated label. Protein content was determined by the BCA analysis.
- (c) The ratio of cpm of ( $^3\text{H}$ )-glucosamine to ug of protein is expressed.

Table 14: Composition of HeLa cell fractions.

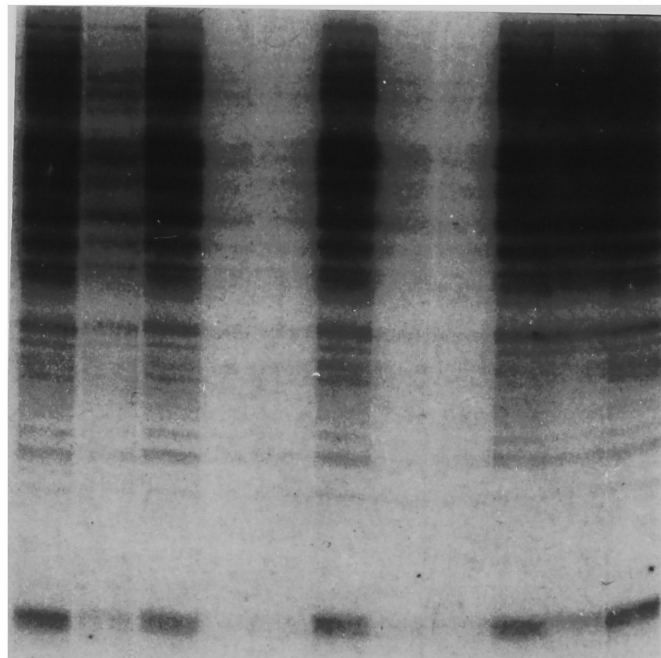
<u>Fraction</u> <sup>(a)</sup>	<u>Composition per 10<sup>6</sup> HeLa Cells</u> <sup>(b)</sup>		Ratio <sup>(c)</sup>
	<u>Glucosamine</u> (cpm)	<u>Protein</u> (ug)	<u>Glucosamine:</u> <u>Protein</u>
Lower Phase	85	13	6.5
Upper Phase	4410	281	15.7
pellet	3840 (87%)		
supernatant	570 (13%)		
Post-Pronase	3586	80	
pellet	586 (16%)	18	32.6
supernatant	3000 (84%)	62	48.4
Post-Dialysis	2936	23	126.0

chemical compositions of the fractions are summarized in Table 14. It has been reported (Yurchenco et al., 1978) that 65% to 100% of tritiated glucosamine is incorporated into HeLa S3 cells as the same compound and thus, it serves as a useful marker for oligosaccharides. The majority of radioactivity in the upper phase/interface fraction redistributed from the pellet to the supernatant as a consequence of pronase digestion. Greater than 97% of the radioactivity in the post-pronase supernatant was recovered following dialysis (1000 dalton exclusion limit), whereas only 37% of the protein was retained. The enrichment of radioactivity over protein from the undigested upper phase/interface material to the final dialysate was 8-fold. HeLa cell fractions were tested for ability to inhibit P.IIa binding to HeLa cell macromolecules immobilized on nitrocellulose, and the effects of nuclease and pronase digestion on the inhibitory aqueous/interface fraction was determined (Figure 16). It should be noted that when SDS lysates of HeLa S3 cells were prepared as ligands on Western blots, binding by P.IIa was identical to that observed with HeLa cell monolayers (data not shown).

The lower phase extract, which is expected to contain most neutral glycolipid, lacked significant inhibitory activity for P.IIa binding (Figure 16, Lane 10). Strong inhibition was observed with the upper phase/interface pool, which contained most of the HeLa cell nucleic acid, protein, and glycoprotein (Lanes 7 to 9). The majority of inhibitory activity in this fraction was insoluble in aqueous solution, but could be solubilized by 1.0 % zwitterionic detergent (Lane 8). Nuclease digestion of the upper phase/interface pool had no deleterious effect on P.IIa inhibitory activity (Lanes 4 to 6). Extensive digestion with pronase resulted in solubilization of most of the upper phase/interface

Figure 16: Western blot of HeLa cell protein and glycoprotein, and inhibition of P.IIa binding by HeLa cell fractions of a chloroform:methanol:water extract. P.IIa was used at approximately 50 ng/ml. Inhibitors were tested at  $10^6$  HeLa cell equivalents/ml, unless otherwise indicated. The upper phase represents the methanol:water fraction plus the interfacial material, and the lower phase corresponds to the chloroform fraction. Supernatant and pellet subfractions were prepared by centrifugation at  $15,000 \times g$  for 10 min. Lanes: 1 and 11, no inhibitor; 2, post-pronase digest of upper phase, pellet; 3, post-pronase digest of upper phase, supernatant (glycopeptide-enriched); 4, post-nuclease digest of upper phase, detergent-insoluble pellet; 5, post-nuclease digest of upper phase, detergent-solubilized pellet; 6, post-nuclease digest of upper phase, supernatant; 7, undigested upper phase, detergent-insoluble pellet; 8, undigested upper phase, detergent-solubilized pellet; 9, undigested upper phase, supernatant; 10, lower phase, 5-fold concentrated. Protein and carbohydrate content of inhibitory fractions is presented in Table 14.

1 2 3 4 5 6 7 8 9 10 11



fraction, and glycopeptides were generated (Table 14). However, the inhibitory capacity for P.IIa binding was lost as a result of pronase treatment (Figure 16, Lane 3). Thus, a macromolecule which is both nuclease-resistant and protease-sensitive is capable of inhibiting P.IIa binding. When P.IIa binding was inhibited, the intensity of each individual molecular component diminished proportionately, suggesting that a single mechanism is responsible for P.IIa binding.

The glycoprotein BSM mucin displayed high inhibitory activity for both WGA and P.IIa binding. Generation of mucin glycopeptide by exhaustive digestion with pronase led to a moderate loss (5-fold) in WGA inhibition (Table 15); this loss probably reflects in part the lack of cooperative binding by the dimeric form of WGA with monovalent glycopeptide. In contrast, pronase digestion of mucin resulted in complete loss (greater than 100-fold) of inhibitory activity for P.IIa. These results add further support that the P.IIa inhibitory component is protein and not carbohydrate.

#### 4.2.3 P.IIa Binding to Proteins of Defined Composition

Several glycoproteins and non-glycosylated proteins were tested as ligands for direct P.IIa binding (Figure 17). WGA and ConA serve to identify the glycosylated state of the proteins. Protein IIA exhibited a high affinity for denatured forms of the nonglycosylated proteins BSA, lactic dehydrogenase, and hemoglobin (lanes 4, 6, and 8). Weak P.IIa binding was observed with cytochrome c and ovalbumin (lanes 2 and 5). The major component of ovomucoid failed to bind P.IIa (lane 1). It can

Table 15: Inhibitory activity of intact mucin and pronase digest.

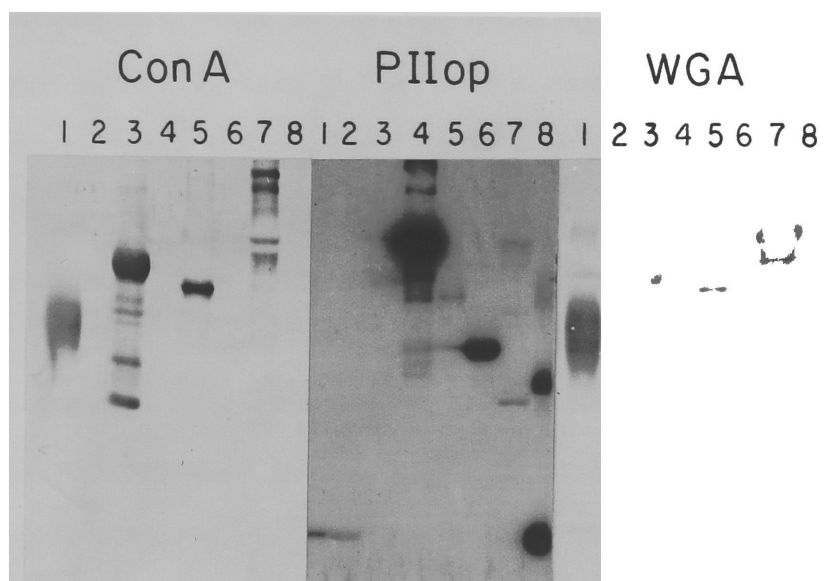
(a) Radioiodinated P.IIa was used at 20 to 50 ng/ml (about 1.0 to 2.5 nM). Inhibition was 3+ (complete), 2+ (partial), 1+ (slight), 0 (none), or ND (not determined). Each score represents a single experiment.



Table 15: Inhibitory activity of intact mucin and pronase digest.

Mucin <u>ug/ml</u>	<u>WGA</u>		<u>P.IIa (a)</u>	
	<u>Intact</u>	<u>Digest</u>	<u>Intact</u>	<u>Digest</u>
5000	ND	ND	ND	0
1000	ND	3+,3+	3+,3+	0,0,0
200	3+	2+,2+	2+,2+,2+	0
40	2+,2+	1+,1+	0,1+,1+	0
8	2+	1+	ND	ND

Figure 17: Western blot of model proteins and glycoproteins, and binding by P.IIa and lectins. Probes: ConA, anti-ConA, and GARIg; radioiodinated P.IIa; and WGA, anti-WGA, and GARIg. Proteins were boiled in SDS and beta-2-mercaptoethanol, subjected to SDS-PAGE (2 ug of protein per lane), and electrophoretically transferred to nitrocellulose. Lanes: ovomucoid (1); cytochrome c (2); rabbit IgG (3); BSA (4); ovalbumin (5); lactic dehydrogenase (6); fetuin (7); hemoglobin (8).



be concluded that while P.IIa may in fact recognize some as yet undetermined oligosaccharide structure, its high affinity interaction is with protein.

Since P.IIa interacted with serum albumin with high affinity, this interaction was studied in greater depth. Figure 18 shows that P.IIa bound to the reduced and unfolded configurations of HSA and BSA, but not to the native (fast-migrating, unreduced) forms. The unreduced form of carboxymethylated BSA (MBSA) was fast-migrating on SDS-PAGE, but unlike native and unreduced BSA, MBSA was avidly bound by P.IIa. In addition, BSA and HSA each failed to bind P.IIa in the native configuration, irrespective of their fatty acid content (data not shown). Serum albumins contain a highly reactive sulfhydryl group which is normally buried in the native molecule, but becomes accessible in certain conformational forms (Foster, 1977). Experiments were designed to determine whether P.IIa formed a covalent disulfide bond with albumin. Binding of P.IIa to albumins was not impaired in the presence of high concentrations of the reducing agent dithiothreitol (DTT) (Figure 18c). To the contrary, the P.IIa recognition site became exposed on the unreduced, native albumin following in situ modification with DTT.

The native forms of proteins to which P.IIa bound directly were tested for their ability to inhibit binding of P.IIa to HeLa cell macromolecules (Figure 19). Nondenatured hemoglobin was a potent inhibitor of P.IIa binding, and good inhibition was also observed with lactic dehydrogenase, fetuin, and mucin. The native form of BSA was noninhibitory. Methylated BSA was a highly effective blocker of P.IIa binding to most (but not to all) HeLa cell macromolecules; in this regard MBSA

Figure 18: SDS-PAGE and Western blot of serum albumins and binding by P.IIa. Gels were stained with Coomassie blue (a) or were blotted to nitrocellulose (b and c). Each lane contained 2.5 ug of the following albumin form: HSA nonreduced (lane 4) and reduced with beta-2-mercaptoethanol (lane 1); BSA nonreduced (lane 5) and reduced (lane 2); MBSA nonreduced (lane 6) and reduced (lane 3). Western blots were incubated with radioiodinated P.IIa in the presence (c) or absence (b) of 100 mM dithiothreitol.

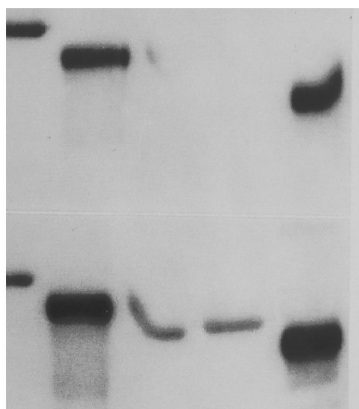
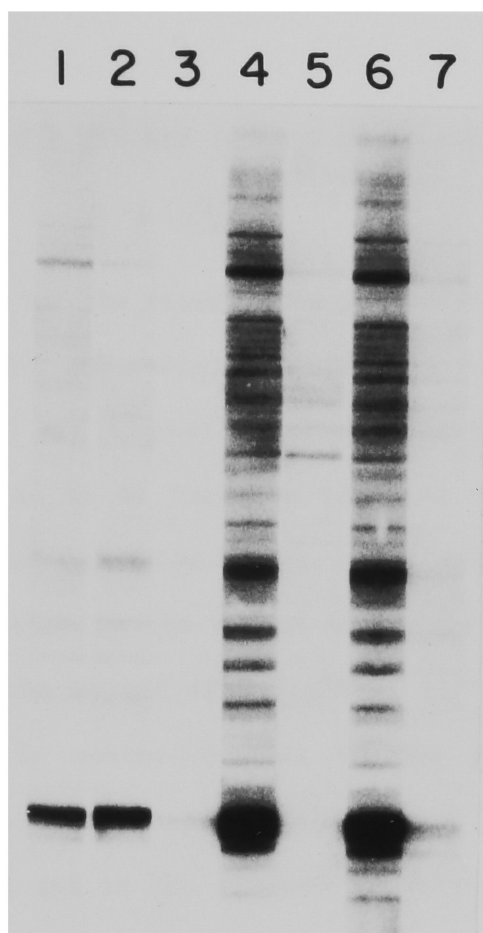


Figure 19: Western blot of HeLa cell protein and glycoprotein, and inhibition of P.IIa binding by proteins. P.IIa was used at approximately 200 ng/ml. Inhibitors were present at 200 ug/ml: lactic dehydrogenase (Lane 1); fetuin (2); hemoglobin (3); no inhibitor (4); MBSA (5); BSA (6); and mucin (7).





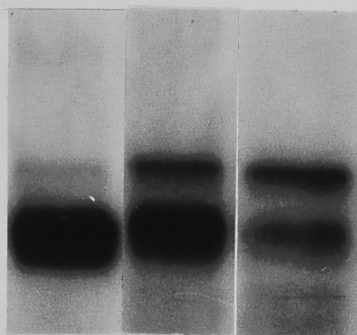
differed from other inhibitors of P.IIa binding, which lacked band selectivity. In conclusion, direct binding of P.IIa to a given ligand correlated well with the ability of that ligand to act as an inhibitor of P.IIa binding to HeLa cell macromolecules.

#### 4.2.4 Covalent Modification of P.IIa and Effects on Binding

The P.IIa probe employed in the experiments presented thus far was covalently modified as a result of radioiodination. A 100-fold molar excess of carrier KI (0.1 mM) was typically included in the iodination reaction mixture because it led to an increase of up to 5-fold in the specific radioactivity of the iodinated probe. The extent of P.IIa derivatization could be modulated by the amount of carrier KI added to the reaction mix. Based on the amount of radiolabel incorporated into TCA-precipitable counts, it is estimated that in the presence of a 100-fold molar excess of KI, an average of 20 molecules of  $I^-$  are incorporated per molecule of P.IIa, and in the absence of carrier an average of 1 in 23 P.IIa molecules are iodinated at a single site. A radioiodinated P.IIa probe was prepared in the absence of carrier KI, and subsequently re-iodinated in the presence of Iodobeads and cold KI only for various periods of time, leading to the generation of P.IIa probes with different degrees of derivatization but without change in specific radioactivity. Figure 20B shows that extensively derivatized P.IIa (lane 1) displayed a higher binding affinity for HeLa cell components than the lesser derivatized probe (lane 3), although the same HeLa cell components were bound by both P.II probes. In addition, the ability of P.IIa to undergo thermal denaturation (heat modification) was diminished

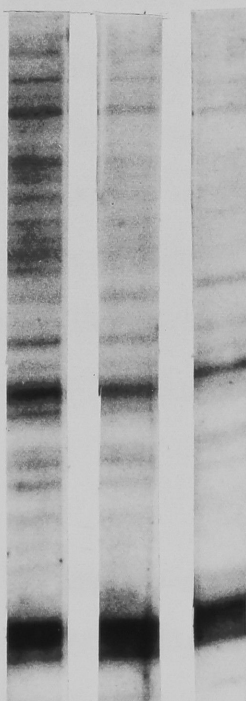
Figure 20: Effect of derivatization of P.IIa with iodine. Purified P.IIa was radioiodinated as usual, except carrier KI was omitted. The radiolabeled molecule was desalted as usual, re-iodinated in 0.1 mM cold KI and iodobeads only, and the reaction stopped at 0 (lanes 3), 1 (lanes 2), and 5 (lanes 1) min with sodium metabisulfite. The derivatized probes were boiled and subjected to SDS-PAGE plus autoradiography (Panel A). The probes were tested for binding to HeLa cell components immobilized on nitrocellulose (Panel B).

**A**



1 2 3

**B**



with increased derivatization (Figure 20A), suggesting that covalent modification at these sites results in stabilization of the native conformation of P.IIa.

Binding of radioiodinated P.IIa to HeLa cell components was inhibited in the presence of noniodinated, cold P.IIa. Nitrocellulose strips containing immobilized HeLa cell components were preincubated with cold P.IIa for 1 h, after which time either extensively or lightly iodinated P.IIa probes were added and allowed to bind (Figure 21). Zwittergent 3-14 was included in the binding mixture as a control since the detergent in itself slightly diminished P.IIa binding (lanes 2, 5, and 7). The extensively derivatized P.IIa probe was partially inhibited in the presence of a 50-molar excess of cold P.IIa (compare lanes 4 and 5). However, inhibition of the lesser derivatized probe was near complete in the presence of a 10-molar excess of cold P.IIa (compare lanes 7 and 8). These results add further support that P.IIa binding affinity is influenced by the degree of derivatization with iodine. In addition, cold P.IIa competes with radioiodinated P.IIa for binding sites on HeLa cell components.

A direct comparison was made between binding to HeLa cell components of radioiodinated P.IIa and cold P.IIa detected by antibody (Figure 22). The majority of bands bound by P.IIa monitored with antibody were also bound by  $^{125}\text{I}$ -P.IIa, and thus for the most part, the reactivity of P.IIa was not impaired by iodination. The cause for differential binding to many other HeLa cell components by  $^{125}\text{I}$ -P.IIa versus P.IIa plus antibody was not determined, but could be due to the large difference in probe sensitivities. The binding of cold P.IIa to

Figure 21: Inhibition of radioiodinated P.IIa binding by cold P.IIa. Extensively derivatized P.IIa (lanes 1 to 5) as shown in Figure 11, and lightly derivatized P.IIa (lanes 6 to 8) as shown in Figure 20 A (lane 3), were tested for binding to HeLa cell components. HeLa cell components immobilized on nitrocellulose were incubated in buffer containing the following additions for 1 h prior to adding radioiodinated P.IIa probes (about 200 ng/ml): no addition (lanes 1 and 6); 0.01 % Zwittergent 3-14 (lane 5); 0.01 % Zwittergent 3-14 plus 50-molar excess (10 ug/ml) cold P.IIa (lane 4); 0.002 % Zwittergent 3-14 (lanes 2 and 7); and 0.002 % Zwittergent 3-14 plus 10-molar excess (2 ug/ml) cold P.IIa (lanes 3 and 8).

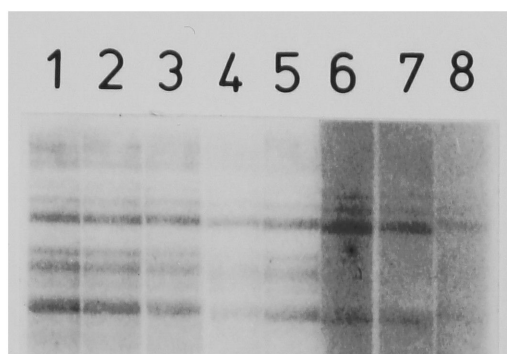


Figure 22: Western blot of HeLa cell protein and glycoprotein, and binding by radioiodinated and cold P.IIa. Lanes 1, radioiodinated P.IIa (20 ng/ml) detected by autoradiography; 2, P.IIa (20 ug/ml) detected with rabbit anti-P.II sera and GARIg conjugated to alkaline phosphatase.

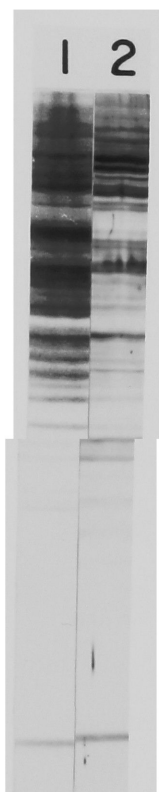
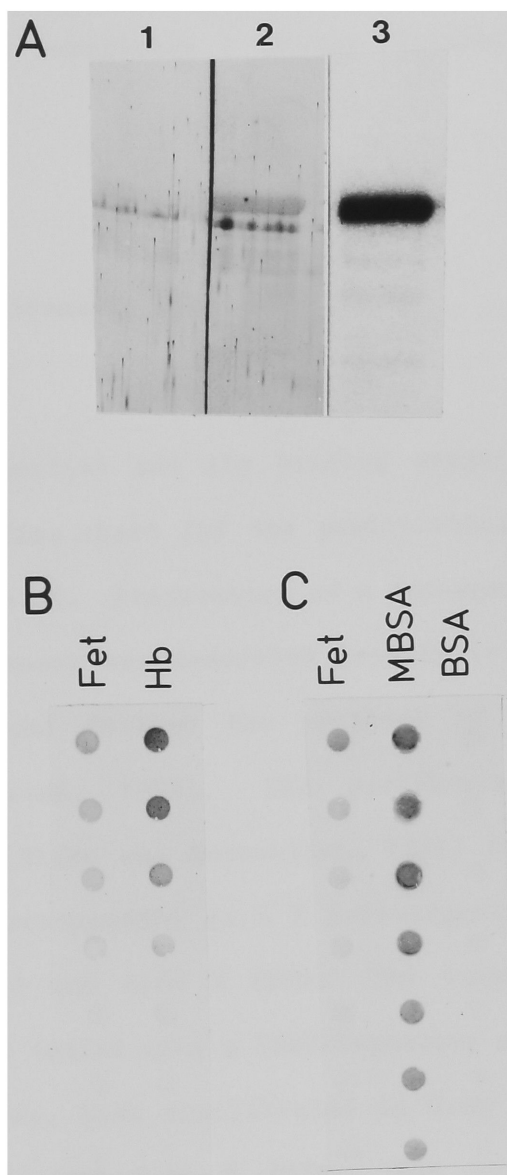




Figure 23: Binding of nonradioiodinated P.IIa to proteins of defined composition. In Panel A, BSA was reduced in beta-mercaptoethanol, subjected to SDS-PAGE (2.5 ug/lane), Western blotting, and incubated with radioiodinated P.IIa (lane 3), cold P.IIa detected with anti-P.II sera plus GARIg conjugated to alkaline phosphatase (lane 2), and anti-P.II sera plus GARIg only (lane 1). In Panels B and C, proteins of defined composition were immobilized on dot blots (20 ug per dot in first row, and diluted 2-fold), and incubated with cold P.IIa detected with anti-P.II sera plus GARIg.



proteins of defined composition correlated well with binding by radioiodinated P.IIa (Figure 23). Cold P.IIa bound to the reduced form of BSA on Western blots (Figure 23A). Nondenatured proteins immobilized on dot blots were bound by cold P.IIop in the following order: MBSA > fetuin > BSA (no binding), and hemoglobin > fetuin (Figures 23B and 23C).

#### 4.2.5 Comparative Study of Functionally Distinct P.IIops

The P.IIc molecule was purified and its binding properties were compared to purified P.IIa. A flow chart for the purification of P.IIa and P.IIc is presented in Figure 24. Preparation of a detergent extract from overnight cultures of gonococci was described briefly in Materials and Methods (Section 4.1.), and follows the protocol of Blake and Gotschlich (Blake and Gotschlich, 1984). The purification scheme reported herein differs from (Blake and Gotschlich, 1984) in that the final ethanol precipitate was resuspended in 5 % Zwittergent 3-14 containing 0.05 M Tris-HCl pH 8.1 and 0.01 M EDTA. The suspension was clarified by centrifugation and loaded onto a DEAE-Sepharose column connected in tandem to CM-Sepharose, both equilibrated in 0.05 % Zwittergent, 0.05 M Tris-HCl pH 8.1, and 0.01 M EDTA. The columns were separated and individually eluted with linear gradients of increasing [NaCl], and the eluate and fall through fractions were analyzed by Coomassie blue staining on SDS-PAGE. Nearly all P.IIa bound strongly to CM-Sepharose, and following elution it could be further purified from residual quantities of the P.I trimer by molecular sieve chromatography on Sephacryl S-200; however, this step was typically accompanied by a

Figure 24: Flow chart of purification scheme for P.IIa and P.IIc.

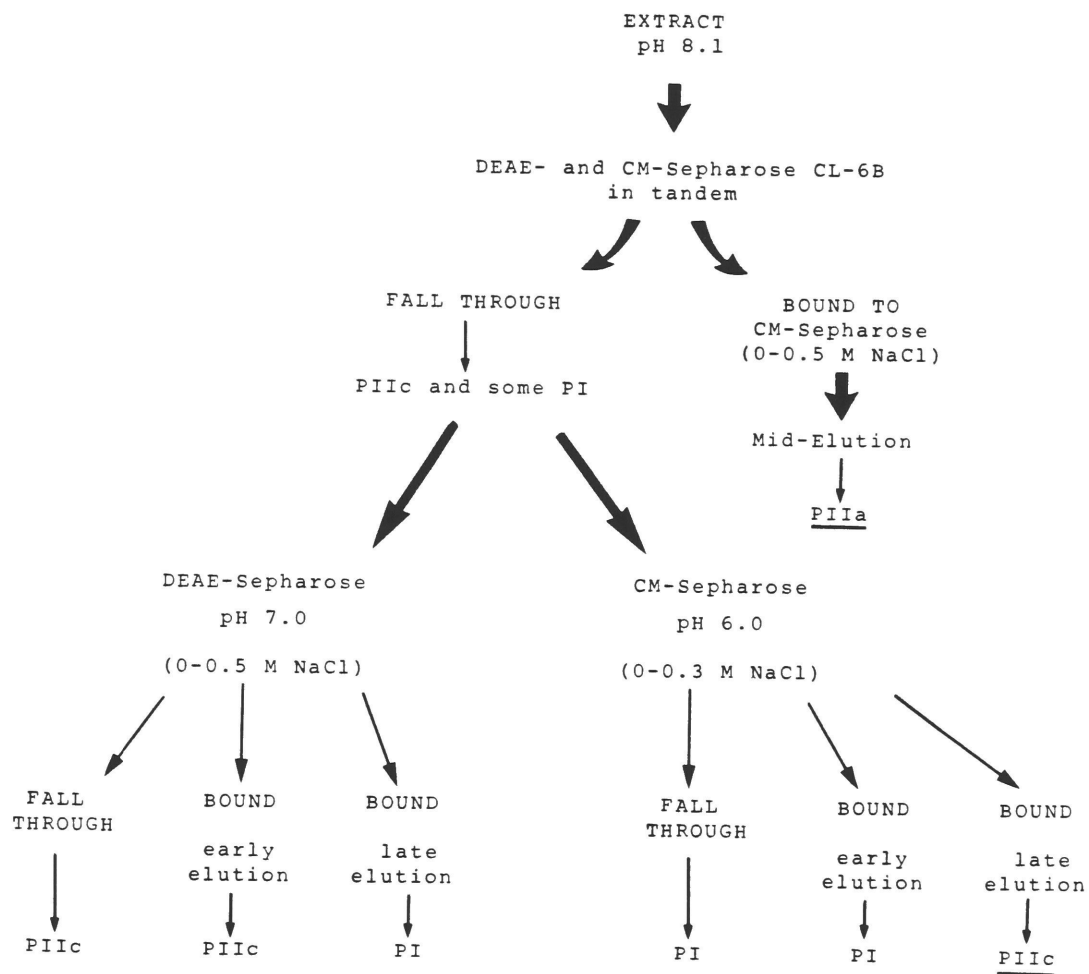
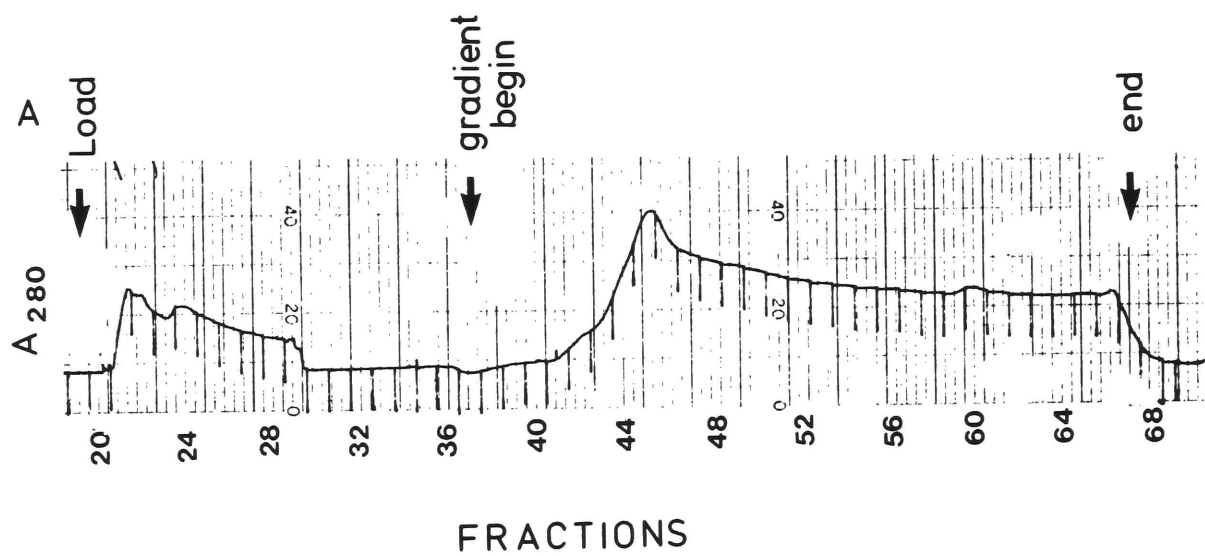


Figure 25: Elution of P.IIc from CM-Sepharose at pH 6.0. Absorbance of column fractions at 280 nm was measured (A); a 0 to 0.3 M linear gradient of NaCl was applied to the column as indicated. An aliquot of the column fractions indicated was precipitated in ethanol at pH 5, solubilized at 37 °C, and subjected to SDS-PAGE followed by staining with Coomassie blue (B). Additional samples are shown: detergent extract loaded onto DEAE-Sepharose connected in tandem to CM-Sepharose, solubilized at 100°C (lane a) and 37°C (lane b); and sample loaded onto CM-Sepharose at pH 6.0 solubilized at 100°C (lane c) and 37°C (lane d). Note P.I migrating at 35 kDa (lanes a and b).

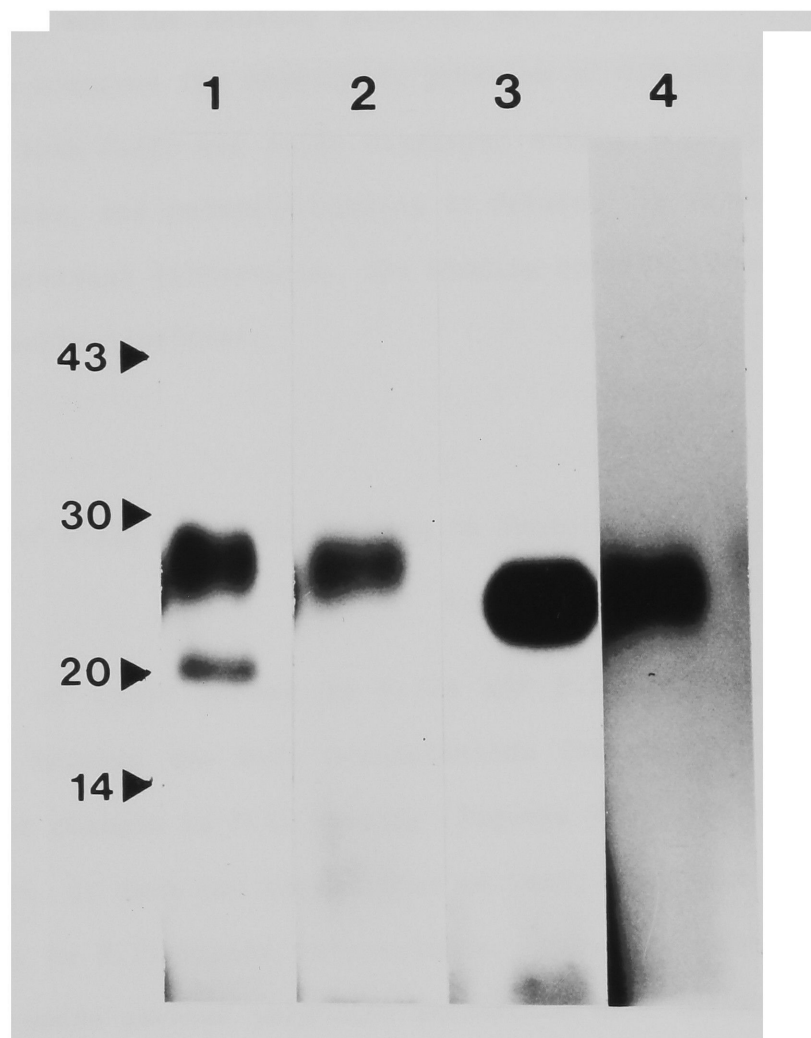


3-fold loss in yield and for this reason, was not employed if sufficient quantities of pure material were obtained by ion exchange chromatography. The majority of P.IIc and some P.I failed to bind either column and appeared in the fall through fractions, which were pooled and dialyzed against 0.05% Zwittergent in the equilibration buffer of the next column. P.IIc and P.I could be separated by one of two methods. P.I bound weakly to DEAE-Sepharose in 0.01 M Tris-HCl pH 7.0, whereas the majority of P.IIc collected in the fall through fractions. On CM-Sepharose equilibrated in 0.01 M 2-[N-morpholino]-ethane sulfonic acid (MES) at pH 6.0, P.I either failed to bind or bound weakly, whereas P.IIc bound more strongly and could be eluted from the column free of P.I with a shallow gradient of NaCl. The optical density at 280 nm and SDS-PAGE of the fractions collected from CM-Sepharose at pH 6.0 is presented in Figure 25. P.IIc was purified substantially-free of P.I and other Coomassie blue staining proteins (fractions 48 to 50), and the P.IIc obtained by this approach was radioiodinated and used in the experiments described below.

Fractions containing purified P.IIc were radioiodinated and subjected to SDS-PAGE and autoradiography (Figure 26, lane 1). Two major bands were observed: one corresponding to the Coomassie blue staining P.IIc protein eluted from CM-Sepharose, and a second band migrating at 21 kDa. The impure P.IIc probe preparation bound strongly to MBSA, and could be eluted in 1.0 % SDS from MBSA immobilized on a dot blot. When subjected to SDS-PAGE and autoradiography, the eluate displayed a single major band corresponding in position to P.IIc (lane 2). Similarly, elution of P.IIa from MBSA confirmed the purity of this probe (lanes 3 and 4). Although the radiolabeled P.IIc probe was contaminated by another



Figure 26: Purity of radioiodinated P.IIa and P.IIc probes. Samples were subjected to SDS-PAGE and autoradiography. Lanes: 1, P.IIc; 2, P.IIc eluted from MBSA in 1.0 % SDS; 3, P.IIa; 4, P.IIa eluted from MBSA in 1.0 % SDS.



molecule, elution of the bound material demonstrates that binding is attributable largely to the P.IIc molecule.

Binding of P.IIc to HeLa cell components was compared to P.IIa binding (Figure 27), and the banding patterns were nearly identical. The P.IIop species were compared for binding to proteins of defined composition (Figure 28). Both P.IIc and P.IIa displayed strong binding to MBSA, hemoglobin and mucin, and moderate binding to fetuin. In spite of some structural and functional differences, the binding specificities of P.IIa and P.IIc were nearly identical.

#### 4.2.6 Chemical Basis of P.IIa and P.IIc Binding to Protein

The contribution of ionic forces in P.IIa and P.IIc binding to ligand was assessed. Raising the NaCl concentration from 0 to 0.5 M caused only very slight changes in P.II binding (Figures 29 [lanes 9 and 10] and 30). Therefore, it does not appear that an ionic component significantly contributes to P.II-ligand interactions. This was supported by additional studies where several polyionic substances were tested for their ability to inhibit P.IIa binding to HeLa cell proteins and glycoproteins. Gonococcal DNA (100 ug/ml), dextran sulfate (200 ug/ml), and polyphosphate (10 ug/ml) each failed to inhibit. The polycations poly-L-lysine and polymyxin B were noninhibitory at concentrations of 200 ug/ml. In addition, divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) and chelating agents (EDTA, EGTA) had no influence on P.IIa binding. Not only did increased concentrations of NaCl fail to diminish P.IIa and P.IIc binding, it failed to enhance binding as well. This salt tends to disrupt

Figure 27: Western blot of HeLa cell protein and glycoprotein, and binding by radioiodinated P.IIa and P.IIc. Approximately equivalent amounts of P.IIa-specific and P.IIc-specific radioactivity was tested for binding.

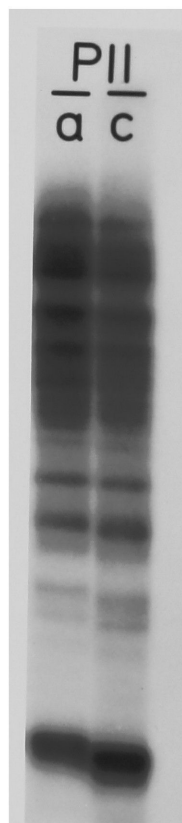


Figure 28: Comparison of P.IIa and P.IIc binding to proteins of defined composition. Proteins were immobilized on dot blots (20 ug per dot), incubated with either P.IIa (solid) or P.IIc (hatched), and the amount of probe bound to each protein was determined by densitometric tracing of the autoradiogram. Relative peak heights are given, and maximal binding of P.IIa and P.IIc set to a value of 10.

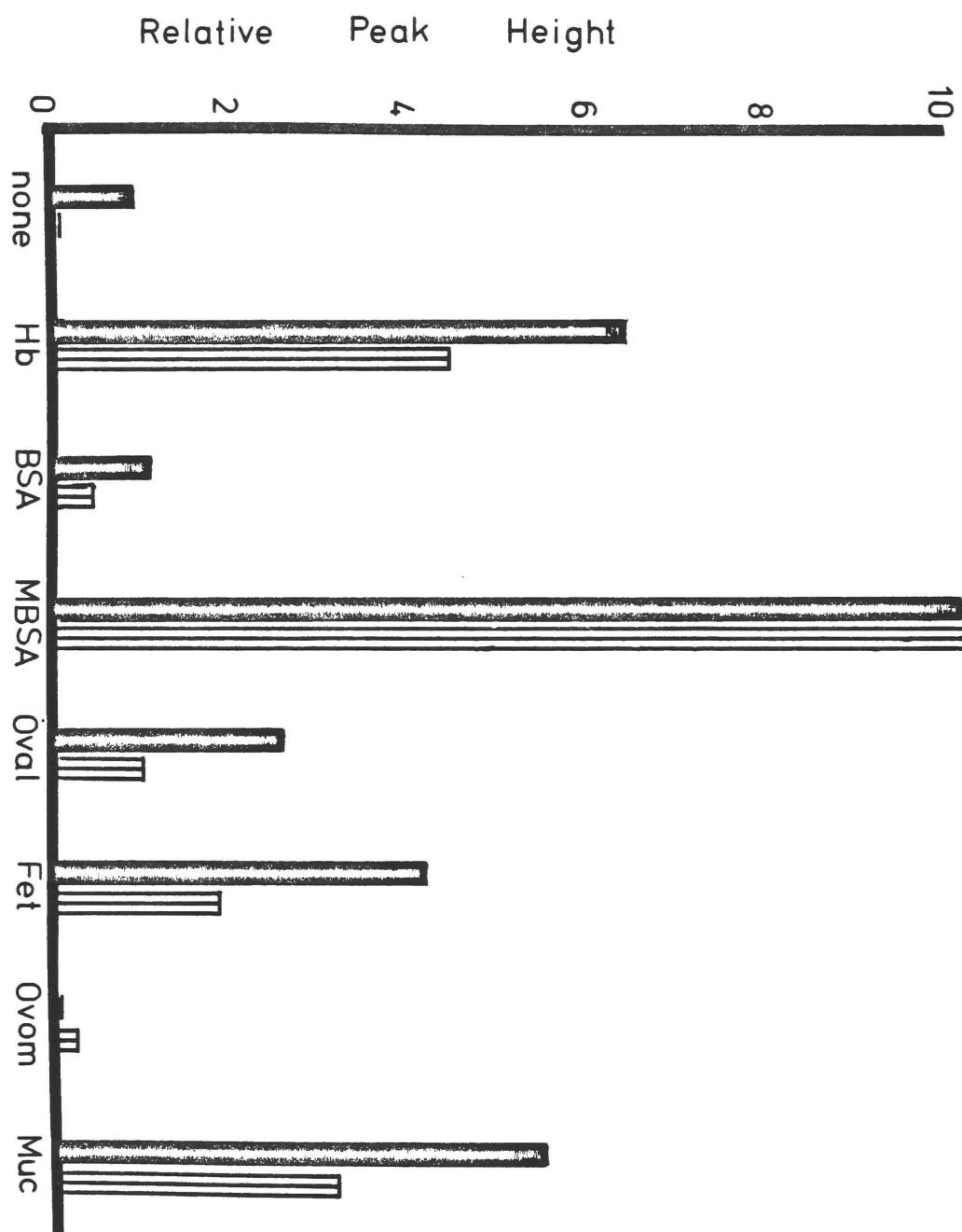


Figure 29: Effect of salts and denaturants on P.IIa binding to HeLa cell protein and glycoprotein on Western blots. Radioiodinated P.IIa bound to HeLa cell components in the 50 mM Tris-HCl pH 8.1 and 0.05% Tween 20 in the presence (odd-numbered lanes) or absence (even-numbered lanes) of 0.5 M NaCl, and in addition: 0.5 M NaSCN (lanes 1 and 2); 0.5 M LiBr (lanes 3 and 4); 8 M urea (lanes 5 and 6); 6 M guanidine HCl (lanes 7 and 8); no addition (lanes 9 and 10); and 0.1 % SDS (lanes 11 and 12).



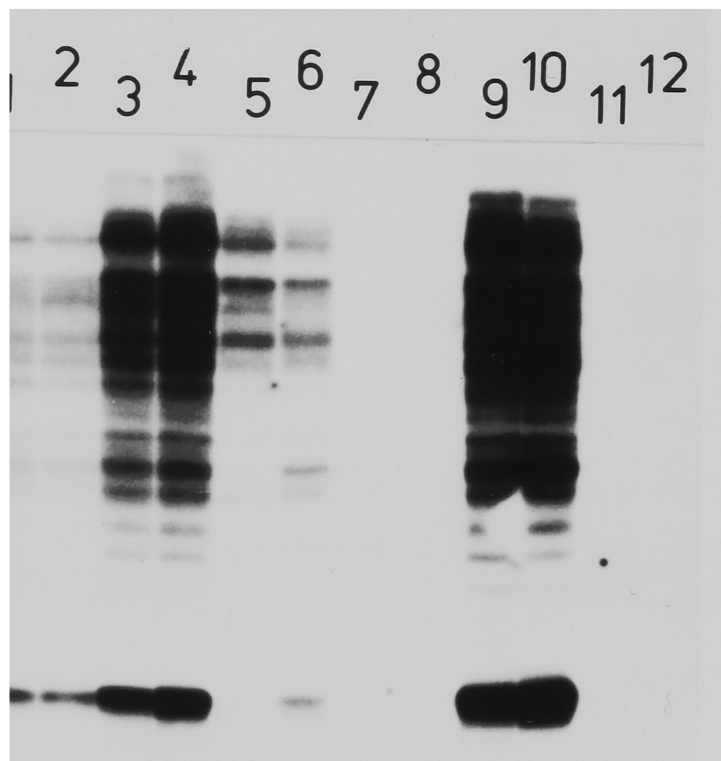
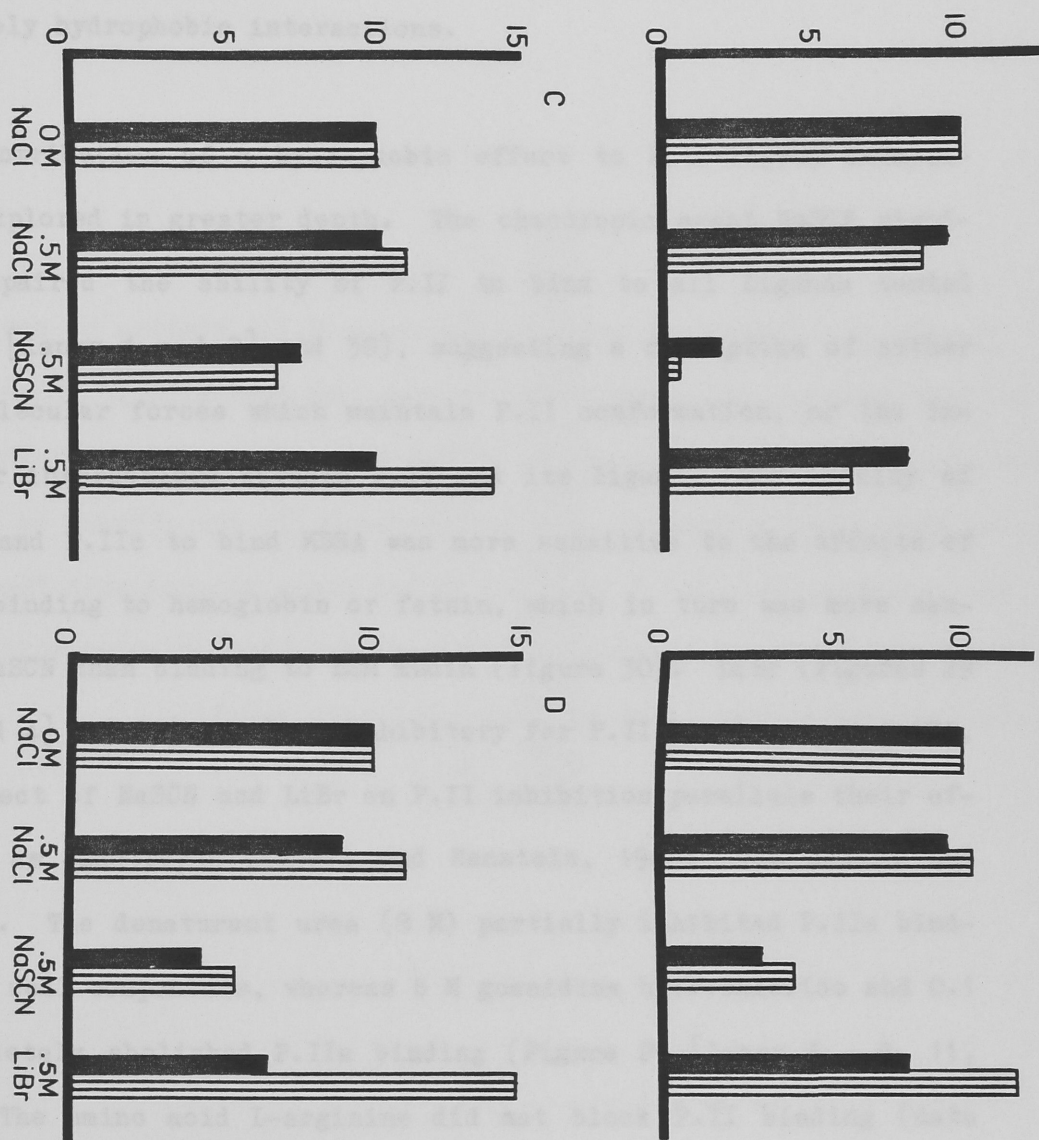


Figure 30: Effects of salts on P.IIa and P.IIc binding to proteins of defined composition. Proteins were immobilized on dot blots (20 ug per dot), incubated with either radioiodinated P.IIa (solid) or P.IIc (hatched), and the amount of probe bound to each protein was determined by densitometric tracing of the autoradiogram. Relative peak heights are given and binding of P.IIa and P.IIc in 0 M NaCl is set to a value of 10. Panel A, MBSA; panel B, hemoglobin; panel C, BSM mucin; panel D, fetuin.

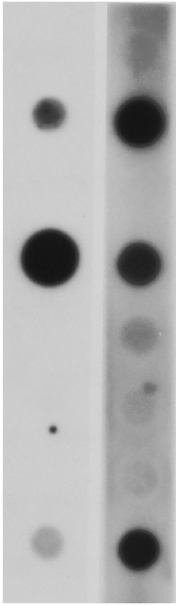
# RELATIVE PEAK HEIGHT



interactions between apolar groups and water, thereby promoting associations among apolar groups ("hydrophobic effect") (Hatefi and Hanstein, 1969; von Hippel and Wong, 1964; Kauzmann, 1959). Thus, it appears that P.II-ligand interactions are not promoted under conditions which augment purely hydrophobic interactions.

The contribution of a hydrophobic effect to P.II-ligand interactions was explored in greater depth. The chaotropic agent NaSCN significantly impaired the ability of P.II to bind to all ligands tested (Figures 29 [lanes 1 and 2] and 30), suggesting a disruption of either the intramolecular forces which maintain P.II conformation, or the intermolecular interactions between P.II and its ligand. The ability of both P.IIa and P.IIc to bind MBSA was more sensitive to the effects of NaSCN than binding to hemoglobin or fetuin, which in turn was more sensitive to NaSCN than binding to BSM mucin (Figure 30). LiBr (Figures 29 [lanes 3 and 4] and 30) was less inhibitory for P.II binding than NaSCN, and the effect of NaSCN and LiBr on P.II inhibition parallels their effectiveness as chaotropes (Hatefi and Hanstein, 1969; von Hippel and Wong, 1964). The denaturant urea (8 M) partially inhibited P.IIa binding to HeLa cell components, whereas 6 M guanidine hydrochloride and 0.1 % SDS completely abolished P.IIa binding (Figure 29 [lanes 5 - 8, 11, and 12]). The amino acid L-arginine did not block P.II binding (data not shown). Hydrophobic interactions influence the ability of P.II to bind protein, however it is unclear as to whether they play a role in maintaining the proper conformation of P.II and/or they affect the intermolecular interactions between P.II and its ligand.

Figure 31: Effect of blocking agents on P.IIa binding to proteins of defined composition. From top to bottom, the following proteins were immobilized (20 ug/dot): none, hemoglobin, BSA, MBSA, ovalbumin, fetuin, BSM mucin, ovomucoid. Radioiodinated P.IIa was allowed to bind in the presence of 0.5 % Tween 20 to dot blots blocked with Tween 20 (left panel), or in the absence of Tween, to dot blots blocked with 1 % gelatin and washed free of excess gelatin (right panel).



The interaction between P.II and protein was stable in the nonionic detergent Tween 20, which served to quench unoccupied sites on the nitrocellulose. Non-detergent blocking agents could be substituted for Tween 20, although detergent was far more effective in preventing P.II background binding to pure nitrocellulose (data not shown). The prominent bands resulting from P.IIa binding to HeLa cell components were visible if the reactions were performed in the absence of detergent, but the less intense bands were obscured by the high background. Binding of P.IIa to proteins of defined composition on dot blots was compared in the presence and absence of Tween 20 (Figure 31). Blots were blocked in 2 % gelatin, washed to remove unbound gelatin, and P.IIa was added in the absence of any additional blocking agent (right lane). Strong binding was observed to hemoglobin, BSM mucin, and MBSA, although binding to MBSA was somewhat diminished compared to quenching and binding in the presence of Tween 20 (left lane). In conclusion, the omission of detergent did not lead to the generation of major new binding sites, did not block P.II binding, nor for the most part, promote P.II binding.

#### 4.2.7 Effect of Ligands on Attachment by P.II-Bearing Gonococci

Attempts were made to correlate purified P.II-ligand interactions with P.II-specific binding by viable gonococci. Gonococci were tested for direct binding to ligands immobilized on nitrocellulose disks (Table 16), or ligands were used as inhibitors of gonococcal adherence to HeLa cell monolayers (Table 17). Both opaque and transparent organisms bound to unquenched nitrocellulose disks, with an average of 24.8 % and 16.1 %

Table 16: Binding of gonococci to ligand immobilized  
on nitrocellulose.

Opaque gonococci (Gc) were of the P.IIaO<sup>++</sup> or P.IIacO<sup>++</sup> type.  
Non-fat dry milk was used as the source of casein.



Table 16: Binding of gonococci to ligand immobilized  
on nitrocellulose.

<u>Ligand</u>	Blocking <u>Agent</u>	% Ad relative to control	
		<u>Opaque</u>	<u>Transparent</u>
none	none	100%	100%
none	gelatin	31	54
none	BSA	26	70
none	casein	14	34
HeLa	none	63	18
O <sup>++</sup> Gc	none	83	19
HeLa	casein	22	29
O <sup>++</sup> Gc	casein	14	44
MBSA	casein	14	25
BSA	casein	11	34

Table 17: Inhibition of gonococcal binding to HeLa cell monolayers.

The opaque variants tested were either P.IIa0<sup>++</sup> or P.IIac0<sup>++</sup>. Viability of the total population is calculated from the sum of the Ad and nonAd fractions.

Table 17: Inhibition of gonococcal binding to HeLa cell monolayers.

<u>Colony Type</u>	<u>Inhibitor</u>	<u>Conc. (mg/ml)</u>	<u>% Relative to Control</u>	
			<u>Ad</u>	<u>Viability</u>
O <sup>++</sup>	None	0	100%	100%
	Mucin	0.8	61	72
	Mucin	1.0	74	115
	Mucin	1.0	20	102
	Mucin, Tryptic	0.8	89	76
	Mucin, Tryptic	1.0	71	140
	Hemoglobin	0.5	108	175
	Hemoglobin	1.0	150	64
	Fetuin	1.0	141	83
	MBSA		aggregates	
O <sup>-</sup>	None	0	100	100
	Mucin	0.8	26	98
	Mucin	1.0	17	42
	Mucin	1.0	50	183
	Mucin, Tryptic	0.8	14	56
	Mucin, Tryptic	1.0	73	57
	Hemoglobin	0.5	134	126
	Hemoglobin	1.0	55	40
	Fetuin	1.0	9	94
	MBSA		aggregates	

of the total population distributing in the Ad phase, respectively. In order to reduce the background binding to the pure nitrocellulose, several blocking agents were tested. The magnitude of inhibition of binding to nitrocellulose by blocking agents was greater for opaque than for transparent organisms, and casein was most effective (Table 16). When HeLa cells or opaque gonococci were immobilized on disks as ligands, binding of opaque organisms was reduced slightly relative to unquenched disks, whereas binding by transparent was reduced about 5-fold. All immobilized ligands (HeLa cells, gonococci, MBSA, BSA) subsequently blocked with casein were bound by opaque and transparent gonococci to the same degree as blank disks blocked with casein. Perhaps the most reasonable interpretation of the data is that HeLa cells and gonococcal ligands saturate nitrocellulose binding sites for both opaque and transparent organisms, but in turn provide new binding sites for opaque gonococci only. It would follow that casein saturates opaque binding sites on both nitrocellulose and immobilized ligand. Since the experiments were inconclusive, they were not extended to measure gonococcal binding to additional ligands, but nevertheless, they are indicative of distinct binding behaviors of opaque and transparent gonococci.

Ligands which were bound strongly by P.IIa and P.IIc were tested as inhibitors for binding of viable gonococci to HeLa cells (Table 17). BSM mucin displayed variable levels of inhibition toward both opaque and transparent gonococci. To ensure that the rheological properties of BSM mucin did not contribute to the inhibition of whole organisms, large glycopeptides of mucin were generated by tryptic digestion (Pigman et al., 1973; Bettelheim, 1963); however, the pattern of inhibition was

unchanged. Attempts to measure the inhibitory capacity of MBSA for gonococcal adherence was confounded by the massive agglutination of organisms induced by MBSA. MBSA has high affinity for polyanions and binds to DNA and acidic polysaccharides, generating insoluble precipitates; it is likely that the polyphosphate present on the gonococcal surface adsorbed MBSA to cause agglutination. Neither hemoglobin nor fetuin displayed opaque-specific inhibition, and it is possible that binding was enhanced instead. The data is complicated by differential viability of gonococci in each of the ligands tested. In summary, there is no evidence to date that the binding specificity of purified P.IIs reflect the P.II-mediated binding of intact gonococci.

#### 4.3 DISCUSSION

Opacity-associated P.IIs appear to act as mediators of adhesion of the gonococcus to epithelial cells, as well as to adjacent gonococci (Swanson, 1983; James et al., 1980; Lambden et al., 1979). The epithelial cell binding site for P.IIop is not chemically defined. The finding that purified P.IIop binds to the oligosaccharide portion of acid-cleaved gonococcal LPS (Blake, 1985) (M. Blake, personal communication), and the speculation that the P.IIop recognition site on the gonococcus mimics the epithelial site in structure, prompted focus on P.IIop interactions with HeLa cell and other eucaryotic-derived glycoproteins.

An assay was established which demonstrated carbohydrate-specific binding by carbohydrate-binding proteins (lectins) to Western blots of HeLa cell protein and glycoprotein. Binding by P.IIa and P.IIc displayed several parallels to binding by the lectins: (i) many distinct molecular species were bound by P.II, suggesting that P.II recognized a feature of HeLa cell macromolecules which occurred at a frequency similar to that of glycosylation; (ii) the concentrations of glycoprotein (fetuin, mucin) necessary for inhibition was similar for P.II and the lectins; and (iii) effective inhibition of P.II and lectin binding led to diminution in the intensity of all bands in a stoichiometric manner, suggesting a single, homogeneous class of binding site(s) on P.II and the lectins. In contrast to the lectins, P.II binding to HeLa cell protein and glycoprotein was not blocked by the monosaccharide form of sugars commonly found on the epithelial cell surface. The inhibitory capacity of HeLa cell extracts and BSM mucin was destroyed by protease treatment. Direct binding of P.II to defined proteins revealed that P.II displayed strong binding reactivity toward glycoprotein as well as to molecules lacking carbohydrate. This study leads to the conclusion that a major binding site for purified P.II is some protein conformation.

The P.II-protein interaction does not appear to be purely electrostatic in nature, based on the observation that binding was virtually unaffected as the NaCl concentration was increased from 0 to 0.5 M. A purely hydrophobic effect would be strengthened by increasing concentrations of NaCl; since binding was not augmented as a result of high [NaCl], it would appear that the physicochemical nature of P.II-protein interactions is more complex than the involvement of purely ionic or

purely hydrophobic forces. The inhibition of P.II binding by the chaotrope NaSCN could be the consequence of denaturation of the native conformation of P.II or disruption of intermolecular interactions between apolar groups of P.II and its protein ligand. The possibility that the guanido groups of guanidine contributed to P.II binding inhibition in a manner independent of its denaturing effects was explored with the larger molecule arginine, and the data indicate that P.II binding does not involve stereospecific recognition of guanido groups. Nonionic detergent (0.5% Tween 20) was present during binding and would be expected to saturate many hydrophobic sites with its hydrocarbon chain. Since P.II binding was stable in the absence of Tween 20, it is improbable that P.II binding occurred through a bridging effect (eg., mixed micelle). No new binding sites were generated in the absence of detergent and therefore potentially important interactions, such as protein-lipid interactions did not appear to be masked by the detergent. In summary, P.II binding to protein does not appear to be purely ionic or purely hydrophobic in nature. The contribution of hydrophobic forces to P.II-ligand interactions is difficult to ascertain because the chaotropic agents which could influence intermolecular associations might also disrupt native protein structure.

The binding specificities of covalently modified P.II (i.e., derivatized to different degrees by iodination), and of unmodified P.II detected by antibody were compared. Light and heavily iodinated P.II displayed the same binding specificities to HeLa cell components, although the overall binding affinity of the heavily derivatized probe was enhanced. Furthermore, binding by radioiodinated (hot) P.II could be blocked by an excess of unlabeled (cold) P.II. However, differences

were noted between direct binding of hot and cold P.II probes to HeLa cell components. Most bands detected by cold P.II were also bound by hot P.II, although the relative intensity of binding to an individual ligand did not always correlate. Additional sites were detected by radioiodinated P.II which were absent with the cold P.II probe. The differences observed between hot and cold P.II binding to total HeLa cell components is not understood, however the large difference in sensitivity for detecting hot and cold P.II probes may have contributed. Since radioiodinated P.II was used in concentrations way below saturation, the intensity of binding would likely be affected by both the quantity of a given ligand and its affinity for P.II. On the other hand, cold P.II was used in significantly higher concentrations. Therefore, the differences between low and high affinity sites would tend to be diminished, and the amount of P.II bound would be more strongly influenced by the amount of ligand present. For example, a ligand with high affinity for P.II but present in small amounts may be below the detection limit of cold P.II. When proteins of defined composition were tested as ligands in equivalent quantities on dot blots, the patterns of relative binding affinity for specific ligands by hot and cold P.II were identical.

The recognition site of P.II is widely distributed among proteins. Based on the frequency of the P.II binding site in total HeLa cell lysates, it was initially thought that P.II recognized oligosaccharide. A clue to understanding the specificity of P.II-protein binding may lie in the frequency of occurrence of particular molecular forms. Arginine residues function as ligands for the hemagglutinins of B. gingivalis and Fusobacterium nucleatum (Inoshita et al., 1986), and while arginine



satisfies the criterion of abundancy, it failed to block P.II binding. Highly reactive cysteine residues are present in serum albumin and hemoglobin, two proteins which avidly bound P.II; however, it appears that disulfide interchange between P.II and albumin did not occur, since the interaction was not inhibited by high concentrations of the reducing agent DTT. Sites on eucaryotic proteins targeted for glycosyltransferase-mediated O- and N-linked glycosylation require a short peptide sequence having a particular secondary structure (beta turn) and specific amino acid side chains (Rose et al., 1985), and these sites occur at a high frequency as illustrated with the lectin binding patterns. The tetrapeptide Arg-Gly-Asp-Ser has been found on several proteins and appears to play a role in cellular recognition. It is present in fibronectin in a beta-turn, and the peptide is recognized both by a receptor on mammalian cell surfaces and by an attachment factor on Trypanosoma cruzi; it is postulated that the fibronectin dimer serves to bridge T. cruzi to the cell surface (Ouaissi et al., 1986). In addition, the tetrapeptide sequence has been identified in several other proteins including an E. coli outer membrane protein and a viral coat protein. Purified P.II failed to bind directly to human fibronectin on Western blots (data not shown), and thus it is unlikely that P.II recognizes this particular sequence. However, it is possible that P.II recognizes a short peptide sequence, perhaps in a beta-turn, that is present in many different proteins.

The interaction of P.II with one of its ligands (serum albumin) was explored in greater depth. Human and bovine serum albumin contain 17 disulfide bridges per mole, and migration on SDS-PAGE is influenced by the integrity of these bonds; migration of reduced BSA is retarded due

to unfolding of the molecule (Foster, 1977). By aligning adjacent Cys-Cys sequences, a model has been constructed whereby disulfide bridges serve to connect a series of nine double loops, each of which are high in alpha-helical content (Brown, 1977; Peters, 1985). The disulfide bonds do not link distant parts of the peptide chain and therefore do not interfere with the flexibility of the molecule; albumin can display substantial heterogeneity in conformational form (Foster, 1977). P.II bound to fully denatured albumin, but not to the native form. However, P.II bound to the unreduced, fast-migrating configuration if carboxyl groups were methylated. Therefore, binding of P.II to albumin did not require complete unfolding of the molecule. The basic P.IIa molecule interacted strongly with the basic MBSA, overcoming any charge repulsion. One possible mechanism for P.II binding is that it recognizes a specific steric conformation which is widely distributed among many different proteins. It follows that the putative conformation is inaccessible or nonexistent in native albumin, but becomes available in the methylated form, which nevertheless maintains a compact configuration.

Studies by others failed to show P.IIop-specific binding of gonococci to glycolipids immobilized on thin layer chromatograms (Deal et al., 1986). Attempts were made to measure direct binding of gonococci to ligands immobilized on nitrocellulose discs, however these studies were complicated by high background binding of organisms to the unquenched membrane. Attachment of gonococci to HeLa cell monolayers was not blocked by the protein ligands bound strongly by purified P.II. There are a couple of explanations for why competitive inhibition of binding of intact organisms might have failed. Each gonococcus can

express up to  $10^5$  molecules of P.II at a given time and in addition, the shedding of outer membrane blebs leads to high rates of outer membrane protein renewal. P.II on the gonococcal surface presents itself in a multivalent form, and inhibition of a single organism may require saturation of nearly all P.II sites. Secondly, the protein ligands tested as inhibitors are complex molecules which might serve to bridge the gonococcus to the HeLa cell rather than block binding. For example, fetuin has cell-sticking and growth-promoting activity towards HeLa cells, and the latter function depends on sialic acid residues (Graham, 1966). It may be necessary to generate haptenic ligands from the complex protein in order to increase the molarity of the inhibitory piece and/or to prevent possible bridging.

The conformation of P.II appears to influence its binding affinity for protein. P.IIs have the unusual property of requiring high temperatures in addition to SDS in order to completely denature. Presumably the slow-migrating form of P.II on SDS-PAGE is in the open configuration and represents the true molecular weight, whereas the unmodified form migrates faster because it is compact and perhaps binds abnormal quantities of SDS. Gonococcal P.IIs share the property of heat modifiability with outer membrane protein A (OmpA) of E. coli (Schnaitman, 1974). Circular dichroism spectra of OmpA show it to be extremely rich in beta-structure, remaining stable in SDS at room temperature and undergoing a conformational change to alpha-helix upon addition of heat (Nakamura and Mizushima, 1976). Shifting to the high temperature form of OmpA can be partially prevented by addition of 0.3 M NaCl (McMichael and Ou, 1977); perhaps this is indicative of the role of hydrophobic forces in protein folding. Addition of LPS or lipid A to boiled OmpA

causes a renaturation to the fast-migrating form, and it had been suggested that LPS is bound tightly to OmpA and boiling breaks this association thereby causing a shift in electrophoretic mobility (Schweizer et al., 1978). However, attempts to isolate unheated OmpA in complex with LPS on SDS-PAGE have failed, and it is currently believed that heat modifiability of OmpA is best explained by gross conformational changes (Freudl et al., 1986). The basis for heat modifiability of P.II is not known, and there is no evidence that purified P.II is complexed to LPS or to any other molecule. Iodination of P.II rendered it increasingly resistant to denaturation in the presence of SDS and high temperature. The phenolic ring of tyrosine, the main target of iodination by the chloroglycoluril method, is somewhat polar but also has a large negative free energy of transfer from water to organic solvents, indicative of hydrophobicity (Schulz and Schirmer, 1979). It seems likely that iodination would increase the overall hydrophobicity of the aromatic ring, however, it is difficult to predict how the bulky size of iodine would contribute to interactions with other residues. Whether the effects of P.II derivatization with  $I^-$ , leading to increased binding affinity and resistance to thermal denaturation are causally related is not known for certain.

It has been suggested that P.IIops are very basic proteins with pIs above 9.5 whereas P.IInops have isoelectric points of 7.5 to 8.0 (Blake, 1985). However the P.IIop, P.IIc, appears to be an exception to this finding. The binding behavior of native P.IIc and P.I to ion exchange resins under conditions of low salt strongly suggests that P.IIc is slightly more basic than P.I, and that the overall net charge of P.IIc is near 0 at a pH of 7.5 to 8.0 or less. The native form of P.IIa is

significantly more basic than P.IIc, and therefore these P.IIops are chemically distinct in this regard. The binding specificities of purified P.IIa and P.IIc were indistinguishable, and the data suggests that the P.II binding observed in this study involves a conserved portion of the molecule. The relative binding affinities of P.IIa and P.IIc appeared to be of roughly the same magnitude. The relation of the physicochemical properties of P.IIa and P.IIc to the functional attributes they provide the gonococcus is the focus of discussion in Chapter 5.

In summary, the data indicates that a bacterial protein which has been implicated as an adhesin, interacts with select proteins and appears not to interact with eucaryotic carbohydrate. This interaction is not dominated by electrostatic forces and evidence is lacking for a purely hydrophobic effect. The fact that P.II binds to many distinct proteins leads to the possibility that it recognizes an epitope which is widely distributed among proteins. Whether P.II binding to protein involves recognition of a particular stereochemical form or whether P.II provides less specific adhesive properties has not been resolved.

#### 4.4 ADDENDUM

##### 4.4.1 Structural Analysis of Two P.IIs from Strain MS11

The complete DNA sequence of 2 P.II genes from strain MS11 has been recently published (Stern et al., 1986), and based on this report structural analyses were performed. Chou-Fasman analysis of the P.II VO sequence revealed a minimum of 36 % of the amino acid residues in a beta-sheet conformation. This amount of beta-sheet is well above the average for globular proteins but is not an unusual quantity (Chou and Fasman, 1974). The P.II proteins derived from the VO and V28 genes are 236 and 234 amino acid residues in length, respectively; the 2 major hypervariable regions are located between residues 86 to 100 (HV1) and 154 to 183 (HV2) (Stern et al., 1986). Hydropathy plots showed the HV1 region of both molecules and the HV2 region of V28 to be hydrophilic. However, a significant portion of the HV2 region of VO contains hydrophobic beta-sheet structure. Whether the HV1 and HV2 regions defined by amino acid sequence correspond to the immunodominant, hypervariable regions identified by antibodies is not known for certain. Nor is it known whether the hydrophilic, surface-exposed peptides unique to individual P.II molecules correspond to the HV1 and HV2 regions.

The P.II VO gene product displayed significant amino acid sequence homology to the OmpA protein of E. coli, with 21 % identity in a 134 amino acid overlap corresponding to the C-terminal half of the P.II molecule. The 7 C-terminal residues of P.II showed complete identity (except for a Arg-Ser substitution) with a portion of OmpA believed to

be buried in the outer membrane (Morona et al., 1984). A 12 residue stretch of beta-sheet structure in P.II VO (194-205) also displayed strong homology to OmpA. One can only speculate as to whether the homologous regions of P.II and OmpA contribute to the shared property of heat modifiability. Mild digestion of the C-terminal end of the P.IIa probe with carboxypeptidase Y had no effect on its ability to bind ligand (data not shown).

## CHAPTER 5

### STRUCTURE-FUNCTION CORRELATES OF GONOCOCCAL PROTEIN IIS



The differential binding of P.II-bearing organisms to eucaryotic cells in vitro and the preponderance of opaque gonococci at certain anatomical sites, has led several investigators to suggest that P.II-mediated attachment is a determinant of tissue tropism. Environmental pressures (eg., antibodies, proteases) probably contribute to the ability of gonococci which express or do not express P.IIs to persist at particular sites. In this chapter, data is presented which demonstrates an environmental influence on the growth of gonococci expressing P.IIa, P.IIc, both, or neither. In the General Discussion of Chapters 3, 4, and 5, P.II content is related to opacity and other functional properties of the gonococcus.

## 5.1 RESULTS

### 5.1.1 Environmental Influences on P.IIa and P.IIc Expression

An observation was made that when gonococci were suspended in DME lacking zeta serum and plated onto GC agar, there was a substantial loss in the expected number of opaque CFUs. In the past, GC agar medium has been problematic for growth of opaque gonococci and the amount of trypticase (a pancreatic digest of casein) was cut back to facilitate growth (Swanson, 1978a); the trypticase content was reduced for these studies as well. Zeta serum could be replaced with BSA and Table 18 demonstrates that the CFU yields of gonococci expressing P.IIa gave significantly lower CFU yields in the absence of BSA, whereas O<sup>-</sup> and P.IIcO<sup>+</sup> organisms were unaffected by BSA. The effect on P.IIa-bearing organisms appeared to be independent of the degree of opacity and

Table 18: Survival of gonococci on GC agar.

Gonococci were suspended in DME containing 10 % zeta serum or 0.1 % BSA, or in DME only and plated onto GC agar. The increase in CFUs is determined by dividing the yield in DME with BSA or serum additive by the yield in DME only.

Table 18: Survival of gonococci on GC agar.

<u>Colony Type</u>	<u>Increase in CFUs Due to Serum</u>	<u>Increase in CFUs Due to BSA</u>
$P^-O^-$	0.998	1.1
$P^+O^-$	1.3	ND
$P^-P.IIaO^{++}$	9.8	ND
$P^-P.IIaO^+$	31.0	35.0
$P^-P.IIacO^{++}$	ND	22.2
$P^-P.IIcO^+$	ND	1.3

furthermore, P.IIa was the dominant phenotype in gonococci coexpressing P.IIa and P.IIc. These findings led to two hypotheses. The first suggested that BSA induced clumps of P.IIa-bearing gonococci to disaggregate, leading to increased (and accurate) CFU yields. The second postulated that a toxic factor, present in the GC agar or produced by the gonococcus, selectively killed P.IIa-bearing organisms and BSA protected them against death. The disaggregation hypothesis appeared to be unlikely based on the following (data not shown): (i) P.IIa-bearing gonococci vigorously vortexed in the absence of BSA displayed CFU yields similar to that of unvortexed organisms; (ii) mild trypsinization failed to increase the yield of P.IIa-bearing organisms; and (iii) gonococci which had been passed through 1.0  $\mu$ m filters retained complete responsiveness to subsequent addition of BSA. The inability to dissociate clumps that may or may not have been present provided evidence in favor of the alternative hypothesis, that an environmental factor induces the selective death of P.IIa-bearing gonococci.

Two main candidates for P.II-specific toxic factors are proteolytic enzymes and fatty acids. Opaque gonococci display increased sensitivity to trypsin (Swanson, 1978a; Swanson, 1978b), and there has been suggestion that some opaque gonococci are more sensitive than others to growth retardation by oleic acid (James and Swanson, 1978a). CFU measurements on GC agar of gonococci suspended in a 10-fold concentrated solution of trypticase in place of DME led to an additional reduction in yield of P.IIaC<sup>0++</sup> organisms with no effect on O<sup>-</sup> or P.IIcC<sup>0+</sup> gonococci (data not shown). The trypticase present in GC agar had residual proteolytic activity as determined by digestion of casein in agarose, and attempts to block this activity with a variety of protease inhibitors was

Table 19: Survival of gonococci in detergents and fatty acids.

The additives caprylic acid ( $C_8$ ), sodium deoxycholate (DOC), and NP-40 were included in GC agar at the concentrations indicated. Gonococci were suspended in DME and plated. The decrease in CFUs was determined by dividing the yield on GC agar by the yield on GC agar containing additive.

Table 19: Survival of gonococci in detergents and fatty acids.

<u>Additive</u>	<u>Decrease in CFUs Due to Additive</u>		
	<u>P.IIacO<sup>++</sup></u>	<u>P.IIcO<sup>+</sup></u>	<u>O<sup>-</sup></u>
None	1.00	1.00	1.00
0.01% C <sub>8</sub>	1.19	0.93	0.95
0.05% C <sub>8</sub>	>100.0	>100.0	>100.0
0.005% DDC	0.97	1.00	0.96
0.05% DDC	>100.0	>100.0	>100.0
0.005% NP-40	1.22	1.06	1.05
0.01% NP-40	4.77	1.15	1.11
0.02% NP-40	10.4	1.39	1.10
0.04% NP-40	14.8	1.74	1.07

unsuccessful. Inclusion of fatty acids and detergents in GC agar, and their effects on gonococcal growth is presented in Table 19. Neither the fatty acid caprylic acid nor the bile salt sodium deoxycholate had a P.II-specific effect on gonococcal growth at the concentrations tested. The nonionic detergent NP-40 inhibited growth of P.IIacO<sup>++</sup> organisms in a dose-dependent fashion, and P.IIcO<sup>+</sup> gonococci were retarded only at higher concentrations of NP-40. Growth retardation of gonococci by 0.005 % NP-40 correlated with both the presence of P.IIa and the degree of opacity. Organisms expressing abundant quantities of P.IIc, although diminished in opacity, were more resistant to the effects of both trypticase and nonionic detergent.

In summary, conditions can be established whereby gonococci expressing a particular P.II are at a survival disadvantage. Proteolytic enzymes or lipophilic agents may be responsible for growth retardation. The converse situation was also observed, whereby a contaminant present in certain preparations of dextran selectively killed O<sup>-</sup> organisms, and P.IIacO<sup>++</sup> were more resistant than P.IIcO<sup>+</sup> (data not shown). Although the identity of the toxic agents and their mechanisms of action are not defined, one can conclude that the environmental milieu appears to be a critical determinant of P.II expression.

## 5.2 GENERAL DISCUSSION OF CHAPTERS 3, 4, AND 5

The degree of opacity was determined by both the amount and the type of P.II. In addition to opacity, gonococci bearing different types and quantities of P.II molecules were distinct from one another in terms of adherence to HeLa cells, and in their growth response to environmental factors. The structural and functional properties attributable to P.IIa and P.IIc are summarized in Table 20.

Stern et al. (1986) report that P.II genes are constitutively transcribed. The colony types P.IIaO<sup>+</sup> and P.IIaO<sup>++</sup> differed in the quantity of P.IIa expressed. There is no obvious distinction between these 2 "P.IIa" molecules based on molecular weight and conversion temperature, however, complete identity can only be established by DNA sequence analysis. If in fact the P.IIa molecules are identical, then the differential expression of P.IIa by variants P.IIaO<sup>+</sup> and P.IIaO<sup>++</sup> suggests that either (i) some gonococci have two copies of the same P.II gene, and translation of each copy to the mature protein is independently controlled; or (ii) expression of the mature P.II product is regulated at another level, in addition to translational control through CTCTT elements. The size of the leader peptide and the efficiency of transport across the cytoplasmic membrane is one such possibility.

Gonococci producing equivalent amounts of P.IIa or P.IIc differed in several respects. The degree of colonial opacity tended to increase with increasing quantities of P.IIa, with maximal P.IIa synthesis yielding the O<sup>++</sup> phenotype. In contrast, P.IIc never attained the same extent of opacity as did P.IIa, and yielded O<sup>+</sup> colonies at best; thus,



Table 20: Structure-function correlates of gonococcal P.IIs.

Abbreviations used: inter (intermediate), mod (moderate), ND (not determined), NR (not relevant).

TABLE 20: Structure - function correlates of gonococcal PIIs

	<u>PIIa/PIIc</u>	<u>PIIa</u>		<u>PIIc</u>	<u>None</u>
Quantity Expressed	high/high	high	mod	high	NR
Intensity of Opacity	+++	++	+	+	0
Nomenclature	P.IIaO <sup>++</sup>	P.IIaO <sup>++</sup>	P.IIaO <sup>+</sup>	P.IIcO <sup>+</sup>	O <sup>-</sup>
Net Charge of P.II	basic/neutral	basic	basic	neutral	NR
Net Adherence	high	high	mod (high)	mod	low
Detachment	low	ND	ND	mod	high
Replication	highest	ND	ND	inter	lowest
Survival on GC Agar					
w/o BSA	0	0	0	+++	+++
w/ BSA	+++	+++	+++	+++	+++
Sensitivity to NP-40	high	ND	ND	mod-low	low

the overall affinities of P.IIa and P.IIc for adjacent gonococci were quite different. Since hundreds of distinct P.IIop molecules exist in nature, each of which is capable of imparting opacity to the gonococcus, it is likely that a highly conserved receptor-ligand interaction is required for generating a minimal degree of opacity. It seems reasonable that different intensities of opacity which arise from equal quantities of a given P.II is the result of a combined effect of a primary site recognized by all P.IIops and a secondary site which further stabilizes select P.IIs. The observed difference between the affinities of P.IIa and P.IIc for adjacent gonococci might be the result of secondary, binding sites involving the positively-charged P.IIa molecule.

Whether the portion of P.IIop molecules engaged in generating a minimal measure of opacity can be separated from other functionally important sites on P.II is not known. For example it is unclear whether the portion of the P.II molecule which recognizes the purported primary site for opacity is identical to the site which functions as an adhesin in attaching to HeLa cells. For the most part, overall gonococcal adherence paralleled opacity; thus, it is not entirely clear as to whether gonococcal adherence is directly related to P.II content, or directly related to opacity. On the other hand, diminished survival of gonococci on GC agar correlated more directly with P.IIa and was less dependent on opacity. Protection provided by BSA and serum resulted in the largest survival increase by P.IIa0<sup>+</sup> organisms expressing only moderate levels of P.IIa. Growth of gonococci expressing abundant quantities of P.IIa (P.IIa0<sup>++</sup>) was retarded to a lesser degree in the absence of serum. There is suggestive evidence that residual protease activity in the GC agar contributed to the death of P.IIa-bearing organisms, and

perhaps P.IIa is susceptible to a particular proteolytic activity whereas P.IIc is not. Although the actual mechanism for growth retardation of certain gonococcal variants was not extensively investigated here, nevertheless, it can be concluded that environmental factors distinguish between gonococci bearing distinct P.IIop molecules in a manner independent of opacity.

In conclusion, the tissue tropism determinant(s) of P.II may function in two independent ways: one influenced by the environment at particular anatomical sites and the other providing adhesion to specific cell types.

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