Pathogenesis of a Neisserial infection: adhesion, invasion, and survival within epithelial cells

by

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Abstract

Neisseria gonorrhoeae (GC) and *Neisseria menigitidis* (MC) are genetically closely related. They are both human pathogens. GC causes gonorrhea and MC causes meningitis. They have similar outermembrane components (except capsules in MC) and use similar mechanisms to colonize host epithelial cells. Porin, pili, Opa, PilC, and LOS are major outermembrane components and are involved in interactions with human epithelial cells, including adhesion, invasion, and evading host immune systems.

Piliated bacteria bind to cells two to three logs better than nonpiliated bacteria. However, P^- bacteria do bind to cells under certain circumstances which suggests that there are nonpilus associated adhesin contributing to the adhesion.

We have identified a DNA sequence from GC which when expressed in *E.coli* causes it to bind to Hec-1-B cells 16-20 fold and A431 cells 60 fold better than its vector control. This new gene is designated *nad1* and encodes a potential membrane protein. P^- GC *Nad1* mutants decrease invasion 70% compared to their P^- parental strain. Our data suggest that *nad1* gene product may be involved in interaction of GC with epithelial cells.

After initial colonization, GC and MC take about 18 to 36 hours to cross the epithelial monolayer. During the time span while they reside in the cell, the bacteria must survive intracellularly. We discovered that the Neisserial IgA protease can increase the turnover rate of LAMP1 probably by cleaving LAMP1 at its IgA like hinge region. LAMP1 is a major component of the lysosomal membrane and presumably serves to protect the integrity of the lysosome membrane from the lysosomal enzymes. GC *iga* mutants can not decrease LAMP1 contents and fail to replicate intracellularly. Our data suggest that besides cleaving human IgA, Neisserial IgA protease plays another important role in promoting the bacterial survival inside the epithelial cells.

Literature Review

Diseases caused by MC and GC

Neisseria gonorrhoeae (GC) and *Neisseria meningitidis* (MC) are the two pathogenic members of the Neisseriae family of gram-negative bacteria. Humans are the only host for these two pathogens. Although GC and MC have similar DNA sequences, they colonize different mucosal surfaces and cause very different diseases.

MC is the second leading cause of bacterial meningitis in the United States and is the leading cause in many countries (Peltola, 1983). MC colonizes the nasopharynx and spreads from person to person probably via respiratory droplets. Most of time, MC infection of normal humans are asymptomatic (Wenger and Broome, 1991). However the reasons for the carrier stage are not clear. In rare cases, the localized infection may disseminate to cause fatal sepsis, disseminated intravascular coagulation and meningitis.

GC is transmitted primary via sexual contact. GC usually colonizes the epithelium of the genitourinary tract and, less frequently, that of the rectum, oropharynx, and conjunctiva. In the male host, urethal infection leads to a symptomatic exudative inflammatory response (Hook et al., 1985). In contrast, the infection of endocervix may not be as obvious. Although GC are non-motile, in rare cases, they migrate to the fallopian tubes and cause salpingitis which usually leads to woman infertility due to tubal blockage. Less commonly, GC infection causes disseminated gonococcal infection (DGI) which affects the skin, synovium, and joints. Together with *Chlamydia trachomatis* , GC is one of the most common causes of infertility in women worldwide.

Although MC and GC are highly related at the genetic level, the reasons for the striking difference in the pathogenesis of the two microorganisms remain unclear. Until now the MC capsule is the only major bacterial component known to be present in MC but not in GC. Isolates obtained from patients with MC disease are usually capsulated, suggesting the importance of the capsule to bacteria virulence (Jarvis and Vedros, 1987, Devoe and Gilchrist, 1975).

Neisserial infection is a multifactorial process

Neisserial infection involves a series of interactions between the bacteria and target cells (Fig.1A). First, MC and GC must adhere to the host without being washed away. MC colonizes the epithelial cells lining the mucosal surfaces of the nasopharynx. While GC primarily colonizes the epithelial lining of the genitourinary tract. After the initial adherence, the bacteria form a close association with host the cell membrane and subsequently invade the host cells (Fig.1B EM). While inside the cells, the bacteria must also survive the hostile intracellular environments. After crossing this epithelial barrier, MC enters and replicates in the bloodstream. MC may also cross the blood brain barrier to enter the cerebrospinal fluid, where it can cause meningitis. GC, on the other hand, does not usually disseminate but causes an intense local inflammatory response.

Colonization and Entry

Major outer membrane proteins PI, pili and the family of Opa proteins (also called PII) are important in bacterial adhesion and/or invasion of epithelial cells. Antigenic variation of pili and Opa is also to play an important role in helping the bacteria escape from host immune systems. The major outer membrane

Attachment

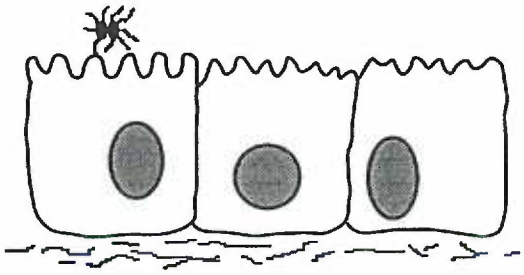
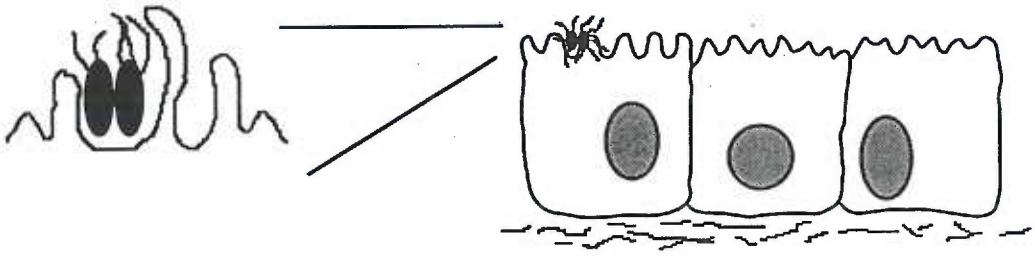
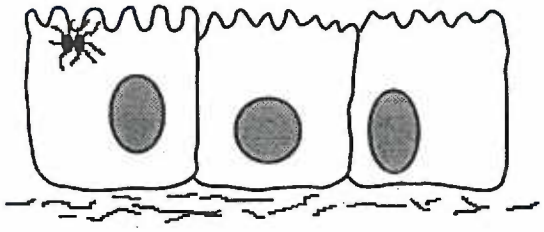


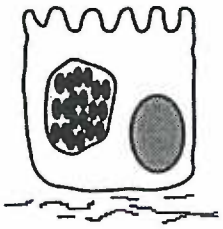
Figure 1A. Critical events of a localized Neisserial infection. The bacteria first attach to epithelial through their pili. After the initial attachment, bacteria form close association with epithelial cell membrane and subsequently enter the cells, possibly multiply within the cells and eventually transcytotically pass to subepithelial cells.

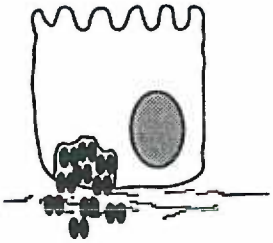
Close Association



Internalization/Invasion







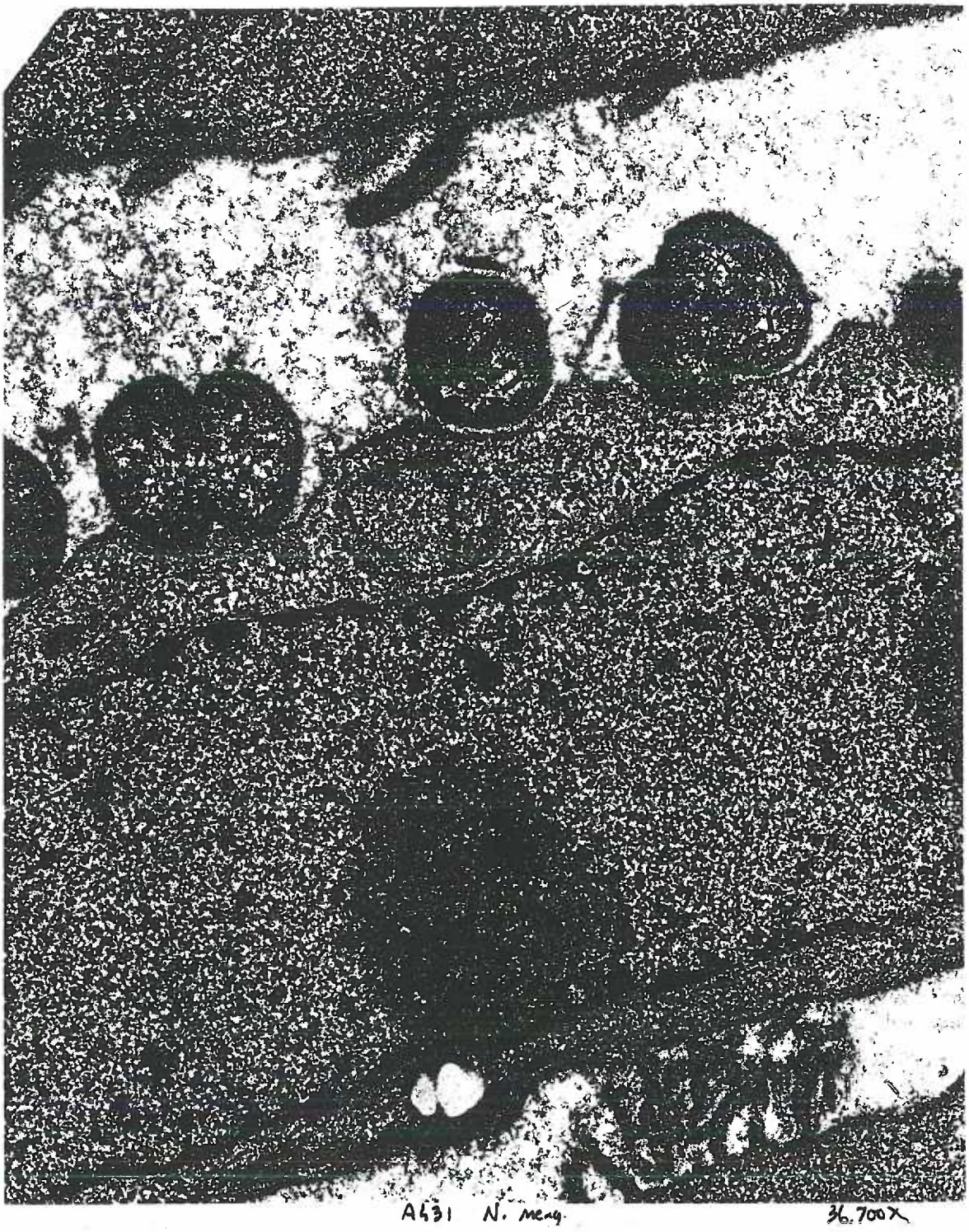


Figure 1B. EM shows the MC forming close association with epithelial cells A431 at the site of attachment.

TABLE 1. Major attributes of pathogenic neisseriae for interaction with nonphagocytic cells

Attribute	*N. meningitidis*	*N. gonorrhoeae*
Pilus-mediated adhesion	Adhesion to all cell types but not invasion	Adhesion but not invasion
Pilin antigenic variation	Modulation of adhesiveness through formation of bundles of pili Cell-binding specificity	Modulation of adhesiveness Cell-binding specificity
PilC proteins	PilC1 likely to be the adhesin	Adhesins localized at the tip of the pilus
Opa/class 5 proteins	Adhesion and invasion of epithelial cells; only certains variants	Adhesion and invasion; only certain variants
Opc	Adhesion and invasion Very efficient in endothelial cells	
Capsule	Inhibition of Opc- and Opa-mediated interactions	
LOS	Inhibition of Opc- and Opa-mediated interactions by sialylation	Inhibition of Opa-mediated interactions by sialylation Binding to the asialoglycoprotein receptor

Source: Nassif, N. and So, M., 1995. Interaction of Pathogenic Neisseriae with Nonphagocytic Cells. Clin. Micro. Rev. 8, 376-388.

proteins and their main functions are summarized in Table I which was nicely reviewed by X. Nassif (X.Nassif and M. So, 1995).

Porin (P.I)

The 40 kD outer membrane of *Neisseria gonorrhoeae* and *Neisseria meningitidis* contains a principle protein P.I that by itself accounts for more than 60% of the outer membrane protein (Frash et al. 1974). P.I is a porin which selectively allows certain hydrophilic molecules to pass across the cell wall into the periplasm (Lutenberg and Alphen, 1983; McDade and Johnston, 1980). Neisserial porins consist of three identical polypeptide chains (Jones et al., 1980; Judd, 1988) and those belonging to a particular strain are antigenically conserved (Swanson, 1978; Lambden and Heckels, 1979). There are two structurally different forms of porin, P.IA and P.IB. P.IA subunits have one terminus exposed on the bacterial surface, while P.IB subunits hembrane (Swanson et al. 1984). Strains expressing P.IA are mostly isolated from DGI (disseminated gonococcal infection). Strains expressing P.IB are predominantly isolated from patients with a local infection (Sandstrom et al., 1984). The reasons for these differences are unclear.

Neisserial porins have also been shown to translocate into artificial lipid bilayers and plasma membranes of mammalian cells (Lynch et al., 1984; Mauro et al., 1988). The rate of P.IA translocation into a foreign membrane is much higher than that of P.IB (Lynch et al., 1984). The translocation of P.I into host cell membranes results in a transient change in membrane potential (Haines et al.,1988; Meyer et al,1996) and an inhibition of exocytosis of GC from human neutrophils (Haines et al., 1988). These events suggest that P.I may be important for the entry and intracellular trafficking of the bacteria and for their interaction with phagocytes.

cells 2-3 logs less than MS11A P+ wild type (this study). Previous work in this lab has proven that certain pilin sequences confer a more adhesive phenotype than others (Nassif, 1993).

A single GC or MC cell is genetically capable of producing antigenically different pilin variants. The GC chromosome contains numerous copies of variant *pil* loci (*pilS*) which lack promoters and 5' coding sequences. Pilin expression is controlled at the *pilE* locus, which contains an intact pilin gene and promoter sequences. Pilin antigenic variation is the result of nonreciprocal recombination of a *pilS* copy with the *pil* gene in *pilE* (Gibbs et al.,1989; Serfeit et al., 1988; Swanson et al., 1987). In MC, antigenic variation of Class I pilin is thought to occur by the similar mechanisms as in GC.

Pili antigenic variation have been shown to play a role in regulating adhesion to host cells during different infection stage and variation in tropism for different cell types (Jonsson et al., 1994; Virji and Alexandrescu et al., 1992; Virji and Kayhty et al., 1991; Virji and Saunders et al., 1993).

Pili protruding from the bacteria surface are easy targets for the host immune defense systems. Therefore, pilin antigenic variation is very likely to play an important role in helping the bacteria to escape the host immune response.

Pilin also undergoes phase variation. Several factors could affect this biphasic on-off switch in GC: imprecise recombination between *pilS* and *pilE* can generate a premature translational termination signal (Bergstrom et al., 1986; Swanson et al., 1986), or transcriptional regulation by *pilA* and *pilB* (Taha et al., 1991; Taha and So et al., 1988). *pilA* and *pilB* are members of two component regulatory systems that regulate expression of genes in response to environmental changes in bacteria. *PilA* represses the pilin promoter in the presence of *PilB*, and activates it in its absence. The reason for the on-off switch in pilin expression is not known. It is clear that pili are important for

The Opa/Class 5 proteins of pathogenic Neisseriae also play an important role in adherence and invasion. These basic outermembrane proteins have a monomeric MW of ~28kDa, and their migration on polyacrylamide gels is heat-modifiable (Swanson, 1980).

Like pilins, Opa proteins undergo high frequency phase and antigenic variation (Sparling et al., 1986; Stern et al., 1986; Stern et al., 1984). The GC chromosome contains 11 variant *opa* genes (Bhat et al., 1991; Connel et al., 1990; Dempsey and cannon, 1991; Dempsey and Litaker t al., 1991). Unlike variant silent *pil* genes, each variant *opa* gene and promoter sequences are intact. Variable expression of *opa* genes is due to slipped strand mispairing during replication of a pentameric repeat (CTCTT) region within the signal sequence-encoding segment of the *opa* gene(Belland t al., 1989; Murphy et al., 1989). A correct number of pentameric repeats within an opa gene results in the correct open reading frame, leading to the production of a full length Opa product; an incorrect number of repeats results in the generation of a premature termination codon which in turn leads to a truncated protein product. Therefore, a cell can express none to several opa genes at the same time. There are only 3 to 4 opa genes in the MC chromosome (Aho et al., 1991). They have a similar gene structure and their products are thought to function in a similar manner as the GC Opas.

Opa proteins promote bacterial interactions with host cells. In GC, certain Opa variants (OpaA, OpaB, OpaD) increase bacterial adherence to epithelial cells (Lambden et al., 1979; Swanson, 1979; Makino and Meyers, 1991). On the other hand, Opa variants OpaA (Kupsch et al., 1993) and OpaH (Walderbeser et al., 1994) promote invasion of the bacteria into the cells. Other Opa proteins such as OpaB (Naids and Rest, 1991) OpaE and Opal (Belland and Fischer, 1992) have the ability to bind to neutrophils. Certain Opa proteins may also

function in bacteria clumping (Blake, 1985). In MC, OpaB and Opc promote adherence to endothelial cells (Virji et al, 1993). Overall, Opa proteins are involved in cell tropism, adhesion, invasion, and interbacteria adhesion.

Other possible adhesin or invasin?

Numerous early studies using naturally occurring piliated (P^+) and nonpiliated (P^-) variants have shown that pili play an important role in initiation of GC disease (Punsalang and Sawyer, 1973; Stephens et al., 1984; Stephens and McGee, 1981; Swanson, 1973). Recent studies examining adhesion of genetically defined P^+ and P^- mutants showed that P^+ bacteria adhere to eukaryotic cells several logs better than P^- variants (this study, Nassif et al., 1994; Rudel et al., 1992; Verji et al., 1992; Verji and Kayhty et al., 1991; Verji and Saunders et al., 1993). In the case of capsulated MC, loss of piliation abolished all cellular interactions (Nassif unpublished observasion). However, a small fraction of P^- GC and P^- unencapsulated MC do attach to cells, indicating that bacterial components other than pili also contribute to bacterial-cell interactions.

To identify GC gene products involved in attachment to host cells, Richard Ajioka in our lab constructed a gene bank derived from a clinical isolate of GC (15063G) and isolated eight clones which had the capacity to adhere to the human endometrial adenocarcinoma tissue-culture line Hec-1-B. Among these eight clones, seven of them overlapped. The gene in these clones that is responsible for adherence to Hec1B cells was subsequently identified as an opa variant. Further studies identified *OpaH* in MS11A as contributing to epithelial invasion (Walderbeser et al.,1994). The eighth clone (G2), has been characterized as part of this thesis project (see manuscript II).

Withstanding the extracellular environment

Capsule

The capsule influences MC-host interactions (Stephens et al., 1993). As mentioned above the capsule decreases Opa- and Opc-mediated adhesion and invasion. This inhibitory effect of the capsule may due to modification of surface charge or hydrophobicity, or to the physical masking of adhesins. The fact that strains isolated from the blood or the cerebrospinal fluid (CSF) are usually encapsulated (Devoe and Gilchrist, 1975) suggests that inaccessibility of these adhesins on the MC cell surface may be important during later stages of the infection, when bacteria have invaded the bloodstream in the CSF. Recently, the capsules of Group B MC were shown to inhibit binding of bacteria to macrophages (Read et al., 1996). This result suggests that the MC capsule is involved in inhibiting phagocytosis of MC by macrophages.

Lipooligosaccharide

There are two forms of LOS (lipooligosaccharide) produced by Neisseriae, short or long. The long LOS is due to the additional carbohydrate residues which can be sialylated by bacterial sialytransferase. Sialylation requires host $CMP^{-}NANA$ as the sialic acid donor (Smith 1991, Van Putten 1993). This modification leads to the generation of an epitope that mimics one present on host cells (Mandrell et al., 1993). This mimicry may help the bacteria resist the host humoral immune response.

Variation of LOS structure seems to be regulated by environmental factors (Brener et al., 1981). Human volunteer studies with GC indicated that bacteria isolated early in infection have a short LOS, while bacteria isolated after the

initiation of the inflammatory response have long LOS (Schneider et al., 1991). In GC, LOS switching has been correlated with modification of the invasive phenotype (van Putten, 1993). Studies using LOS variants with and without the sialic acid acceptor site showed that GC with large amounts of sialic acid on their LOS are not internalized, whereas the non-sialiated variants are. The GC LOS is able to mediate adherence *in vitro* .

In MC, the majority (70%) of bacteria isolated from the nasopharynx of carriers are unencapsulated and preferentially express a short LOS species (Broome 1986). In the disease state, 97% of the isolates from blood and CSF express long LOS (Jones et al.,1992). In MC, LOS sialylation also appears to modify adhesion and invasion (Verji and Makrepeace et al., 1993). In the case of MC this effect of sialylation has been observed only in unencapsulated strains. The mechanisms by which LOS sialylation affects invasiveness is unknown, but as in the case of capsule, this effect could be due to the masking of adhesins or to an increase in negative charges at the bacterial cell surface.

Like pili and Opa in the pathogenic Neisseriae, LOS is also subject to antigenic variation (Schneider et al., 1984).

IgA protease

Another virulence factor which is thought to play a role in the early phase of infection is IgA protease. All pathogenic Neisseriae produce and secrete human IgA1 protease, an enzyme that cleaves human IgA1 in the proline-rich hinge region (Mulks and Shoberg, 1994). There are two types of IgA1 proteases, type 1 and type 2, which differs slightly in their cleavage specificity. Only one type of protease is produced per strain. Many other mucosal pathogens produce IgA1 proteases as well. These include *Haemophilus influenzae*, *Streptococcus pnumoniae*; several oral microorganisms such as *S.*

sanguis, *S. mitis*, *S. orlis*, *Prevotella (Bacteroides)* sp., and *Capnocytophago* sp.; as well as *Ureaplasma urealyticum* and *Clostridium ramosum*.

The Neisserial IgA1 protease is encoded by the *iga* gene, which is present in single copy on the chromosome. IgA1 protease is first produced as a polyprotein consisting of five distinct domains. Secretion and extracelluar maturation of IgA1 protease occurs via an autoproteolytic process (Pohlner et al. 1987). Although this protease has been studied during the last decade, direct evidence for an important role involving pathogenesis is missing. Organ culture infection studies using isogenic *iga*$^+$ and *iga*$^-$ strains indicated that the protease does not play a role in the early steps (~12 hour) of adherence or invasion (Cooper et al. 1984). The work done in the first part of this thesis has revealed a major function for the Neisserial IgA1 protease (see manuscript I).

What happens after GC and MC invasion?

Adapting a polarized cell line to study the trafficking of Neisseriae

So far, the early event, such as adhesion and invasion of Neisserial infection has been extensively studied. However, the intracellular behavior of GC and MC remains largely a mystery.

The biochemistry of GC and MC invasion is unknown. After colonization of and entry into the epithelial cell monolayer, Neisseriae traffic through the monolayer, exit the cell and enter the subepithelial matrix. Since humans are the only host of Neisseria, obtaining and maintaining primary organ cultures to study transcytosis behavior is inconvenient. The most common approach used *in vitro* is infection of cultured cells. However, transformed cells are too degenerated to be representative of the natural infection. In addition, the nature of the culture system does not allow studies of bacterial transcytosis. As an alternative, a

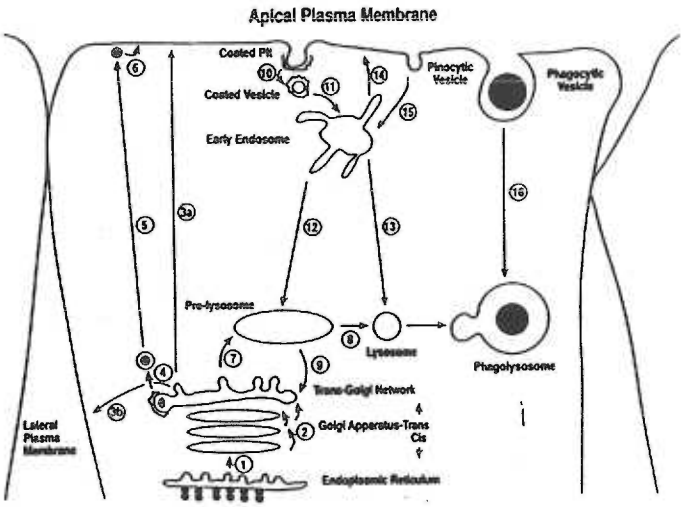
polarized epithelial cells system was adapted to serve this purpose. So far, the most successful polarized epithelial cell line used to study GC and MC transcytosis is T84 cells .

Merz et al. in our lab successfully adapted the polarized T84 cells (derived from colorectal polarized epithelium) to study GC and MC trafficking. Their results show that the bacteria traversed the monolayer at different times, and the times of trafficking were distinct for each Neisserial species. MC traversed the monolayer 12 and 24 hours post-inoculation while GC took 36 to 48 hours. These trafficking times are identical to those reported for these bacteria in *ex vivo* organ culture infection studies (McGee et al., 1981). Interestingly, the one nonpathogenic *N. perflava* assayed in these studies did not traffic through the monolayer (Merz et al., 1996). This suggested that the ability to traverse monolayer may be a requirement for pathogenesity of Neisseriae. Currently, more trafficking studies using the T84 polarized monolayer model are being conducted in this lab.

General trafficking pathways in eukaryotic cells (Figure 2)

There are two major vesicular pathways in a typical eukaryotic cell - exocytosis and endocytosis. Exocytosis is used in organelle biosynthesis and transport of secretory proteins. Endocytosis is used in bringing particles from outside into the cells.

Exocytosis pathway starts from the ER where the proteins are synthesized. Newly sythesized proteins are then transported into the Golgi and modified. From there, the proteins are transported directly to the plasma membranes, or to secretory storage granules. These granules are then transported to the plasma membrane region where they await the stimuli for secretion of there cargo. The newly sythesized proteins, most of which are lysosomal, can also be modified



rab proteins involved in vesicular trafficking

rab1: transport proteins between ER and Golgi
rab2: located in intermediate compartments between ER,Golgi apparatus and ciscisternae, early part of exocytosis
rab3: exocytosis
rab4: recycle receptors between EE and cell surface, early sorting
rab5: regulate endosome fusion
rab7: late endosome, large vesicle and vacule transport
rab8: TGN to basolateral PM
rab9: lysosome biogenesis, recycle M6PR to TGN

Figure 2 Vesicular traffic in a eukaryotic cell. The following steps are discussed in the text: 1. Transport from the endoplasmic reticulum to the Golgi. 2. Movement between cisternae of the Golgi. 3. Transport of constitutively transported proteins to the plasma membrane. In polarized cells the route is split between 3a. Apical delivery and 3b. Basal/Lateral delivery. 4. Formation of secretory storage granules. 5. Delivery of secretory granules to the plasma membrane region. 6. Stimulated fusion of secretory granules to the plasma membrane. 7. Delivery of lysosomal constituents to a prelysosomal compartment. 8. Delivery of lysosomal constituents from the prelysosomal compartment to the mature lysosome. 9. Recycling from the prelysosomal compartment to the *trans*-Golgi network, for example of the mannose-phosphate receptor. 10. Formation of a coated vesicle from a coated pit during receptor mediated endocytosis. 11. Fusion of an endocytic vesicle with an early endosome. 12. Sorting of an endocytosed molecule to the pre-lysosomal compartment. 13. Sorting of an endocytosed molecule to the lysosome. The exact route to the lysosome may be direct or may be through the pre-lysosomal compartment. 14. Recycling from the early endosome to the plasma membrane. 15. Delivery of a pinocytic vesicle to the early endosome. 16. Delivery and fusion of a phagocytic vesicle with a lysosome.

Picture source: B. Wattenberg. Vesicular Traffic in Eukaryotic Cells. Book: The structure of Biological membranes, Chapter 22, 997-1046.

and transported to prelysosomes. From there, they can be delivered to lysosomes and phagolysosomes.

Three major processes are involved in endocytosis. Pinocytosis is the nonspecific uptake of fluid and membrane-bound substance from outside the cell in small vesicles. Endocytosis describes the receptor-mediated uptake of molecules bound to cell surface receptors. Phagocytosis applies to the internalization of large particles. Most bacteria invade cells through phagocytosis. At the site where the bacteria form close association with cells, the bacteria induce host cell cytoskeletal rearrangement resulting in phagocytosis.

Endocytic vesicles then deliver their contents to early endosomes and prelysosomes through fusion. Some membranes containing surface receptor are recycled to the plasma membrane. Others follow the same pathway as endosomes and become prelysosomes and eventually lysosomes. The engulfed material within the vesicles is ultimately digested by lysosomal enzymes.

After endocytosis, endosomes are directed to their specific destinations through sorting and fusion. The trafficking of these membrane bound organelles appears to be regulated by a family of ras-like GTPase proteins: rab GTPases. Rab GTPases are localized on the surfaces of distinct vesicles. They function in the process by which transport vesicles dock and/or fuse with target vesicle membranes.

So far, over thirty rab GTPases have been found. Fig 2 summerizes some of these proteins and the vesicular transport events that they are thought to regulate.

The mature lysosome has a pH around 5.0. The high concerntration of H^+ inside the lumen is maitained by a proton pump H^+-ATPase that resides on the lysosomal membranes. Once lysosomal enzymes are synthesized in the ER and modified in the Golgi, they can be delivered to prelysosomes through mannose-6-phosphate receptor (M6P) dependent or independent pathways. In the M6P dependent pathway, the lysosomal enzymes bind to the M6P receptor in the Golgi and are transported to prelysosomes where they disassociate from the M6P receptor under acidic pH and enter the lysosome lumen. Most of these enzymes are only active at low pH. After delivering the lysosomal enzymes to their destination, M6PRs are recycled back to Golgi for the next delivery.

Lysosomal glycoproteins

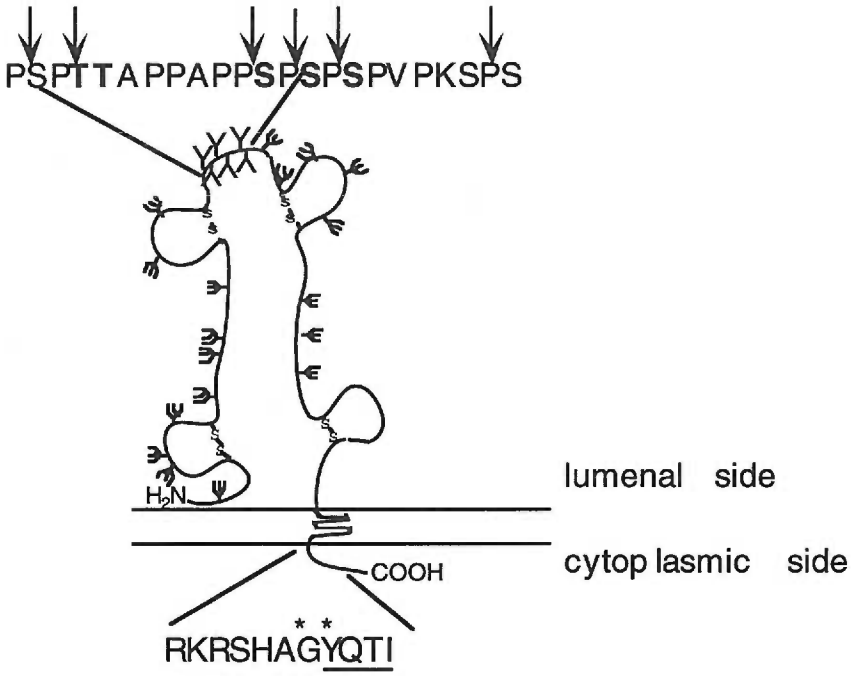
The lysosomal membrane plays an important role in the proper functions of lysosomes by sequestering numerous acid hydrolases (table 2) from the rest of the cytoplasmic compartments. Besides maintaining a low pH in the lysosomes, the lysosomal membrane is presumably involved in several important functions of lysosomes, such as its resistance to degradation by lysosomal hydrolases and its ability to interact and fuse specifically with other membrane organelles, including endosomes, phagosomes, and plasma membranes.

The major components found on the lysosomal membrane are glycoprotein LAMP1, LAMP2, CD63 (LAMP3) and LimpII. Among them, LAMP1 and LAMP2 are represented in highest number. A typical vacuole has an average of 25,000 to 30,000 LAMP1 present on the membrane which allows them to form a coat covering the vacuole membrane. Both LAMP1 and LAMP2 have core proteins with Mr about 45,000 kD. The glycosylated LAMP1 and LAMP2 have Mr up to 110kD to 120kD (Carlsson et al., 1988). These carbohydrates are presumably playing a critical role in maintaining the stability and integrity of the lysosomal

Table 2 Acid hydrolases that have been located in lysosomes

Enzyme	Natural substrate
PHOSPHATASES	
Acid phosphatase	Most phosphomonoesters
Acid phosphodiesterase	Oligonucleotides and other phosphodiesters
NUCLEASES	
Acid ribonuclease	RNA
Acid deoxyribonuclease	DNA
PROTEASES	
Cathepsin(s)	Proteins
Collagenase	Collagen
Peptidases	Peptides
POLYSACCHARIDE- AND MUCOPOLYSACCHARIDE-HYDROLYZING ENZYMES	
β-galactosidase	Galactosides
α-glucosidase	Glycogen
α-mannosidase	Mannosides, glycoproteins
β-hexosaminidase	Glycolipids
Glucocerebrosidase	Glycolipids
β-glucuronidase	Polysaccharides and mucopolysaccharides
Lysozyme	Bacterial cell walls and mucopolysaccharides
Hyaluronidase	Hyaluronic acids; chondroitin sulfates
Arylsulfatase	Organic sulfates
LIPID-DEGRADING ENZYMES	
Esterase(s)	Fatty acyl esters
Phospholipase(s)	Phospholipids

SOURCE: D. Pitt, 1975, *Lysosomes and Cell Function*, Longman.



↓ IgA protease cleavage site

Ψ N-linked oligosaccharide

Y O-linked oligosaccharide

Figure 3. The predicted hLAMP1 structure. N-linked glycans and O-linked glycans are indicated by Ψ and Y , respectively. O-linked glycans are almost exclusively attached to the flexible hinge region which has high homology to human IgA. The possible IgA protease cleavage sites are indicated by ↓ .The cytoplsmic tail has a conserved GY motif as indicated by *. The tyrosine sorting motif YXXI motif is underlined.

membrane from the hydrolases and proteases inside the lumen, since tunicamycin treatment result in the rapid degradation of newly synthesized LAMPs (Barriocanal et al., 1986). Although LAMP1 and LAMP2 have similar molecular weights, about 110kDa, they are encoded by separate genes located on different chromosomes and have a identity of 36.7%. Previous results suggest that LAMP1 and LAMP2 have distinctly separate functions. The cytoplasmic tails of LAMPs are 11-13 amino acids long. There is a conserved GY motif among the LAMPs and also in the acid phosphatase cytoplasmic tail (Cha et al., 1990). The space between the YXXI motif and the transmembrane domain is critical for targeting LAMP1 to dense lysosomes (Rohrer et al., 1996). The structure of LAMP1 is shown in Fig. 3. LAMP1 has 18 N-glycans and 6 O-glycans (Carlsson et al., 1990). The hinge region of LAMP1 is flexible and has homology to the human IgA hinge region (Viitala et al., 1988). The first part of this thesis showed that by secreting IgA protease which can cleave host cell LAMP1, pathogenic *Neisseria* promote their survival within their host (see manuscript I).

Ways pathogens use to avoid lysosome killing

After a pathogen enters the host cell by phagocytosis, it resides in a phagosome which fuses with a lysosome to form a phagolysosome. The Lysosome plays an important role in defending the body against invading microbes. Many intracellular pathogens have developed various means to escape the inhospitable environment of lysosomes. For instance, *Salmonella typhimurium, Chlamydia psittaci* and *Legionella pneumophila* block phagosome-lysosome fusion (Zeichner et al., 1983; Horwitz, 1983). *Listeria monocytogenes* produces a toxin that lyses the endosomal membrane and allows the bacteria to escape into the cell cytoplasm.

Manuscript I:

The IgA1 protease of pathogenic Neisseriae increases LAMP1 turnover and promotes survival of bacteria in epithelial cells

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Running title: IgA1 protease-mediated cleavage of LAMP1 promotes intracellular survival of bacteria

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Summary

Infection of epithelial cells by the pathogenic Neisseriae results in a reduction of LAMP1 and CD63 levels and acid phosphatase activity. The bacteria reduce LAMP1 levels by increasing its turnover rate. Several lines of evidence indicate that the Neisserial IgA1 protease is directly responsible for LAMP1 turnover. LAMP1 contains an IgA1-like hinge region with potential cleavage sites for the Neisserial type 1 and type 2 IgA1 proteases. Purified Neisserial type 2 IgA1 protease cleaves LAMP1 in vitro. Unlike its WT isogenic parent, an *iga*- mutant of *N.gonorrhoeae* cannot affect LAMP1 turnover and its growth rate in epithelial cells is dramatically reduced. Thus, IgA1 protease plays an important role in promoting intracellular survival of pathogenic Neisseriae.

Introduction

Neisseria meningitidis (MC) and *Neisseria gonorrhoeae* (GC) are the two pathogenic members of the Neisseria family of bacteria. They are closely related, sharing >80% homology at the nucleic acid level. MC is a major cause of bacterial meningitis in young children and adults; GC is the agent of gonorrhea. MC and GC colonize the mucosal epithelium, while MC is thought to interact also with brain endothelium as it can cross the blood brain barrier.

Studies of early events in Neisseria/host interactions using organ cultures (McGee et al., 1981; Stephens et al., 1981; Stephens and McGee, 1983) have identified key early events in a Neisserial infection. MC and GC adhere to nonciliated cells of the epithelium and induce elongation of the microvilli at the site of contact. They form a close association with the host surface and the region of contact enlarges until the bacteria are surrounded by host membrane. The bacteria enter the apical side of the cells, traffic through the cell and exit into the subepithelial stroma. Shortly after colonization, the cilia of neighboring uncolonized ciliated cells stop beating and the cells slough from the tissue. This cell type-specific toxicity is thought to be mediated by TNFα produced by secretory cells in response to shed bacterial components such as lipooligosaccharide and peptidoglycan (Gregg , Melly et al., 1981; Melly et al., 1981; Melly et al., 1984).

Several bacterial components are important in Neisseria/host cell interactions. Piliation, *per se*, and PilC, a high molecular weight pilus-associated protein, are important for adhesion of GC and MC to host cells (Rudel et al., 1992; Nassif et al., 1993, 1994; Rudel et al., 1995). Certain

Class I pilin variants (Lambden et al., 1980; Rudel et al., 1992; Nassif et al., 1993) and Opa/Class 5 variants (Virji, Alexandrescu, 1992; Kupsch et al., 1993; Virji et al., 1993; Waldbeser et al., 1994) promote adhesion and/or invasion, while Opc, an Opa-like protein present in some MC strains, enhances adhesion and contributes to tissue tropism (Virji, Makepeace et al., 1992; Virji et al., 1993).

All pathogenic Neisseriae constitutively express and secrete one of two closely related types of IgA1 proteases with different specificities for the hinge region of the human IgA1 subclass of immunoglobulins (Plaut et al., 1975; Pohlner et al., 1987; Mulks and Shoberg, 1994). Type 1 protease cleaves at a specific Pro/Ser bond, while type 2 protease cleaves at a Pro/Thr bond in the IgA1 hinge. A given strain produces only one type of IgA1 protease. The specificity of this enzyme for mucosal immunoglobulins and their production only by the pathogenic Neisseriae argue for their importance in bacterial virulence. Infection studies using human Fallopian tube tissue segments with a GC *iga-* mutant indicated that the IgA1 protease does not influence the early phases of adherence or invasion in the absence of secretory IgA1 (Cooper et al., 1984). This mutant was not assessed for any role it might play at later stages of infection.

Neisseriae appear to enter cells by a phagocytic mechanism, although the precise nature of the invasion process is unknown. Most phagosomes are targetted to fuse with lysosomes, which are acidic, terminal degradative compartments in the endocytic route. This compartment contains hydrolytic enzymes and other microbicidal compounds and plays

an important role in defending the body against invading microbes. Many intracellular pathogens have developed various means to modify their phagosomal environments in order to avoid lysosome killing. *Salmonella typhimurium, Chlamydia psittaci* and *Legionella pneumophila* block phagosome-lysosome fusion (Buchmeier et al., 1991, Zeichner et al., 1983; Horwitz, 1983). *L. pneumophila* (Horwitz et al., 1984), *S. typhimurium* (Alpuche-Aranda et al., 1992), *Toxoplasma gondii* (Sibley et al., 1985), and *Mycobacterium avium* (Sturgill-Koszycki et al., 1994) attenuate the pH of their phagosomal environment. To date, the molecular mechanisms used by these microbes to modify their phagosomes are unknown.

We have determined how Neisseriae escape lysosome killing in epithelial cells. Infection of epithelial cells by the pathogenic Neisseriae results in the reduction of three lysosomal markers, LAMP1, CD63 and acid phosphatase. The Neisserial IgA1 protease cleaves LAMP1 at its IgA1-like hinge region, thereby increasing its turnover rate. Purified Neisserial type 2 IgA1 protease cleaves LAMP1 in vitro. Unlike its WT isogenic parent, an *iga-* mutant of *N.gonorrhoeae* cannot affect LAMP1 turnover and its growth rate in epithelial cells is dramatically reduced. Thus, the IgA1 protease plays an important role in promoting intracellular survival of pathogenic Neisseriae.

Results

LAMP1 levels are decreased in Neisseria infected cells

Immunofluorescence microscopy studies were initiated to examine interactions between the pathogenic Neisseriae and LAMP1, a major component of late endosomes and lysosomes. A431 cells (a human epithelial cell line derived from columnar epithelium of the endocervix) were infected with MC strain 8013.6 for 4, 8 and 22 hrs, and examined by microscopy. Results indicate that the amount of LAMP1-stainable material decreased in infected cells compared to the uninfected control. This decrease, first visible at 8 hrs post-infection (Figure 1C, Panel I), was very evident by 22 hrs post-infection (Figure 1E, Panel I). This reduction in the LAMP1 signal is due to a general decrease in the amount of LAMP1 protein, not to any alteration of the MAb reactive epitope (see below). Infected cells were healthy, as judged by several parameters (see below). Similar results were also observed in cells infected with three other MC strains and five GC strains (Table 1). In addition, Neisseria infection resulted in decreased LAMP levels in a second human epithelial cell line, HEC-1-B (data not shown). These data strongly suggest that reduction of LAMP1 levels is an important biological activity of the Neisseriae.

To quantitate LAMP1 levels, A431 cells were infected for 22 hrs with MC 8013.6 and total cellular proteins were Western blotted using the LAMP1 MAb. As an internal control, the blot was also probed with an anti-b-tubulin MAb. Results from this experiment support our earlier microscopy observations and indicate that the amount of LAMP1 is indeed decreased in infected cultures compared to uninfected controls (Figure 2). The

signals were scanned and quantitated using the NIH Image System program. A comparison of LAMP1 values, after normalization to their respective b-tubulin values, indicated that, in this experiment, the amount of LAMP1 was decreased ~40% in MC infected cells. Analysis of a series of blots from several experiments indicate that, depending on the multiplicity of infection (MOI; the number of infecting bacteria per cell), LAMP1 is decreased by 20%-50% in infected cultures compared to uninfected controls.

MC and GC do not infect all cells in a culture. Depending on the MOI, age of the inoculum and other undefined conditions, the extent of infection in any given experiment varies between 40 and 90%. This variation in infectivity explains the variation in LAMP1 values observed. In the experiment shown in Figure 2, LAMP1 was decreased ~40% compared to the uninfected control and the level of infection was ~60%. Therefore in a given infected cell LAMP1 would be reduced by more than 40%. The dramatic decrease in LAMP1 in an infected cell can be observed by microscopy (Figure 1G, Panel I). The amount of b-tubulin was identical in infected and uninfected cultures, strongly suggesting that MC infection does not affect the microtubule network (see below).

Neisseria infection does not affect the general morphology of, nor cause extensive damage to, infected cells

To detemine whether the decrease in LAMP1-stainable material in Neisseria-infected cells is due to an indirect toxic effect of the bacteria, A431 cells were infected with MC 8013.6 for 22 hrs, then trypsinized and stained with Trypan Blue. The number of stained and unstained cells

were quantitated. In these studies, the percentage of cells excluding Trypan Blue was nearly identical in infected and uninfected cells (>85%), indicating that the plasma membrane of infected cells are intact (data not shown).

The effect of MC on the morphology of actin and microtubule networks was investigated. The actin- and microtubule-based cytoskeletal systems are important for the structural integrity of the cell and play important roles in organelle position and movement (Langford, 1995; Lippincott-Schwartz et al., 1995). Actin is involved in adhesion of the cell to the substratum, maintainance of cell polarity and cell mobility (Hitt et al., 1994; Mays et al., 1994). Microtubules and their motor proteins maintain the spatial organization of various organelles and facilitate the delivery of transport intermediates between these organelles (Luby et al., 1994; Terasaki et al., 1986; Cooper et al., 1988; Lippincott-Schwartz et al., 1991). A431 cells were infected with MC 8013.6 for 22 hrs, stained for actin using phalloidin (Figure 3A,B) or for microtubules using a MAb to ß-tubulin (Figure 3C,D). Examination of stained cells by confocal microscopy at a focal plane near the center of the cells revealed that the gross arrangement of both cytoskeletal actin and microtubules was identical in infected and uninfected cells. Taken together, our results indicate that the decrease in LAMP1-stainable material in infected cells is not due to the loss of structural integrity of the cell.

Neisseria infection affects the half-life of LAMP1

The decrease in LAMP1 levels in a Neisseria-infected cell may be caused by perturbations in the biosynthetic or turnover rate of this protein. The

synthesis of LAMP1 was first examined. A431 cells were infected with MC 8013.6 for 22 hrs and labelled with ^{35}S-methionine. At various times the cells were lysed and the labelled LAMP1 was immunoprecipitated and counted. Results (Figure 4A) show that the biosynthetic rate of LAMP1 is identical in infected and uninfected cells.

The turnover rate of LAMP1 was next examined. A431 cells were infected with MC 8013.6 at an MOI of 10. In these experiments the high MOI and length of incubation resulted in infection of ~90% of the cells in culture. At 22 hrs post-infection, cells were labelled with ^{35}S-methionine for 90 minutes and chased for various lengths of time. LAMP1 was immunoprecipitated from the lysates, separated by SDS-PAGE and autoradiographed. Signals from the gel were quantitated and the LAMP1 values in infected and uninfected cells were expressed relative to the 0 hr value (Figure 4B). Results indicate that the rate of LAMP1 turnover in infected cultures was increased significantly compared to uninfected controls and that it followed second order kinetics. In the uninfected control, the LAMP1 turnover rate was constant over the entire chase period. In infected cells, this rate was nearly twice as rapid in the first 30 minutes of the chase period compared to the control. Thereafter it was similar to that for uninfected cells. In an infected culture, the rate of LAMP1 turnover is a reflection of the turnover in infected and uninfected cells. The increased rate at the beginning of the chase period mostly reflects the rate of turnover in the large population (90%) of infected cells. The rate observed at later time points mostly reflects the rate of turnover in the uninfected population of cells. The high MOI used in these experiments would explain the nearly one-log difference in LAMP1 levels between

infected and uninfected cells at the later time points. Taken together, our results strongly suggest that the decrease in the cellular level of LAMP1 in MC-infected cells is due to increased turnover of this glycoprotein.

IgA1 production correlates with the ability to reduce LAMP1 levels

Many Neisserial virulence factors could potentially reduce LAMP1 levels. 12 Neisseria strains differing in the production of a series of virulence factors were examined for their ability to reduce LAMP1 levels in A431 cells (Table 1). These strains differed in pilin type and in pilus, Opa, Opc and capsule expression. Two strains were nonpathogenic. Of the virulence factors examined, only the production of IgA1 protease correlates with the ability of the organisms to decrease LAMP1 levels (Table 1).

The Neisseria IgA1 protease cleaves LAMP1 in vitro

LAMP1 is a ~120kD protein present in the membrane of late endosomes and lysosomes (reviewed by Fukuda, 1991). It is heavily glycosylated and the carbohydrate groups are thought to play an important role in protecting the protein and ultimately the compartments from degradation by lysosomal acid hydrolases (Barriocanal et al., 1986; Fukuda, 1991). The glycosylated residues, clustered in two regions of the protein, are separated by a proline-serine rich region with striking homology to the hinge region of the human IgA1 subclass of immunoglobulins (Mattei et al., 1990). All pathogenic Neisseriae secrete one of two types of IgA1 protease. Present in the human LAMP1 hinge region are potential sites for the type 1 and type 2 Neisserial IgA1 proteases (reviewed by Mulks

and Shoberg, 1994). Moreover, the LAMP1 hinge region is situated in the lumenal face of the organelle and thus is a potential target for these proteases.

Human IgA1 hinge region: CPVPSTPPTPSPSTPP$_2$TPSP$_1$SCC

hLAMP1 IgA1 hinge region: P$_1$SP$_2$TTAPPAPP$_1$SP$_1$SP$_1$SPVPKSP$_1$S

(1) predicted type 1 IgA1 protease cleavage site
(2) predicted type 2 IgA1 protease cleavage site

The presence of potential IgA1 protease cleavage sites in LAMP1 and the location of these sites in the inner (lumenal) face of late endosomes and lysosomes strongly suggest that the Neisserial IgA1 protease may play a role in the rapid turnover of this protein.

The ability of the Neisserial IgA1 protease to cleave LAMP1 was investigated. Purified LAMP1 was incubated with Neisseria type 2 protease in pH 7.5, 6.5, or 5.0 buffer, or with buffer alone, at 37°C for 17 hours. The samples were seperated by 10% SDS-PAGE and LAMP1 was detected byt Western blot using a rabbit polyclonal antibody to LAMP1. In all samples containing IgA protease the antibody reacted with a broad band migrating at ~60kD (Figure 5). The hing region in LAMP1 situated near the center of the molecule (Mattei et al., 1990) and IgA protease cleavage at this site should generate two fragments of similar molecular weights. Therefore, the 60kD cross-reactive band most likely contains both IgA1 protease cleavage products. Cleavage of LAMP1 was specific as the protease did not cleave b-tubulin or bovine serum albumen

(data not shown). These results demonstrate that in vitro IgA1 protease cleaves LAMP1 at the hinge region and that the enzyme is active on the cell surface (pH7.5) and in late endosomes (pH6.5) and lysosomes (pH5.0).

A GC *iga-* mutant cannot reduce LAMP1 levels in infected cells

The involvement of the IgA1 protease in LAMP1 turnover predicts that an IgA1 protease deficient strain would not be able to affect LAMP1 levels in vivo. An *iga*⁻ mutant was assessed for its ability to decrease LAMP1 levels. GCM740 is a type 2 IgA1 protease-producing GC strain and GCM740Δ4 is its isogenic protease-deficient mutant. Both are piliated and transparent (does not produce Opa). In GCM740Δ4, a 4.3kbp deletion in the extreme 5' region of the *iga* gene has removed ~93% of the *iga* coding sequence. GCM740Δ4 has no IgA1 protease activity and the outermembrane protein profiles of both parent and mutant are identical (Shoberg and Mulks, 1991). A431 cells were infected with GCM740 and GCM740Δ4 for 22 hrs. Total cellular proteins were detected on Western blots using the anti-LAMP1 and anti-b-tubulin Mabs. The signals were quantitated and the LAMP1 values from each strain were normalized to their respective internal b-tubulin values and compared. As expected, LAMP1 levels in GCM740-infected cells were reduced (~20%; Figure 6C). In contrast, LAMP1 levels in GCM740Δ4 infected cells were nearly identical to those in uninfected cells. The same results were obtained in HEC1-B cells infected with GCM740 and GCM740Δ4 (data not shown). These results demonstrate the importance of IgA1 protease in LAMP1 turnover in vivo.

IgA1 protease has access to LAMP1

The infection experiments clearly shows that the IgA1 protease has access to LAMP1. There are two possible pathways by which the enzyme can be delivered. Most reports indicate that intracellular MC and GC are surrounded by a phagosomal membrane (Nassif and So, 1995; Stephens et al, 1983; Weel et al, 1991) although one study found no such evidence (Shaw and Falkow, 1988). If intracellular Neisseriae are indeed inside phagosomes, and if these vesicles are capable of fusion with late endosomes and lysosomes, then the IgA1 protease secreted by intracellular bacteria would gain access to LAMP1 upon fusion of the phagosome with these compartments. This method of delivery would require bacteria within LAMP1 compartments. Compartments containing bacteria and intact LAMP1 would likely be transient in nature. IgA1 protease is a secreted enzyme (Pohlner et al., 1987); it is very active and is produced in large quantities by the pathogenic Neisseriae (Mulks and Shoberg, 1994). LAMP1 is very likely cleaved as soon as the bacterium enters this compartment. Thus, compartments containing bacteria and intact LAMP1 might be detected at early but not later stages of infection. Laser scanning confocal microscopy was used to visualize such a compartment. A431 cells were infected with MC strain 8013.6 for 12 or 22 hrs and the samples examined by confocal microscopy. Examination of successive, apical to basolateral 1mm-thick optical sections of infected cells showed that compartments containing bacteria and intact LAMP1 were observed more frequently at 12 hr post-infection than at 22 hrs post infection (Figure 7, compare Panels I and II). The same results were obtained with cells infected with GC strain MS11A (data not shown).

IgA1 protease may also gain access to LAMP1 compartments by the endocytic route. Bacteria adhering to the epithelial cell surface are a very rich source of this enzyme. IgA1 protease secreted by colonizing bacteria may be endocytosed and, like a variety of internalized compounds, delivered to late endosomes and lysosomes via vesicle fusion. The integrity of the endocytic pathway in Neisseria infected cells was next examined. A431 cells were infected with 8013.6 for 22 hrs, pulsed with Lucifer Yellow (LY) and chased and the relative distributions of LAMP1 and LY were examined by confocal microscopy. LY was endocytosed and delivered to LAMP1 compartments in both infected and uninfected cells (Figure 8C,F). As observed previously, the levels of LAMP1 in 22 hr-infected cells (Figure 8B) were reduced compared to the uninfected control (Figure 8E). Similar experiments indicate that low density lipoprotein (LDL) can be endocytosed and delivered to LAMP1 compartments in infected cells (data not shown). These results indicate that Neisseria infection does not compromise bulk flow or receptor mediated endocytosis or the delivery of endocytosed material to LAMP1 compartments.

Neisseria infection affects other lysosomal markers

The effect of a Neisseria infection on other lysosomal markers was also examined. A431 cells were infected for 22 hrs with MC 8013.6 and cell lysates were assayed for the activity of one of the lysosomal hydrolases, acid phosphatase (AP). Results show that AP activity was reduced 20% in infected cells compared to the uninfected control (Figure 9). This value is within the range established for LAMP1 decrease. Immunofluorescence

microscopy studies indicate that CD63 levels were also reduced in A431 cells infected with 8013.6 for 12 hrs (Figure 1, Panel II). CD63 levels could not be quantitated as none of the available anti-CD63 abs are appropriate for Western blots. Thus, in addition to LAMP1, two other lysosomal markers are reduced in Neisseria-infected cells, strongly suggesting that late endosomes and lysosomes are modified in infected cells.

The GC *iga-* mutant colocalizes with LAMP1 and fails to grow intracellularly

GCM740- and GCM740Δ4-infected A431 cells were examined by confocal microscopy for the relative locations of bacteria and LAMP1. After 22 hrs of infection, the vast majority of GCM740 did not colocalize with LAMP1 (Figure 10 D) Like other pathogenic Neisseriae, GCM740 decreased the cellular content of LAMP1. In contrast, in 30 fields of infected cells examined, >85% of intracellular GCM740Δ4 colocalized with LAMP1 (Figure 10E). Furthermore, in these infected cells, LAMP1 levels were similar to those in uninfected cells.

Late endosomes and lysosomes are acid in pH and contain numerous acid hydrolases. Alteration of these compartments is predicted to result in an environment more conducive to bacterial survival (see also Discussion). The intracellular growth rate of the GC *iga*⁻ mutant GCM740Δ4 was therefore assessed. A431 cells were infected with GCM740 and GCM740Δ4 for 12 hrs at which time the cultures were treated with Gentamicin (Gm) to kill extracellular bacteria. At 2-hr intervals after removal of Gm, the cells were washed and lysed and the lysates were plated for intracellular CFUs. Results show that the intracellular

growth rate of the *iga*⁻ mutant, GCM740Δ4, is dramatically decreased compared to its wild type GCM740 parent (Figure 11). The number of intracellular GCM740Δ4 decreased ~15-fold during the incubation time examined (10 hrs). In contrast, the number of intracellular GCM740 increased after an initial decrease. Both strains have identical growth rates in vitro (Shoberg and Mulks, 1991) and adhere to and invade A431 cells at the same frequency (data not shown). The decreased cell counts of GCM740Δ4 shown here therefore represent a decrease in its ability to replicate within cells. At the 0 hr time point (termination of Gm treatment), the actual intracellular CFUs for GCM740 is ~6-fold greater than that for GCM740Δ4. This difference is due to the fact that at this time, the cells have already been infected for 12 hrs and therefore is expected to yield fewer viable GCM740Δ4. The increase in GCM740 CFUs was not due to intracellular bacteria escaping into and subsequently growing in the supernate, as plate counts of supernates at each time point yielded no detectable CFUs. The initial decrease in intracellular GCM740 has been observed by us in other experiments (unpublished data). Taken together our results indicate that IgA1 protease-mediated LAMP1 turnover is important for the intracellular survival of the pathogenic Neisseriae.

Dead MC and nonpathogenic Neisseria are found within LAMP1 containing compartments

MC, after infecting A431 cells, were killed by gentamicin treatment to see whether the transiant colocalization will persist. First, Gentamicin (Gm) was assessed for its ability to kill Neisseria in infected A431 cells. Gm, considered a membrane-impermeant antibiotic, has been used widely in adhesion/invasion assays to kill extracellular bacteria including GC

(Waldbeser et al., 1994). Recently, Gm was shown to kill intracellular *Listeria* when infected cells were incubated for a sufficient length of time with a high enough concentration of the antibiotic (Drevets, Canono, 1994). The Gm killing curve for cell-associated MC was determined. Results indicate that incubation of infected A431 cells with 150 mg/ml Gm for 15 min led to a rapid reduction in the number of live cell-associated bacteria and prolonged (>1 hr) incubation resulted in nearly complete killing of bacteria in the culture (data not shown).

A431 cells were infected with MC 8013.6 for 22 hrs, a time when the effects of MC on LAMP1 were most dramatic. Infected cells were treated with 150 mg/ml Gm for 3 hrs, washed extensively and processed for immunofluorescence microscopy. Cells were stained for bacteria and LAMP1 and examined by confocal microscopy. In these experiments, samples of Gm-treated cells were also plated to monitor efficiency of killing of bacteria. 20-30 fields containing infected cells from each of 6 slides were scored for the relative locations of bacteria and LAMP1. MC were found to colocalize with LAMP1 in >90% of infected cells treated with Gm. In contrast, MC rarely were found within LAMP1 containing compartments in untreated cells. Moreover, many bacteria within LAMP1 compartments had a degraded appearance (Figure 10A). Gm treatment of infected cells resulted in >5 log decrease in CFUs (colony forming units), indicating that the bacteria observed within these compartments are indeed dead. In Gm-treated cells the degree of LAMP1 staining returned to normal within 6 hrs after antibiotic treatment. In contrast, infected A431 cells not treated with Gm contained very few LAMP1 compartments. These results indicate that bacterial interactions with

LAMP1 require live bacteria and that infection does not cause irreversible damage to cells over the time period examined (22 hrs).

As a control, a nonpathogenic Neisseria strain *N.perflava*, was used to infect A431 the same way as the infection using MC. Infected samples were fixed and labeled with a-N.perflava and a-LAMP1 and examined by laser scanning confocal microscopy (Fig. 10B and C). Results showed that the nonpathogenic Neisseria were found within LAMP1 containing compartments at high frequency (>80%) and they also had a degraded appearance. These results suggest that dead MC and nonpathogenic Neisseriae enter the LAMP1 containing compartment and reside in these compartment; eventually, these bacteria were degraded.

Discussion

We have presented evidence that Neisseria infection of epithelial cells leads to a reduction in three late endosomal/lysosomal markers: LAMP1, CD63 and AP. The decrease in LAMP1 level is due to increased turnover of this glycoprotein. LAMP1 contains an IgA1-like hinge region with potential cleavage sites for the Neisserial type 1 and type 2 IgA1 proteases. Purified Neisserial type 2 IgA1 protease cleaves LAMP1 at the hinge region in vitro; a genetically-defined *iga*-mutant does not reduce the amount of LAMP1 in infected cells and its intracellular growth rate is dramatically decreased compared to its protease-producing parent strain. These data indicate that the IgA1 protease is important for the survival of the pathogenic Neisseria within epithelial cells.

Our infection studies clearly show that the protease has access to LAMP1 compartments. It could gain access by three pathways. The existence of compartments containing bacteria and intact LAMP1 indicates that the lysosomal marker can be delivered to bacteria-containing phagosomes. Therefore, we favor a model in which the protease is delivered by bacteria entering LAMP1 compartments. A plausible second pathway involves endocytosis. IgA1 protease is a secreted enzyme and is produced in large amounts by the pathogenic Neisseriae. IgA1 protease secreted by bacteria adhering to the cell surface could be endocytosed and delivered to late endosomes and lysosomes by vesicle fusion. Results show that Neisseria infected cells can undergo bulk flow and receptor mediated endocytosis and that endocytosed material can be delivered to LAMP1 compartments. Finally, LAMP1 may be cleaved by IgA1 protease at the cell surface. The majority of newly synthesized LAMP1 is transported from the Golgi directly to late

endosomes and lysosomes (Carlsson and fukuda, 1992). A small portion, however, is first transported to the plasma membrane before they are internalized and delivered to lysosomes (Carlsson and fukuda, 1992). The hinge region of surface-located LAMP1 is situated on the extracelluar side of the plasma membrane and therefore is accessible to IgA1 protease secreted by adherent bacteria. It is important to note that the later two methods of delivery do not require bacteria to be inside the cell. These pathways would exert a very localized effect, as uninfected cells in the proximity of an infected cell appeared to have normal LAMP1 levels (data not shown). Future studies should determine which pathway(s) is important for transporting IgA1 protease to LAMP1. It is interesting to note that in vitro IgA protease can cleave LAMP1 at the pH found in all three subcellular sites (pH 7.5, 6.5 and 5.0).

The reduction of three lysosomal markers in infected cells strongly suggest that LAMP1 compartments are modified. Whether the modification is a direct result of increased LAMP1 turnover is unclear. Lysosomes contain acid hydrolases that function in the digestion of a wide variety of macromolecules and are maintained at low pH by the vacuolar proton-ATP pump. The mildly acidic late endosomes also contain LAMP1 and many hydrolases. LAMP1 is thought to play an important role in maintaining the stability of these compartments. Increased LAMP1 turnover could set into motion a series of events that ultimately would promote bacterial survival: a decrease in the integrity of the membranes of late endosomes and lysosomes, perhaps enough to lead to their disintegration; leakage of the contents of these compartments into the cytoplasm; an increase in pH in the compartments and attenuation or inactivation of the acid hydrolases. Modified membranes could account for the contradictory reports regarding whether intracellular Neisseriae are surrounded

by a membrane. Reduced LAMP1 levels could also result in a decrease in the number of late endosomes and lysosomes in a cell.

All pathogenic Neisseriae produce one of two types of IgA1 proteases with slightly different specificities for the IgA1 hinge region. Invasiveness of the isolate is correlated with type 1 enzyme production (Mulks and Knapp, 1987). It is interesting to note that LAMP1 contains five potential type 1 cleavage sites and one type 2 site. Further studies should establish whether the type 1 IgA1 protease also plays a role in LAMP1 turnover.

We have shown that the IgA1 protease is important for the ability of the pathogenic Neisseriae to survive within epithelial cells. It will be interesting to determine whether these proteases also play a role in bacterial survival within other cell types, including phagocytes. Several other mucosal pathogens also produce IgA1 proteases. *Haemophilus influenzae, Ureaplasma urealyticum, Clostridium ramosum, Bacteriodes melaninogenicus* and several Streptococcal species (reviewed by Mulks and Shoberg, 1994). The exact function of these proteases is unknown. Recently, Hap, a *H. influenzae* protein with a region homologous to the catalytic domain of IgA1 protease, was shown to promote bacterial adherence and invasion *in vitro* (St. Geme et al., 1994). It will be interesting to determine whether these bacterial IgA1 proteases play a similar role in the intracellular survival of these pathogens.

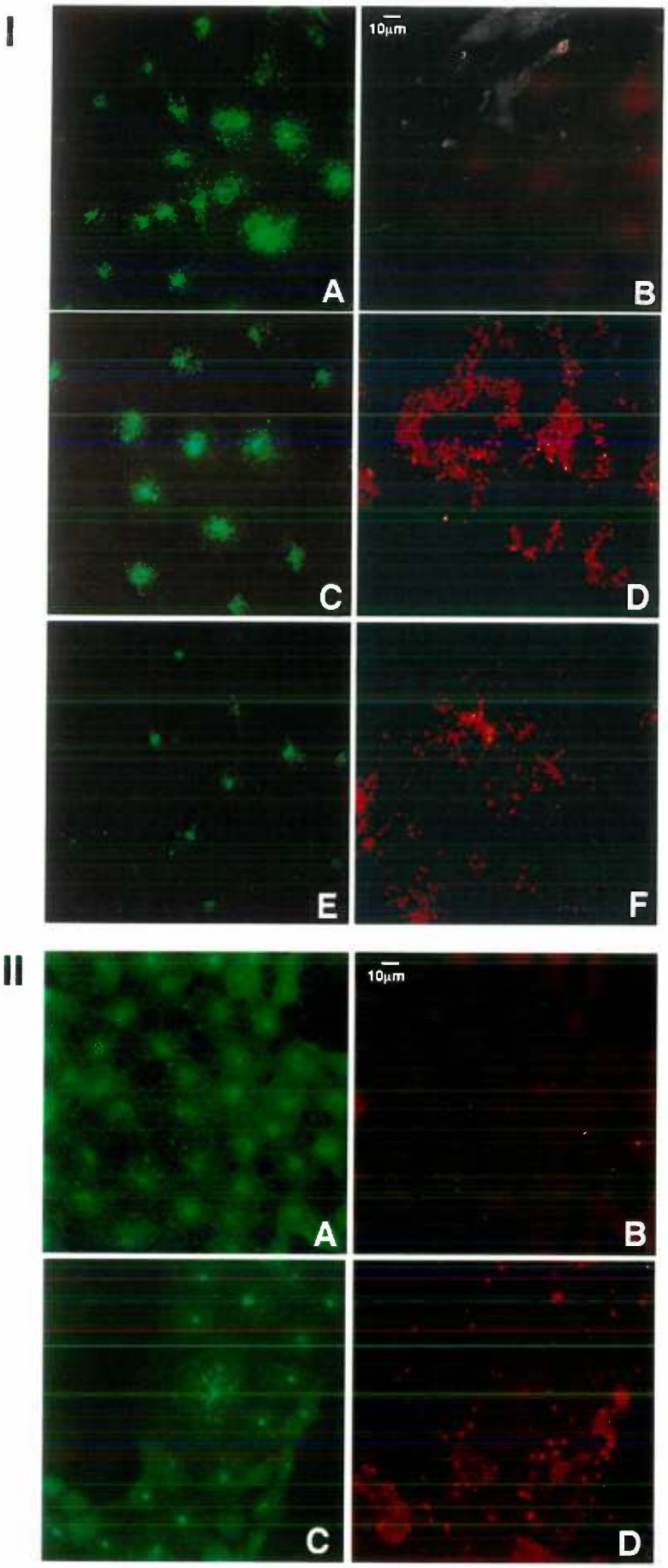

Figure 1. Double immunofluorescence microscopy of MC-infected A431 cells. Panel I: A431 cells were infected with MC strain 8013.6 for 8 hrs (C and D) or 22 hrs (E and F), stained with 1^{o} rabbit anti-MC ab and anti-LAMP1 Mab, and 2^{o} TRITC-conjugated goat anti-rabbit and BODIPY-conjugated goat anti-mouse abs. A, C, and E: LAMP1 staining; B, D and F: MC staining. (A and B): Uninfected cells.

Panel II: Uninfected A431 cells (A and B) and cells infected with MC strain 8013.6 for 12 hrs (C and D) were stained with 1^{o} rabbit anti-MC ab and anti-CD63 Mab, and 2^{o} TRITC-conjugated goat anti-rabbit and BODIPY-conjugated goat anti-mouse abs. A and C: CD63 staining; B and D: MC staining.

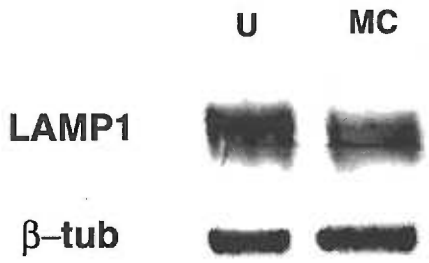

Figure 2. LAMP1 levels in uninfected (U) A431 cells and cells infected with MC strain 8013.6 (MC). Equal amounts of total cell proteins were separated by SDS-PAGE and probed with anti-β-tubulin or anti-LAMP1 Mabs and autoradiographed.

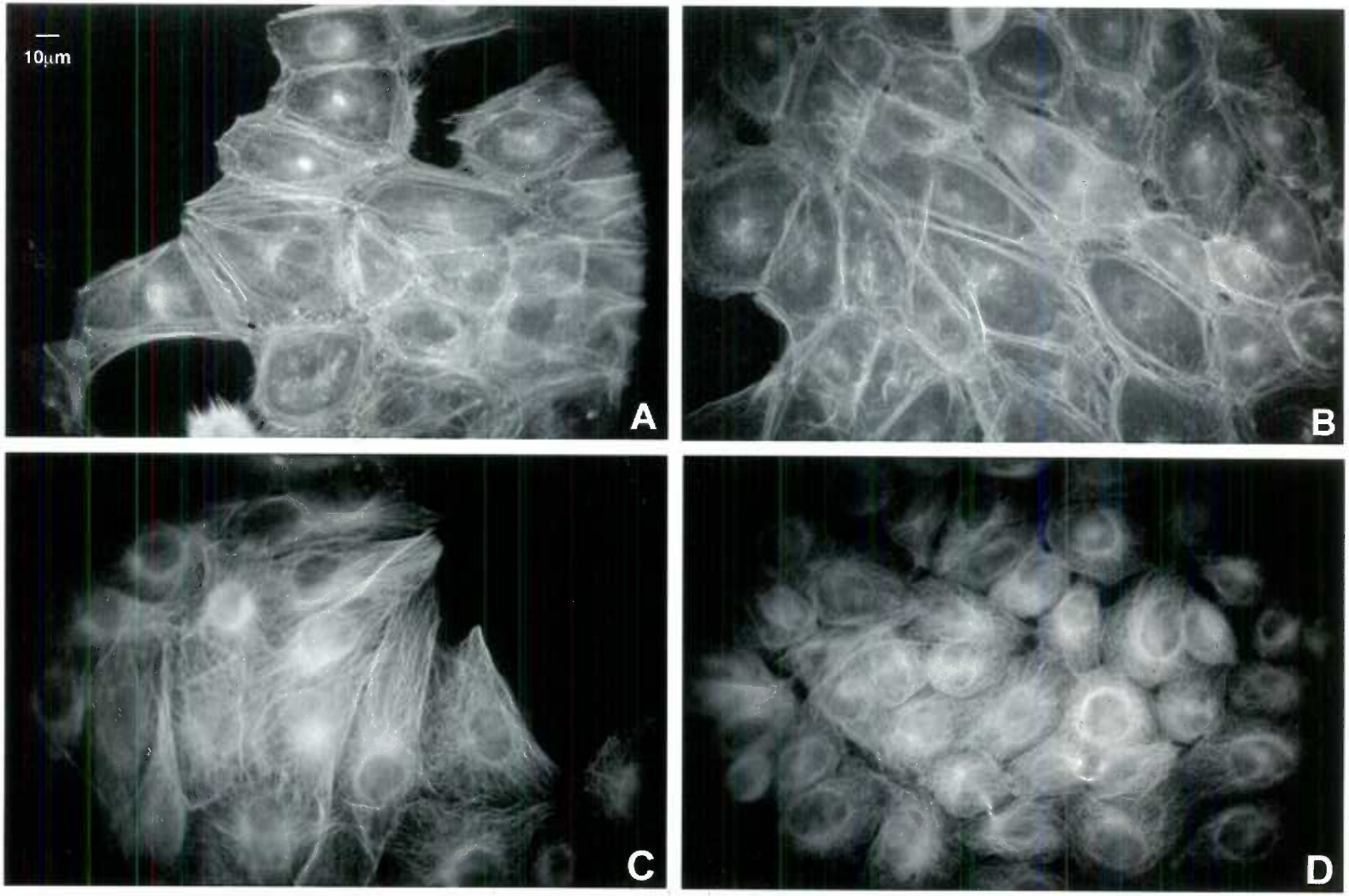

Figure 3. Fluorescence microscopy of cytoskeletal networks in uninfected (B and D) or 22 hr MC infected (A and C) A431 cells. Cells were stained for actin with phalloidin (A and B) or for tubulin with 1^{o} anti-β-tubulin Mab and 2^{o} BODIPY-conjugated goat anti-mouse ab (C and D).

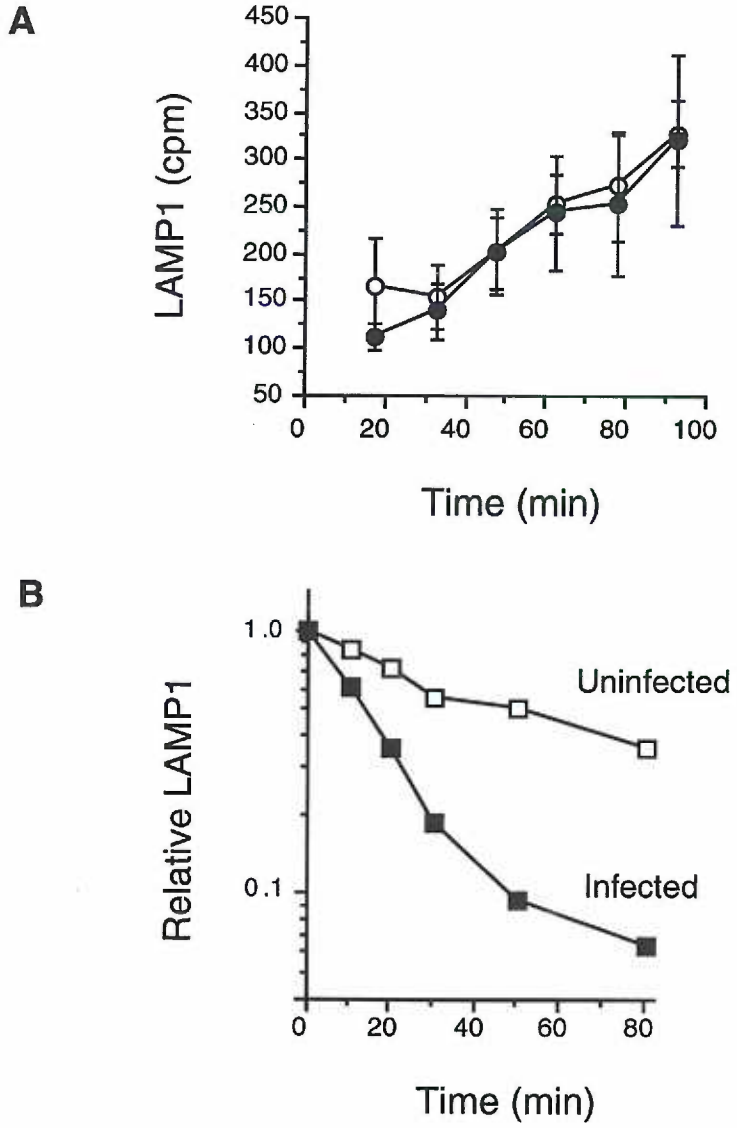

Figure 4. LAMP1 synthesis (A) and turnover (B) in uninfected and MC-infected A431 cells. To determine the effect of infection on LAMP1 biosynthesis, roughly equal numbers of infected cells (infected for 22 hrs, closed circles) and uninfected cells (open circles) were labelled with 35S-methionine for various lengths of time, lysed, and the lysates were immunoprecipitated using the anti-LAMP1 Mab and counted. To determine LAMP1 turnover, approximately equal numbers of infected cells (infected for 22 hrs, closed squares) and uninfected cells were labelled with 35S-methionine for 90 min, chased for the indicated lengths of time, lysed and the lysates immunoprecipitated using the anti-LAMP1 Mab. Equal amounts of each precipitate were separated by SDS-PAGE and autoradiographed. Signals from the gel were quantitated in the PhosphorImager PSI-Mac and the NIH image system V 1.57 program.

Table 1: Relevant characteristics of *Neisseria* strains used in this study.

Strains	Relevant Characteristics					
	Pili	Opa	Opc	Capsule	IgA1 protease type	LAMP1 decrease[1]
MC 8013.6	+[2]	ND	-[3]	Gp C	type 2	+
MC 7972	+[4]	ND	ND	Gp C	type 1	+
MC 8064	+[4]	ND	ND	Gp C	ND	+
MC 130.77	ND	ND	ND	Gp A	ND	+
GC MS11A	+[2]	-	-	-	type 2	+
GC MS11A-300[5]	-	-	-	-	type 2	+
GC MS11A-14.1[6]	+	-	-	-	type 2	+
GC 15063G	+[4]	+[7]	-	-	ND	+
GCM740	+[4]	-	-	-	type 2	+
GCM740Δ4	+[4]	-	-	-	-	-
N. perflava	-	ND	ND	-	-	-
N. polysaccharea	-	ND	ND	-	-	-

1 Colocalization determined at 22 hrs post infection

2 *pilE* sequences in 8013.6 and MS11A differ (Meyer et al., 1982; Nassif et al., 1993).

3 *opc* gene is absent from this strain (X. Nassif, personal comm.).

4 *pilE* sequence not determined.

5 Nonpiliated derivative of MS11A; *pilE1::Erm*; Δ*pilE2* (Merz et al., in press).

6 *opaH*; invasion frequency decreased ~6 fold (Waldbeser et al., 1994).

7 Opa profile not characterized.

ND: Not determined

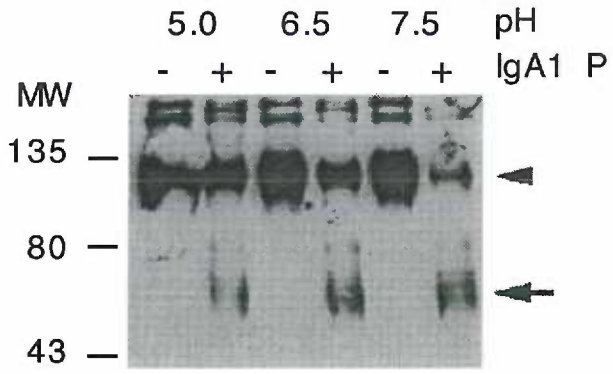

Figure 5. Cleavage of LAMP1 by purified type 2 IgA1 protease in vitro. Microtiter wells containing A431 cells were lysed in pH 6.5 IgA1 protease cleavage buffer containing 0.05% Triton X100 and 0.1 mM PMSF, with (+) or without (-) IgA1 protease. LAMP1 was detected by Western blotting using the anti-LAMP1 MAb (A) or the anti-LAMP1 polyclonal ab (B). The blots were stripped and reprobed with the anti-b-tubulin MAb.

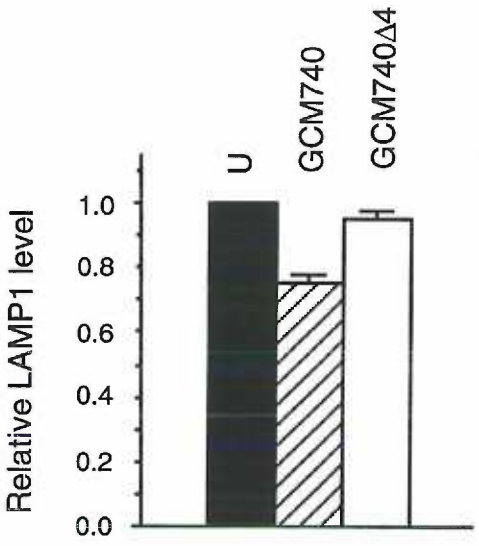

Figure 6. LAMP1 levels in uninfected (U) A431 cells and cells infected for 22 hrs with protease producing GC strain GCM740 and GCM740Δ4, its genetically defined *iga*- mutant. Equal amounts of total cell proteins were separated by SDS-PAGE and probed with anti-b-tubulin or anti-LAMP1 Mabs and autoradiographed. Signals from the blot were scanned and quantitated using the PhosphorImager PSI-Mac and the NIH image system V 1.57 program. LAMP1 values were normalized to their internal b-tubulin signals and values for GCM740 and GCM740D4 were expressed relative to the value for uninfected cells. Results represent the average of five independent experiments. Error bars represent standard deviation.

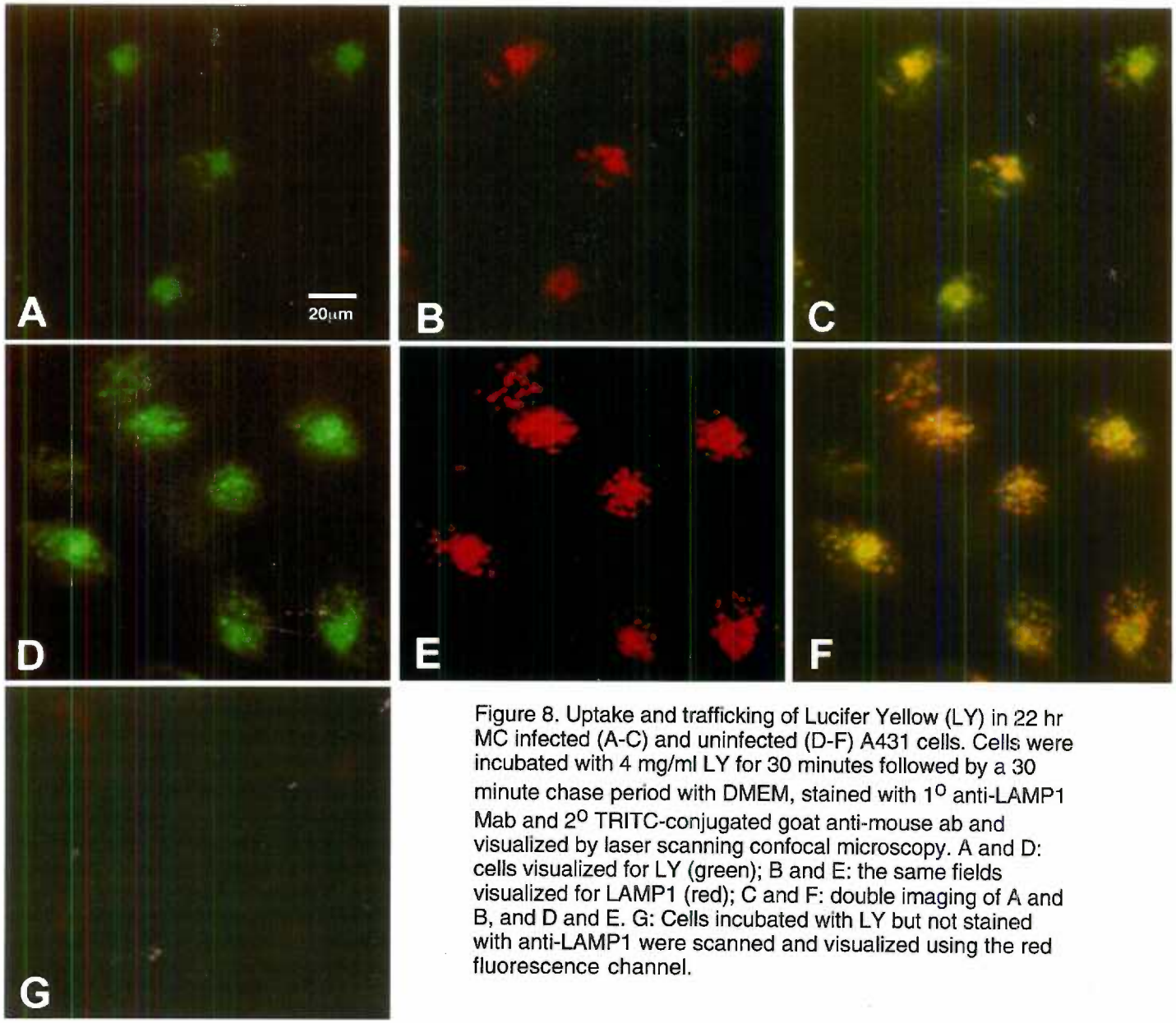

Figure 8. Uptake and trafficking of Lucifer Yellow (LY) in 22 hr MC infected (A-C) and uninfected (D-F) A431 cells. Cells were incubated with 4 mg/ml LY for 30 minutes followed by a 30 minute chase period with DMEM, stained with 1^{O} anti-LAMP1 Mab and 2^{O} TRITC-conjugated goat anti-mouse ab and visualized by laser scanning confocal microscopy. A and D: cells visualized for LY (green); B and E: the same fields visualized for LAMP1 (red); C and F: double imaging of A and B, and D and E. G: Cells incubated with LY but not stained with anti-LAMP1 were scanned and visualized using the red fluorescence channel.

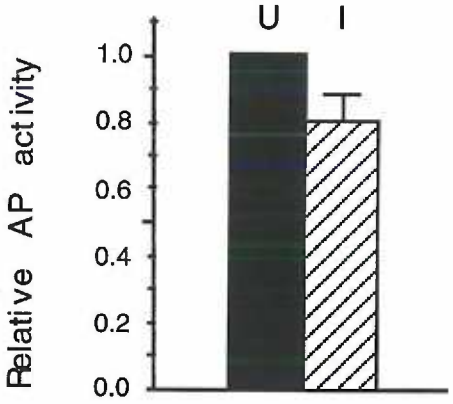

Figure 9. Relative acid phosphatase (AP) activity in uninfected (U; black bar)A431 cells or cells infected with MC strain 8013.6 for 22 hr (I; shaded bar). AP activity for infected cells were normalized to the uninfected control. The data shown above represent the average of four independent experiments. Error bars represent standard deviation.

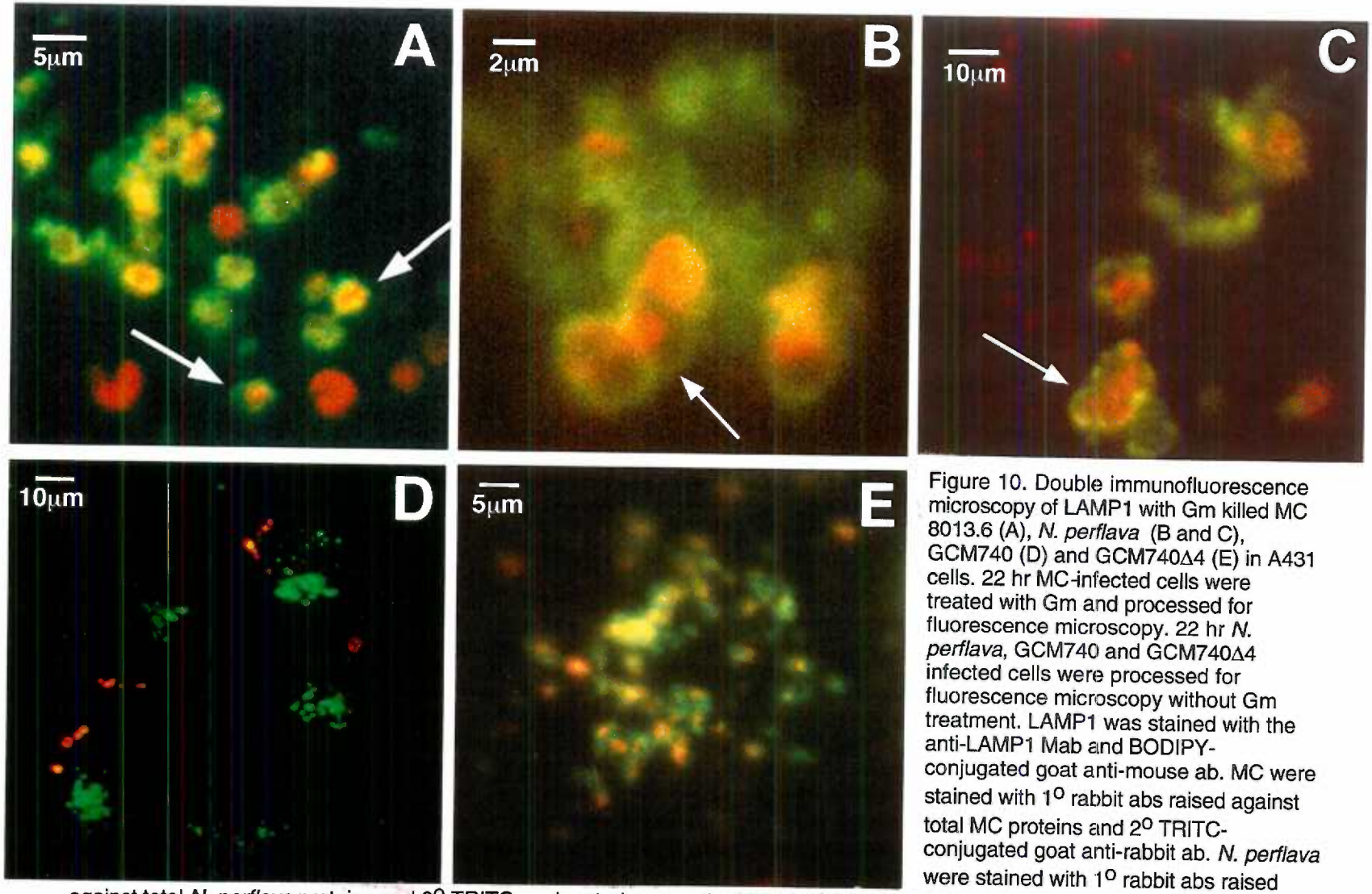

Figure 10. Double immunofluorescence microscopy of LAMP1 with Gm killed MC 8013.6 (A), *N. perflava* (B and C), GCM740 (D) and GCM740Δ4 (E) in A431 cells. 22 hr MC-infected cells were treated with Gm and processed for fluorescence microscopy. 22 hr *N. perflava*, GCM740 and GCM740Δ4 infected cells were processed for fluorescence microscopy without Gm treatment. LAMP1 was stained with the anti-LAMP1 Mab and BODIPY-conjugated goat anti-mouse ab. MC were stained with 1^{o} rabbit abs raised against total MC proteins and 2^{o} TRITC-conjugated goat anti-rabbit ab. *N. perflava* were stained with 1^{o} rabbit abs raised against total *N. perflava* proteins and 2^{o} TRITC-conjugated goat anti-rabbit ab. GCM740 and GCM740Δ4 were stained with 1^{o} rabbit abs raised against total GC strain MS11A abs and 2^{o} Texas Red-conjugated goat anti-rabbit ab. Slides were examined by laser scanning confocal microscopy.

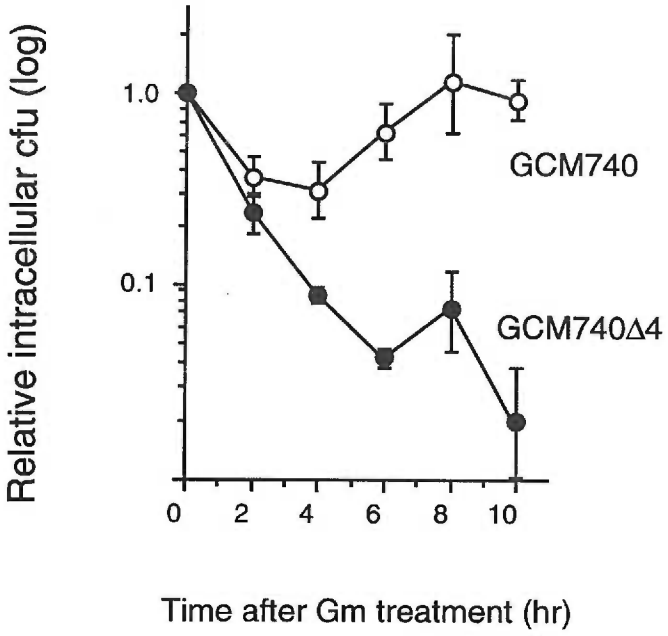

Figure 11. Intracellular growth of GCM740 (WT) and GCM740Δ4 (iga-). A431 cells were infected for 12 hrs with GCM740 (open circles) and GCM740Δ4 (closed circles) at an MOI of 5 then treated with Gm to kill extracellular bacteria. Cells were then reincubated with fresh DMEM without Gm. At various times cells were lysed and lysates were plated for intracellular CFUs. Numbers of viable intracellular bacteria were normalized to the 0-hr value. Note that at 0-hr, the cultures have been infected for 12 hrs and treated with Gm for 90 min. Each point is the average from four independent experiments. Time points for each experiment were done in duplicate. Error bars represent standard deviation; for some time points the standard deviation may be too small to be visible.

Acknowledgements

We wish to thank Alex Merz for innumerable hours of thoughtful discussions, J. Engstrom for her help with the Laser Scanning Confocal Microscopy studies and Michael Moody for his help in digital imaging of confocal micrographs. The anti-LAMP1 polyclonal ab was a generous gift from M. Fukuda. The H4A3 (anti-LAMP1), H5C6 (anti-CD63), and E7 (anti-b-tubulin) monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by the department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD. This work was supported by NIH grant AI32493 awarded to M. So.

Experimental procedures

Cell culture

A431 (obtained from S. Schmid) is a human endocervical epithelial cell line. HEC-1-B (obtained from ATCC) is a human epithelial cell line derived from an adenocarcinoma of the endocervix. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% heat inactivated fetal bovine serum (FBS, Gibco). Cells were used for bacterial infection experiments between the 5th and 20th passages.

Bacterial strains

Information for all strains used in this study are listed in Table 1. All pathogenic strains used in this study, except for GC MS11A-300, are piliated (P^+). *N. meningitidis* MC 130.77, serogroup A, was obtained from the ATCC. MC 7972, MC 8064 and MC 8013.6, belonging to serogroup C, were bloodstream isolates from patients from the Pasteur Hospital (provided by X. Nassif). MC 8013.6 is a capsulated, P^+ strain producing high adhesive pilin (Nassif et al., 1993); its Opa phenotype has not been determined.*N. gonorrhoeae* strain MS11A is P^+ and Opa^- (Segal et al., 1986); 15063G is a highly invasive P^+, Opa^+ clinical isolate (Waldbeser et al., 1994); its Opa proteins have not be characterized. GCM740 is P^+, Opa^-. The construction and characterization of GCM740Δ4, its *iga*$^-$, P^+, Opa^- derivative, has been described in detail (Shoberg and Mulks, 1991). The *iga* mutation was generated by deleting 4.3kb of the 5' end of the *iga* gene, which removed ~93% of the *iga* coding sequence. GCM740Δ4 has no protease activity and the outermembrane protein profiles of GCM740 and GCM740Δ4 are identical. *N. perflava* and *N. polysaccharea* are commensal strains obtained from the Center for Disease Control, Atlanta, GA.

Infection and intracellular growth rate experiments

Bacteria were passed every 18-22 hours on GCB plates (Difco) with supplements (Kellogg et al., 1968) as previously described (Segal et al., 1986). All strains used for infection experiments have been passaged fewer than 10 times. A431 cells were seeded on 18 mm square coverslips at a density of 5-8 X 10^4 cells/well 24-36 hours prior to infection. Cells were washed with PBS, pH7.4, incubated in DMEM/5%FBS and infected with bacteria resuspended in the same medium. Unless otherwise stated, bacteria were added at a multiplicity of infection (MOI) of 10 bacteria/cell for infections less than 10 hours and an MOI of 1 for 22 hour infections. To determine intracellular growth rates of GCM740 and GCM740Δ4, A431 cells were seeded in 24-well plates (Falcon), grown to ~80% confluency, and infected with bacteria for 12 hrs at an MOI of 5. Cells were washed 4X with PBS, pH7.4 and incubated with 0.5 ml fresh DMEM/5%FBS containing 20mg/ml Gm for 90 min at 37°C in 5% CO2. Cultures were then washed 4X with PBS and incubated with fresh DMEM without Gm. At various times, 100 ml of the supernates were plated on supplemented GCB agar for extracellular CFUs. The cells were washed 4X with PBS, lysed with 0.5ml GCB containing 0.5% saponin (Aldrich) at RT for 15 min and the lysates were plated on supplemented GCB agar for intracellular CFUs.

Antibodies

Polyclonal rabbit anti-MC antibodies were raised against whole MC (L.Walbeser and A.Merz; this lab) or whole *N. perflava* (L.Lin; this lab). The H4A3 (anti human LAMP1), H5C6 (anti-CD63), and E7 (anti-ß-tubulin) monoclonal antibodies were obtained from the Developmental Studies

Hybridoma Bank; polyclonal antiserum to LAMP1 was a generous gift from M. Fukuda; BODIPY 581/591-Phalloidin, Lucifer Yellow and BODIPY goat-anti-mouse antibody were obtained from Molecular Probes; TRITC-goat-anti-rabbit antibody was purchased from Pierce; FITC-goat-anti-rabbit and TRITC-goat-anti-mouse antibodies were obtained from Sigma.

Immunofluorescence microscopy

Coverslips with uninfected or infected cells were fixed for 25 min in Zamboni's fixative (PAPF; Zamboni and De Martino, 1967) at RT. After three washes with PBS, samples were permeabilized and blocked in PBS with 3% goat serum, 0.01% azide, and 0.01% saponin (IFB). Primary antibodies were added at the appropriate dilutions and incubated for 3 hours at RT or overnight at 4°C. Cover slips were then washed 3X with PBS, blocked with IFB for 20 min and incubated with secondary antibodies at 1:200 dilution in the dark for 30 min. Cover slips were further washed 5X with PBS and mounted in mounting buffer (5mM Tris, pH8.0, 20 mg/ml n-propyl gallate, 90% glycerol). Stained cells were examined using a Nikon Microphot FX at the stated magnification, or a Leica Confocal Laser Scanning Microscope equipped with a Leitz Fluovert-FU inverted microscope and an argon/krypton laser (CLSM AR/KR Laser) with transmitted light detector DMIR, complete two-channel scanner and a 24-bit TCD System.

Fluid phase uptake of Lucifer Yellow

Uninfected and A431 cells infected with 8013.6 for 22 hrs were washed three times with PBS and incubated with fresh DMEM/5% FBS containing 4 mg/ml Fixable Lucifer Yellow-CH (LY, Molecular Probes) at 37°C in 5% CO2 for 30 min. Cells were washed and incubated 30 min with fresh medium without LY. Cells were washed 3X with PBS and fixed and labelled with anti-LAMP1 and

TRITC-goat-anti-mouse antibodies. Labelled cells were analyzed using a Leica inverted laser-scanning confocal microscope.

Quantitation of LAMP1 by Western blots

Cells were infected with bacteria as described above. Uninfected and infected cells were washed 3X with PBS, lysed with PBS/1% Triton-X100, and cell debris pelleted by centrifuged at 15,000 rpm for 15 min. The proteins in the supernate were separated in an 8% SDS acrylamide gel and transferred to nitrocellulose membrane (Current Protocols in Molecular Biology). The membrane was incubated with the anti-LAMP1 or anti-ß-tubulin monoclonal or the anti-LAMP1 rabbit polyclonal antiserum and developed using goat-anti-mouse or goat anti-rabbit alkaline phosphatase (Boehringer Mannheim) or sheep anti-mouse horseradish peroxidase (ECL Chemiluminescence Kit, Amersham). LAMP1 and ß-tubulin signals from the blots were scanned and quantitated using the NIH Image System V1.57 program. The LAMP1 value of each sample was normalized to the internal ß-tubulin value. Normalized LAMP1 values for infected samples were then expressed relative to the normalized LAMP1 value of the uninfected control.

Acid phosphatase cytochemistry

Cells were infected as described above, washed 3X with PBS, lysed with 1% Triton-X100 and the debris pelleted. Supernatants were assayed for acid phosphatase activity using the phospatase, acid, alkaline and prostatic acid kit (Sigma Diagnostics) as specified by the manufacturer.

LAMP1 synthesis and turnover

To examine LAMP1 biosynthetic rate, uninfected and infected cells were washed 3X in PBS and incubated in DMEM/5% FBS containing 200mCi/ml ^{35}S-Methionine and ^{35}S-Cysteine (Dupont) at 37°C in 5% CO2. At various times, the labelled cells were washed 3X with ice cold PBS and lysed by incubating in ice cold NET buffer (0.01 M Tris, pH7.4, 0.15M NaCl, 0.005 M EDTA) containing 1% Triton X-100 and 1mM PMSF for 5 minutes. Cell lysates were centrifuged at 15,000 rpm for 15 min and the supernatants were collected for LAMP1 immunoprecipitation. To immunoprecipitate LAMP1, supernatants were incubated with mouse monoclonal anti-LAMP1 at 4°C overnight on a shaker. The next day, samples were centrifuged at 15,000 rpm at 4°C, and the supernatants incubated with Protein A-conjugated Sepharose CL-4B (Sigma) for three hours at 4°C on a shaker. Samples were centrifuged at 15,000 rpm for 30 sec and the pellets were washed with washing buffer A (0.1 M Tris pH 8.0, 0.1 M NaCl, 1.0 mM PMSF, 2 mM EDTA pH8.0, 1% Triton) three times and washing buffer C (0.1 M Tris pH 8.0, 0.1 M NaCl) twice. The pellets were then resuspended in 30µl protein sample buffer. 5µl of each sample were counted in a Beckman (LS 5000 TD) liquid scintillation counter.

To examine the LAMP1 turnover rate, cells were infected for 22 hrs with bacteria, then washed with fresh medium. ^{35}S-Methionine (200mCi/ml) was added to the wells and cells incubated for 90 min further. Supernatants were removed and the cells were chased by incubation with label-free DMEM/5% FBS for various lengths of time. (The 0-hr time point in Figure 7B represents the beginning of the chase period.) Cells were lysed, immunoprecipitated with the anti-LAMP1 Mab as described above and the precipitates resuspended in 30 ml SDS-sample buffer. Equal volumes of each precipitate were separated by SDS-PAGE. The gels were processed for autoradiography and the signals on

the gels were quantitated using a PhosphorImager PSI-Mac (Molecular Dynamics) and analyzed using the NIH Image V1.57 program.

IgA1 protease cleavage of LAMP1

A431 cells were seeded into 6-well tissue culture plates at 2 x 10^5/well and grown for 24 hrs. Cells were washed 3X with PBS at RT and lysed directly in 0.8 ml IgA1 protease cleavage buffer (Mulks and Shoberg, 1994), pH6.5, containing 0.05% Triton X100 and 0.1 mM PMSF, with or without 1mg of IgA1 protease purified from an *E. coli* recombinant expressing the GC type2 *iga* gene (Boehringer Mannheim). The lysates were incubated at RT for 5 minutes. 25 ml of the reaction was added to 5 ml of protein sample buffer containing 1 mM DTT and boiled for 10 min . The samples were separated by SDS-PAGE and analyzed by Western blotting using the anti-LAMP1 MAb and the Western blotting kit from Pierce. The blot was subsequently stripped and reprobed with the anti-b-tubulin MAb.

Manuscript II:

A *Neisseria gonorrhoeae* gene involved in epithelial invasion has homology to the similar gene found in *P.denitrificans*, *H.influenzae*, and *E.coli*.

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Summary

Neisseria gonorrhoeae is a gram-negative diplococcus that normally adheres to and invades its human host via the mucosal epithelium of the urogenitial tract. We have identified a new GC gene, *nad1*, that is involved in promoting GC interaction with human epithelial cells *in vitro*. This gene was isolated from a gene bank constructed from a clinical isolate of GC,15063G, in *E.coli* HB101. The bank was enriched for clones that adhered to the human endometrial adenocarcinoma tissue culture line Hec-1-B. The insert of one of the adhesive isolates was subcloned in pBluescript. An *E.coli* strain containing a subclone of this insert adhered to Hec-1-B cells 15 fold better than *E.coli* strain containing vector alone. It also adhered to A431 cells 60 fold better than *E.coli* strain containing vector alone. Analysis of the predicted amino acid sequence of the cloned gene (*nad1*) revealed that it has homology to a predicted outermembrane protein in *Pseudomonas denitrificans*. *Escherichia coli* and *Hemophilus influenzae* also contain genes whose products are homologous to Nad1. A GC MS11A *nad1* mutant in a nonpiliated background, invaded Hec-1-B cells 70% less well than its parental strain. These results suggest that *nad1* plays a role in the invasion of nonpiliated GC to epithelium.

Introduction

The gram-negative diplococcus *Neisseria gonorrhoeae* is the causative agent of gonorrhea. Humans are the only host for this pathogen. In order to produce a successful infection, *Neisseria gonorrhoeae* must attach to and invade mucosal epithelial cells. Pili of GC are important for virulence; they play an essential role in mediating adhesion of GC to human cells. Piliated bacteria adhere to host epithelial cells 2-3 logs better than their P^- variant (this study, Nassif et al., 1994; Rudel et al., 1992; Verji et al., 1992; Verji and Kayhty et al., 1991; Verji and Saunders et al., 1993). Kellogg and his colleagues showed that non-piliated GC failed to infect human volunteers (Kellogg et al, 1968). PilC is a high molecular weight protein which is associated with the pilus fiber. At present, a controversy exists regarding the function of PilC in GC. One group has reported that PilC in GC plays a role in adhesion, and that PilC is located at the tip of the pilus (Rudel et al., 1995). A second group believes that PilC is involved in pilus assembly and that PilC is located on the surface of the bacterial membrane at the pilus base (Jonsson et al., 1991; Jonsson et al., 1992; Jonsson et al, 1995). In *Neisseria meningitidis* (MC), pilin antigenic variation modulates adhesion of MC to human epithelial cells (Nassif et al., 1993). PilC1 in MC functions in adhesion. In addition it also plays a role in pilus assembly. PilC2 plays a role in pilus assembly (Nassif et al, 1994). Although pili are essential for GC and MC to attach to the human host, nonpiliated (P^-) GC and P^- unencapsulated MC do attach to cells which indicates that there may be nonpilus adhesins that contribute to this phenotype.

There are nonpilus associated elements involved in neisserial interactions with human cells. Opa is one of them. Neisserial Opa proteins promote bacterial

interactions with host cells. In GC, certain Opa variants (OpaA, OpaB, OpaD) increase bacterial adherence to epithelial cells (Lambden et al., 1979; Swanson, 1979; Makino and Meyers, 1991). On the other hand, Opa variants OpaA (Kupsch et al., 1993) and OpaH (Walderbeser et al., 1994) promote invasion of the bacteria into the cells. Other Opa proteins such as OpaB (Naids and Rest, 1991) OpaE and Opal (Belland and Fischer, 1992) have the ability to bind to neutrophils. In MC, OpaB and Opc promote adherence to endothelial cells (Virji et al, 1993). Thus attachment to different host tissue cell types may require multiple adhesins.

Nonpilus adhesins have been identified in several other pathogenic strains. In nontypable *H. influenzae*, a family of nonpilus surface-exposed high molecular weight proteins HMW1 and HMW2 have been identified to contribute to the binding of the bacteria to the human epithelium (St. Geme et al., 1993). The F protein produced by the group A Streptococcus *S. pyogenes* binds to the fibronectin of epithelial cells from the human respiratory track (Hanski et al., 1992). Other nonpilus adhesins are filamentous hemagglutinin from *Bordetella pertussis* (Sato. et al.,1983) and mannose-resistant hemagglutinin from *Salmonella typhimurium* (Halula et al.,1987). In addition, Yad A from *Y. pseudotuberculossis* was found to mediate intimate bacterial attachment to and entry into Hep-2 cells (Bliska. et al.,1993). The *inv* genes in Yersinia and Salmonella and the *ipa* genes in Shigella encode proteins which are involved in these invasion pathogens entry of human host.

Although pili and Opa play a major role in Neisseria adhesion, a fraction of P^-, $PilC^-$ and Opa^- bacteria adhere to human epithelial cells as well. This suggests that there are proteins besides pili and Opa which contribute to adhesion and/or

invasion to epithelial cells. The purpose of our study is to identify GC genes whose products contribute to the interaction of the bacteria with human epithelial cells. To do this, we generated a gene bank from GC 15063G, a clinical isolate, in *E.coli*. The bank was enriched for clones that adhered to the human endometrial adenocarcinoma tissue culture line Hec-1-B, a human endometrial cell line commonly used to study Neisseria/host interactions. Eight "adhesive" clones were isolated. Seven of them overlapped and the characterization of these clones have been reported (Waldbeser et al., 1994). Here we characterized the eighth clone (G2) which had a unique restriction pattern. We identified a gene, named *nad1*, in the G2, which when expressed in E.coli, allowed the E.coli to adhere to Hec-1-B cells 15 fold better than the E.coli harboring vector alone. The E.coli strain containing this gene also adhere to A431 cells (a human epidermoid carcinoma cell line) 60 fold better than the E.coli control. Sequence analysis of Nad1 revealed that it has significant homology to a predicted outermembrane protein in *Pseudomonas denitrificans Escherichia coli* and *Hemophelus influenzae* also have genes whose predicted amino acid sequence show homology to Nad1. A *nad1* mutant of GC strain MS11A, in a P^- background, invaded Hec-1-B cells 70% less than its parental strain. These results suggest that *nad1* is involved in the invasion of nonpiliated GC of epithelial cells.

Results

Adhesion Assay of G2

The 15063G gene bank was constructed in the low copy number cosmid vector pCP19 (Darzins and Chakrabarty,1984). The bank was enriched for adhesion to Hec-1-B cells as previously described (Waldbeser et al., 1994). After enrichment, eight independent colonies were isolated. Among them, seven were found to contain overlapping inserts; their characterization have been reported(Waldbeser et al., 1994). The clone with a unique restriction pattern, named G2, was further characterized for adhesion.

Approximately 10^6 bacteria were incubated with a nearly confluent monolayer of Hec-IB cells for 1 hrs at 37°C in 5% CO2, at which time the ratio of cell associated to total colony forming units (cfu) was determined and compared to a strain harboring the vector (pCP19) alone. In these assays (repeated 30 times), adherence of G2 to Hec-1-B cells was consistently 2 fold higher than the control vector (Fig.1A). A t-test with p value of 0.05 showed that the difference in the adherence between G2 and vector control was significant. These data suggest that G2 contains an "adhesin" gene which allowed the *E. coli* to adhere to epithelial cells.

Subcloning of G2

The insert in G2 was mapped for ClaI and EcoRI sites (Fig. 2). Overlapping ClaI fragments were subcloned into a low copy number vector pACYC177. These subclones were named G2Y1-G2Y20. Subcloning these ClaI fragments into a high copy number vector was not successful. However, the 2.0 kb EcoRI

fragment was successfully cloned into a high copy number vector pBluescript. This subclone was named as G2B5 (Fig2).

Adhesion assays of G2 subclones

Adhesion assays were performed on the subclone as mentioned in Methods. Among these subclones, G2Y15 and G2Y17 adhered to Hec-1-B cells 2 fold better than vector control (data not shown). In contrast, G2B5 adhered to Hec-1-B cells 15 times better than vector control and to A431 cells 60 times better than the vector control (Fig. 1B). However, Both G2B5 and its vector control adhered to Hec-1-B cells 10 times better than A431 cells (data not shown). These results suggested that the "adhesin" resides in the 2.0 kb EcoRI fragment of the G2 insert (Fig.2).

There are two explanations for the difference in adhesion frequency between G2B5 and G2Y17. One is the copy number effect. G2B5 is in a high copy number vector while G2Y17 is in a low copy number vector. Thus more "adhesin" may be produced in G2B5 than in G2Y17. Alternatively, G2B5 may contain a full length "adhesin" sequence while G2Y17 may be missing a portion of the coding sequence (Fig.2).

Sequence Analysis of G2B5

The 2.0 kb EcoRI fragment from G2B5 was sequenced (Fig.3) and found to contain one major ORF (open reading frame) of 876 bp. A well-conserved ribosome-binding site (rbs) was present 8 bp upstream of the first ATG codon in this ORF. A well conserved -10 promoter sequence is present 42 bp upstream of the rbs. A well conserved -35 region is also present. This ORF was designated *nad1* (Neisserial adhesin 1). A Blast search of *nad1* revealed

significant homology to *P.denitrificans* cob operon orf9, a gene encoding a hypothetical protein (HI0198) in *H.influenzae* , and to a gene encoding at the 3' of *mepA* region of *E.coli*.

The deduced amino acid sequence of Nad1 was aligned with those from *P. denitrificans*, *H. influenzae* and *E. coli* Nad1 homologous sequences (Fig.4). Nad1 has 50% sequence identity and 70% similarity with the hypothetical protein encoded by the *P. denitrificans* cob operon orf9; 42% identity and 62% similarity with the hypothetical protein from the *E. coli mepA* region and 39% identity and 52% similarity with the hypothetical protein from H. *influenzae* (Fig.4). There are two highly conserved domains at amino acid 8-80 and 150-182. Further analysis of Nad1 using the ISREC-Server TMpred program revealed that Nad1 may have 6 transmembrane domains and that the N-terminus is in the cytoplasm. These predicted transmembrane domains are indicated by the boxes (Fig.4). The same predicted transmembrane domains were described for the hypothetical protein in *P.denitrificans* (Crouzet. et al,1991). Interestingly, one of the three predicted extracellular domains is well conserved among the four proteins (27-80). At present, the functions of these hypothetical proteins are not known.

Generation of *nad1* mutants

In order to determine the function of Nad1 in GC interaction with epithelial cells, a *nad1* mutant MS11A was generated. The *nad1* gene was mutated by transposon mutagenesis using mTnErm (Seifert et al, 1986). The mTnErm had inserted into the region encoding amino acid 115-200. These mutants, all piliated (P^+), were assayed for their ability to adhere to or invade Hec-1-B cells. In these experiments, no significant difference in adhesion or invasion

frequency was found between the mutant strain and its wild type parent strain (data not shown).

A chloramphenicol antibiotic cassette (1.2 kb) from plasmid pCMXX was inserted into the MunI site in the 5' coding region of *nad1* in G2Y17 to yield G2Y17 *nad1*::Cm^r (Fig.2). The insertion interrupted the coding sequence for the conserved predicted extracellular region of Nad1. The mutated DNA was used to disrupt the gene in MS11A by DNA transformation/allelic exchange. Southern blots of the mutant GC chromosomes confirmed that G2Y17 *nad1*::Cm^r had recombined correctly with its homologue on the chromosome and that there were no wild type copies of the *nad1* gene in these mutants (data not shown).

Constructing Gonococcal *nad1* mutants in a P$^-$ background

The pilus plays an essential role in adhesion of GC to epithelial cells. P$^+$ variants adhere to epithelial cells 2-3 logs better than P$^-$ variants. If Nad1 serves as a minor adhesin in GC, its contribution to adhesion may be more noticeable in a P$^-$ background. A *nad1* mutant in a P$^-$ background was generated next. The G2Y17 *nad1*::Cm^r DNA was transformed into MS11C3 which contains an intact *pilE1* (pilin expression site) but is deleted in *pilE2* (second pilin expression site). This transformant was next transformed with the plasmid pGL19 (Fig5A) which has a mutated *pilE1* locus. The final mutant was named MS11L3 which is P$^-$, *nad1*$^-$ (MS11L3 *pilE1*::Kan^r Δ*pilE2 nad1*::Cm^r). pGL19 DNA was also transformed into MS11C3 to yield MS11K3 (MS11K3 *pilE1*::Kan^r Δ*pilE2*). This strain contains a wild type *nad1* and serves as a positive control. DNA from plasmid G2Y17 *nad1*::Cm^r, GC chromosomes of MS11C3, and 16 independent transformants (MS11L3 mutants1-16) were digested with ClaI and southern blotted using the 1.0kb *nad1* ClaI fragment as

the probe (Fig.5B). Results indicate that all transformants now had the Cm^r cassette (1.2 kb) inserted into the 1.0 kb ClaI fragment containing the *nad1* gene. The new ClaI fragment is now 2.2 kb. This indicates the incoming mutant DNA has correctly recombined with the wild type homologue on the chromosome. The mutant MS11A has no WT *nad1* in the chromosome. Further more, *nad1* is not essential for GC survival as the mutant grows as well as its WT parent (data not shown).

Invasion of the GC *nad1* mutant

Adhesion/invasion assays were performed with MS11L3 (P^-, *nad1*$^-$) and MS11K3 (P^-, *Nad1*$^+$) to determine the role played by *nad1* in adhesion or invasion of human epithelial cells. Hec-1-B monolayers were infected with MS11L3 and MS11K3 at an MOI of 20 for 6 hours. Adherence was scored as cell-associated cfu/total inoculum cfu. Invasion was scored as gentamicin resistant cfu/cell-associated cfu. All values were then normalized to their wild type control. Results indicated that the mutant MS11L3 was not affected in its ability to adhere to Hec-1-B cells, although the variation in data between experiments was high (data not shown). However, the invasiveness of mutants was decreased 70% compared to the parent strain (Fig.6). All experiments were done in triplicate. Results presented here are the average from three independent experiments. The error bars represent the standard deviation of the mean (Fig. 6). Taken together, our results indicate that Nad1, in a P^- background, contributes to the invasiveness of GC for Hec-1-B cells.

Discussions:

To cause a successful infection, pathogenic Neisseriae must attach to epithelial cells and subsequently invade these cells. Pili and Opa have long been identified as major elements in the colonization process. However, a small fraction of P^- GC do attach to cells, indicating that bacterial components other than pili and Opa contribute to bacterial-cell interactions.

In order to identify GC gene(s) involved in attachment to and entry of human epithelial cells, we constructed a gene bank from a clinical GC isolate 15063G in *E. coli* and identified adhesive clones by passing the gene bank on Hec-1-B cells. Seven of the eight independently isolated clones contain a gene with strong homology to *opaH* in MS11A. When *opaH* was mutated in MS11A, the mutant invaded Hec-1-B cells 4.3 times less well than its wild type parent but adhered to Hec-1-B cells as well as the wild type parent. These results indicate that OpaH in MS11A is involved in epithelial cell invasion but not adhesion. Moreover, they suggest that GC adhesion and invasion are mechanistically separate events (Waldbeser. et al,1994).

The G2 encoded "adhesin", when subcloned into the high copy number vector pBluscript, adhered to human epithelial cells 15-60 times better than vector controls. This "adhesin" in G2, termed *nad1*, encodes a putative transmembrane protein. A GC *nad1* null mutant, in a P^- background, was decreased 70% in its ability to invade Hec-1-B cells. There was no difference in adhesion between the mutants and wild type GC. These support the earlier observations that adhesion and invasion are separate events in GC and that neisserial adherence and invasion are multifactorial events.

Interruption of *nad1* does not totally abolish GC invasion of epithelial cells. Two possible explanations are: first, there may be other genes in Neisseriae which serve the similar purpose as *nad1.* Mutating one of them would not therefore dramatically decrease the invasiveness of GC. Second, GC colonizes the mucosal lining at various locations in human body, eg. endocervix , urethra, conjunctiva. It has been known that tissue tropism can be governed by the GC variant Opa proteins. Thus it is possible that the effect of *nad1* mutation may be more dramatic if the adhesion/invasion assays are performed using different cell types.

Nad1 has significant homology to hypothetical proteins encoded by genes in *P. denitrificans*, *E. coli,* and *H. influenzae.* The hypothetical protein encoded by *P.denitrificans* cob operon orf9 is predicted to be a membrane protein and it was speculated that this protein may, together with the proteins encoded by orf6, orf7 and orf 8 in the same operon, serve as a transport system necessary for coenzyme B12 synthesis (Crouzet et al., 1991). The hypothetical protein in *E. coli* is at the 3' end of the *mepA* gene, which is the structural gene for the penicillin-insensitive murein endopeptidase. The functions of these hypothetical proteins are not known.

In summary, the data presented here revealed a new GC gene that contributes to bacterial interaction with host cells. It will be interesting to determine the molecular interactions that are mediated by Nad1 and to determine whether the similar genes found in the other bacterial species such as *P. denitrificans*, *H. influenzae*, and *E. coli* function in the same way.

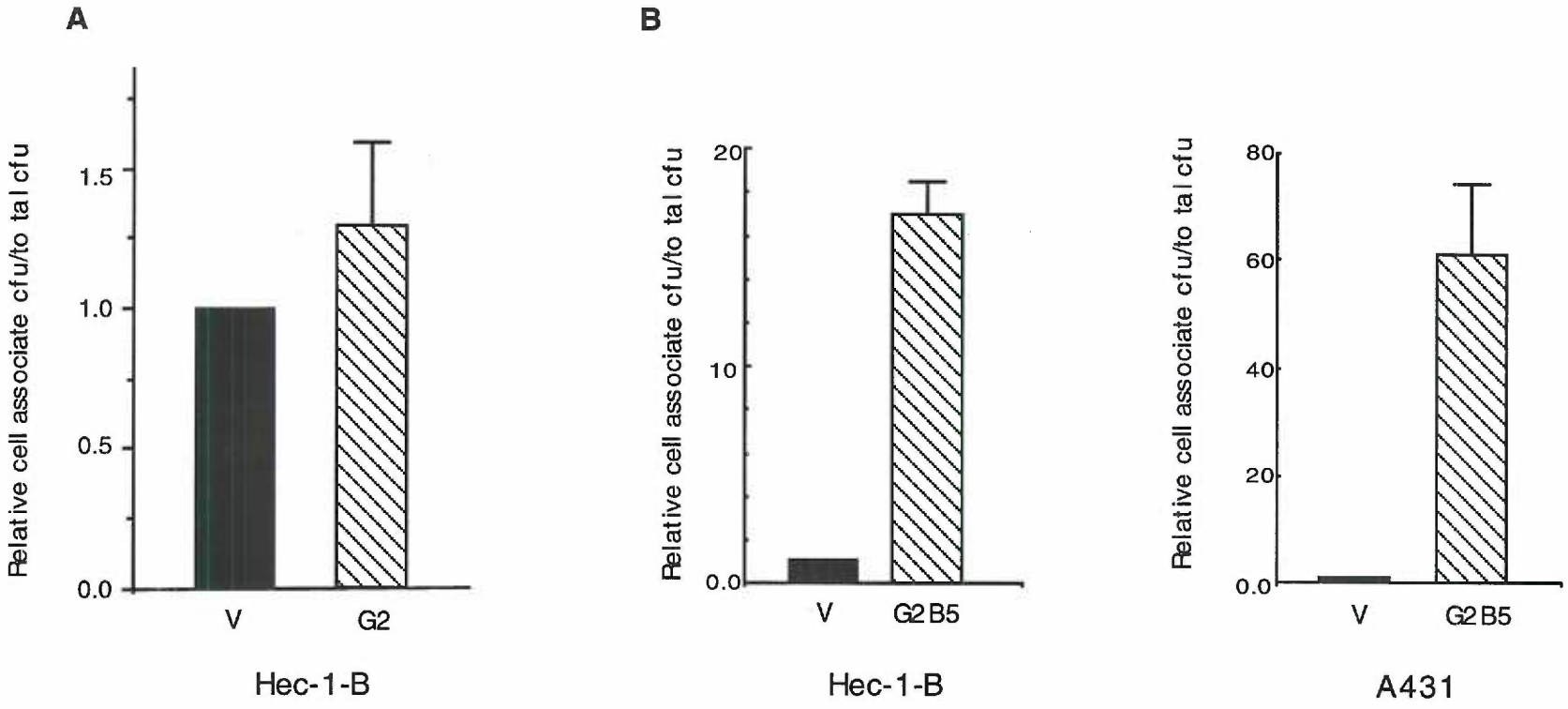


Figure 1: Adhesion to epithelial cells of G2 and its subclones relative to the vector control.
Ratios of cell-associated cfu to total cfu were normalized to the vector control.
A: Relative adhesion of G2. The data shown are the average of thirty independent assays, each performed in triplicate. Error bar represents standard deviations of the mean. A t test with a p value of 0.05 indicated that the difference between G2 and the vector control was significant.
B: Relative adherence of G2 subclone G2B5 to epithelial cells Hec-1-B and A431. The ratio of cell associated cfu / total cfu was normalized to that of vector control. The values represent the mean and standard deviation calculated from three independent experiments, each performed in triplicate.

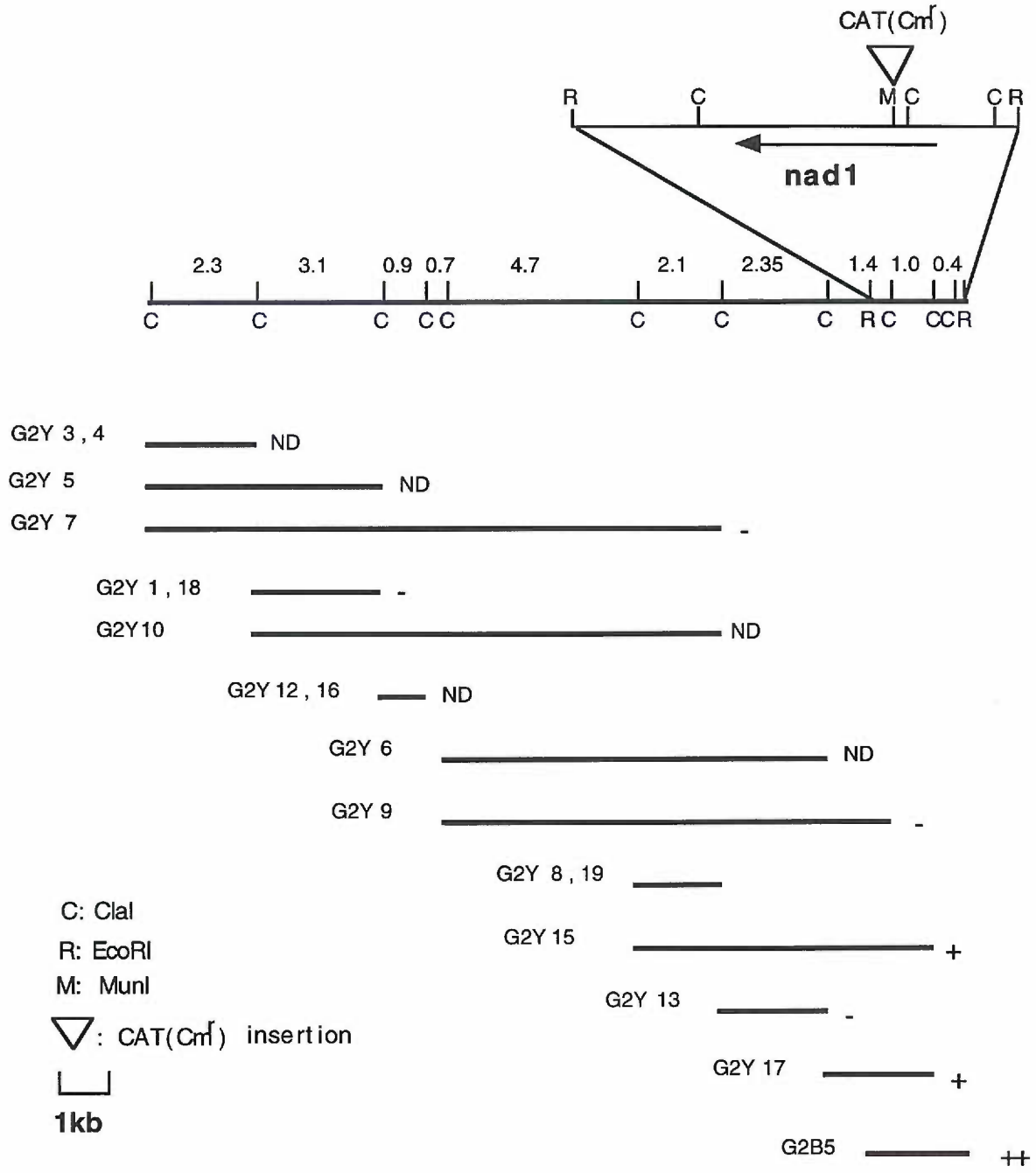
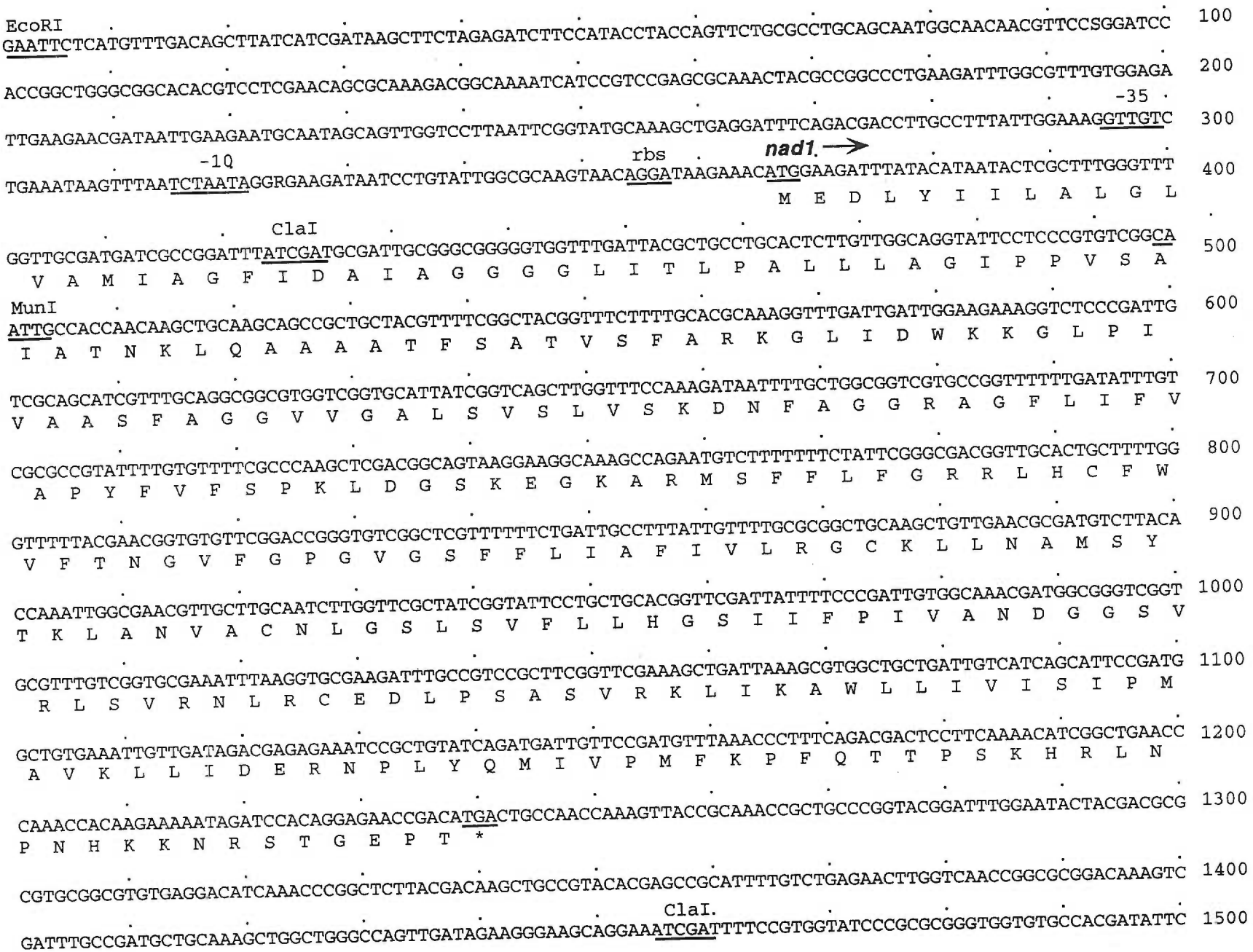


Figure 2: Restriction site map of G2 and G2 subclones.
(C): ClaI, (R): EcoRI, and (M): MunI. "+" indicates that the subclones adhered to epithelial cells better than their vector control. "-" indicates no differences in adhesion between the experim ental and control values. (ND): not assayed. The nad1 orf is indicated by the arrow. A Cm^r gene was inserted into the MunI site of G2Y17 to generate the nad1 mutant as indicated on the diagram.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ACCGGCTGGGCGGCACACGTCCTCGAACAGCGCAAAGACGGCAAAATCATCCGTCCGAGCGCAAACTACGCCGGCCCTGAAGATTTGGCGTTTGTGGAGA 200
-35
TTGAAGAACGATAATTGAAGAATGCAATAGCAGTTGGTCCTTAATTCGGTATGCAAAGCTGAGGATTTCAGACGACCTTGCCTTTATTGGAAAGGTTGTC 300
-10
rbs
nad1.
TGAAATAAGTTTAATCTAATAGGRGAAGATAATCCTGTATTGGCGCAAGTAACAGGATAAGAAACATGGAAGATTTATACATAATACTCGCTTTGGGTTT 400
M E D L Y I I L A L G L
ClaI
GGTTGCGATGATCGCCGGATTTATCGATGCGATTGCGGGCGGGGGTGGTTTGATTACGCTGCCTGCACTCTTGTTGGCAGGTATTCCTCCCGTGTCGGCA 500
V A M I A G F I D A I A G G G G L I T L P A L L L A G I P P V S A
MunI
ATTGCCACCAACAAGCTGCAAGCAGCCGCTGCTACGTTTTCGGCTACGGTTTCTTTTGCACGCAAAGGTTTGATTGATTGGAAGAAAGGTCTCCCGATTG 600
I A T N K L Q A A A A T F S A T V S F A R K G L I D W K K G L P I
TCGCAGCATCGTTTGCAGGCGGCGTGGTCGGTGCATTATCGGTCAGCTTGGTTTCCAAAGATAATTTTGCTGGCGGTCGTGCCGGTTTTTTGATATTTGT 700
V A A S F A G G V V G A L S V S L V S K D N F A G G R A G F L I F V
CGCGCCGTATTTTGTGTTTTCGCCCAAGCTCGACGGCAGTAAGGAAGGCAAAGCCAGAATGTCTTTTTTTCTATTCGGGCGACGGTTGCACTGCTTTTGG 800
A P Y F V F S P K L D G S K E G K A R M S F F L F G R R L H C F W
GTTTTTACGAACGGTGTGTTCGGACCGGGTGTCGGCTCGTTTTTTCTGATTGCCTTTATTGTTTTGCGCGGCTGCAAGCTGTTGAACGCGATGTCTTACA 900
V F T N G V F G P G V G S F F L I A F I V L R G C K L L N A M S Y
CCAAATTGGCGAACGTTGCTTGCAATCTTGGTTCGCTATCGGTATTCCTGCTGCACGGTTCGATTATTTTCCCGATTGTGGCAAACGATGGCGGGTCGGT 1000
T K L A N V A C N L G S L S V F L L H G S I I F P I V A N D G G S V
GCGTTTGTCGGTGCGAAATTTAAGGTGCGAAGATTTGCCGTCCGCTTCGGTTCGAAAGCTGATTAAAGCGTGGCTGCTGATTGTCATCAGCATTCCGATG 1100
R L S V R N L R C E D L P S A S V R K L I K A W L L I V I S I P M
GCTGTGAAATTGTTGATAGACGAGAGAAATCCGCTGTATCAGATGATTGTTCCGATGTTTAAACCCTTTCAGACGACTCCTTCAAAACATCGGCTGAACC 1200
A V K L L I D E R N P L Y Q M I V P M F K P F Q T T P S K H R L N
CAAACCACAAGAAAAATAGATCCACAGGAGAACCGACATGACTGCCAACCAAAGTTACCGCAAACCGCTGCCCGGTACGGATTTGGAATACTACGACGCG 1300
P N H K K N R S T G E P T *
CGTGCGGCGTGTGAGGACATCAAACCCGGCTCTTACGACAAGCTGCCGTACACGAGCCGCATTTTGTCTGAGAACTTGGTCAACCGGCGCGGACAAAGTC 1400
ClaI.
GATTTGCCGATGCTGCAAAGCTGGCTGGGCCAGTTGATAGAAGGGAAGCAGGAAATCGATTTTCCGTGGTATCCCGCGCGGGTGGTGTGCCACGATATTC 1500

```
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TTTCACTTCATCAACTGGACAAAAACCGCATTTGAAAATGTGGACGTGATTCCGCGGGCAACGGCATCATGCACCAAATCAATCTTGGAAAAAATGTCGC 1800
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CGTGGGCGGTTTGGAAGCGGAAACCGTGATGCTGGGACGCGCGTCCATGATGCGCCTGCCCGATATTGTCGGCGTGGAGCTGACGGGCAAACGGCAGGCG 2000
                    EcoRI
GGCATTACTGCCACAGACATCGTGTTGGCACTGACCGAATTC
```

Figure 3: Nucleotide sequence of the G2B5 insert.
Relevant restriction sites are labeled. The nad1 open reading frame spans nucleotides 366 to 1249. The -10 and -35 promoter sequences as well as the ribosome-binding-site (rbs) are underlined. The deduced amino acid sequence is shown below the DNA sequence.

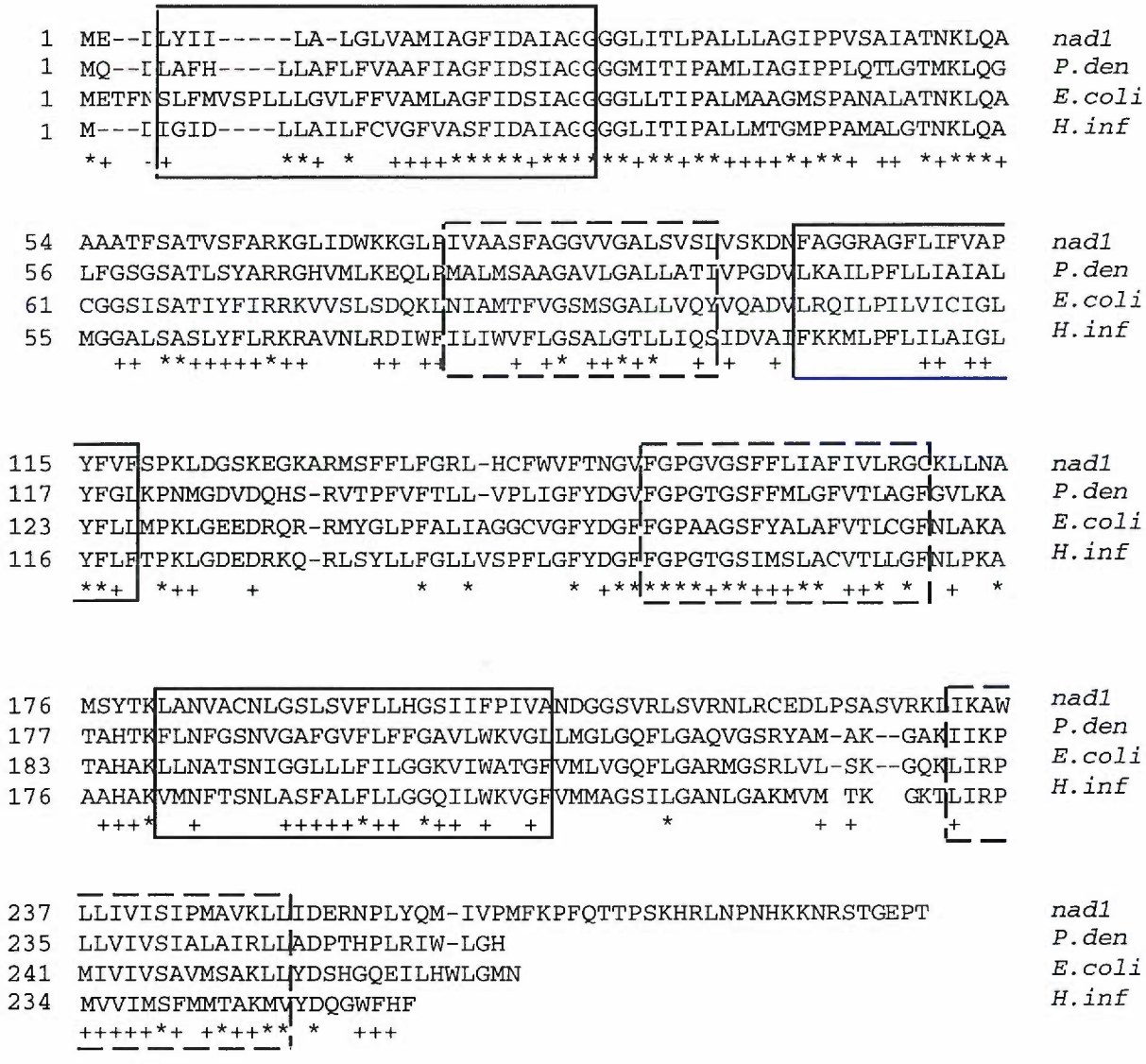
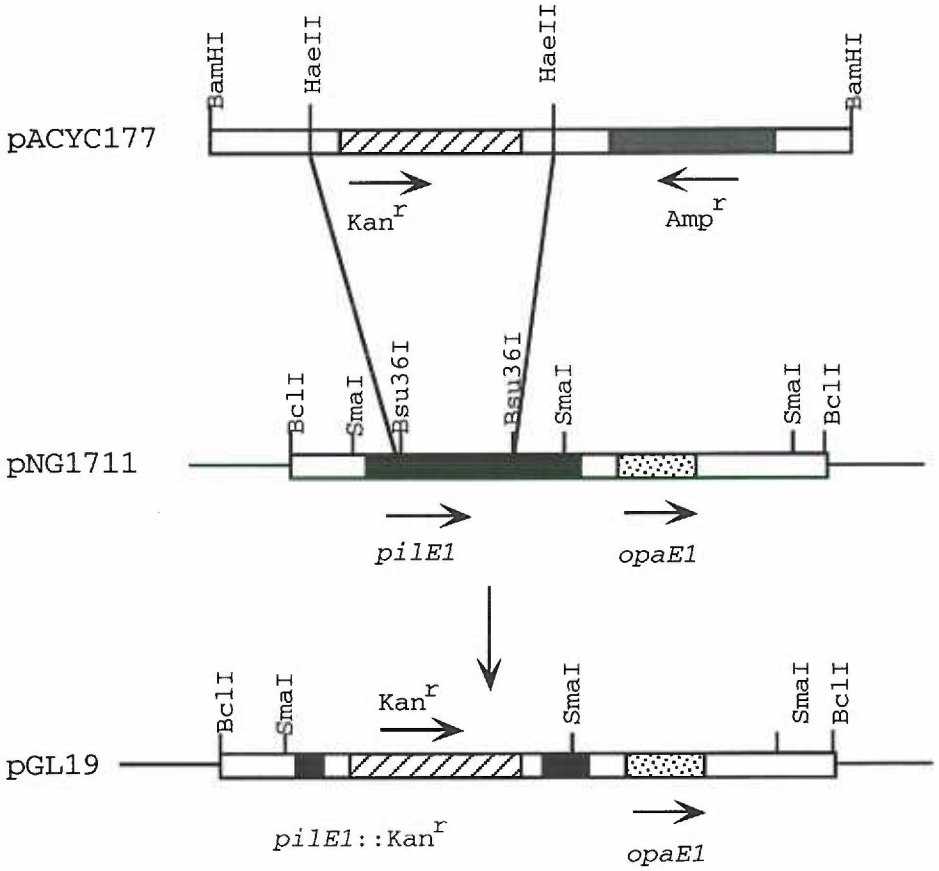


Figure 4. Amino acid sequence alignment of Nad1 with three related bacteria proteins. Identical amino acid between the four protein sequences are indicated by "*" and similar ones are indicated by "+". The solid boxed regions denote predicted transmembrane domains with N-termini being the cytoplasmic domain. The dashed boxed regions denote predicted transmembrane domains with the N-termini being the extracellular domain.

A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BamHI
HaeII
HaeII
BamHI
Kan^r
Amp^r
pNG1711
BclI
SmaI
Bsu36I
Bsu36I
SmaI
SmaI
BclI
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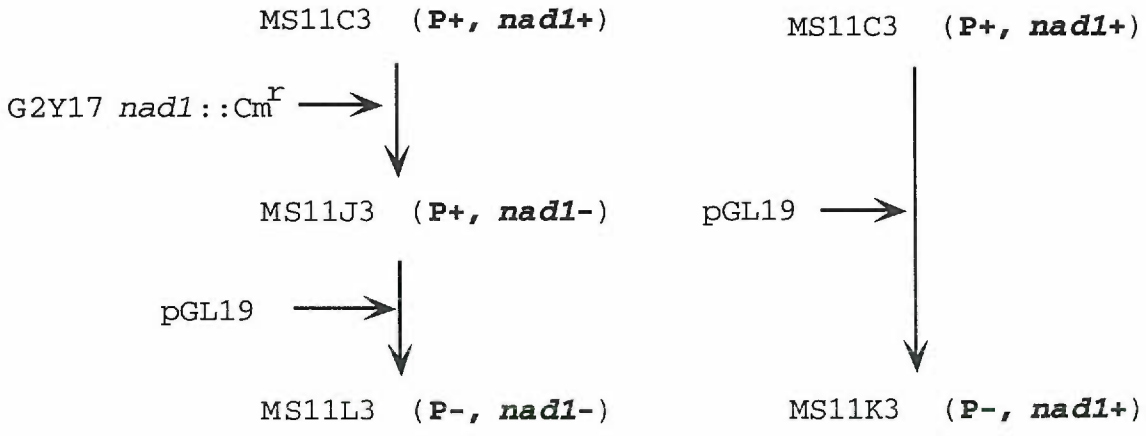
BclI
pilE1::Kan^r
opaE1


MS11C3 (P+, nad1+)
G2Y17 nad1::Cm^r
MS11J3 (P+, nad1-)
pGL19
MS11L3 (P-, nad1-)
MS11C3 (P+, nad1+)
pGL19
MS11K3 (P-, nad1+)

B

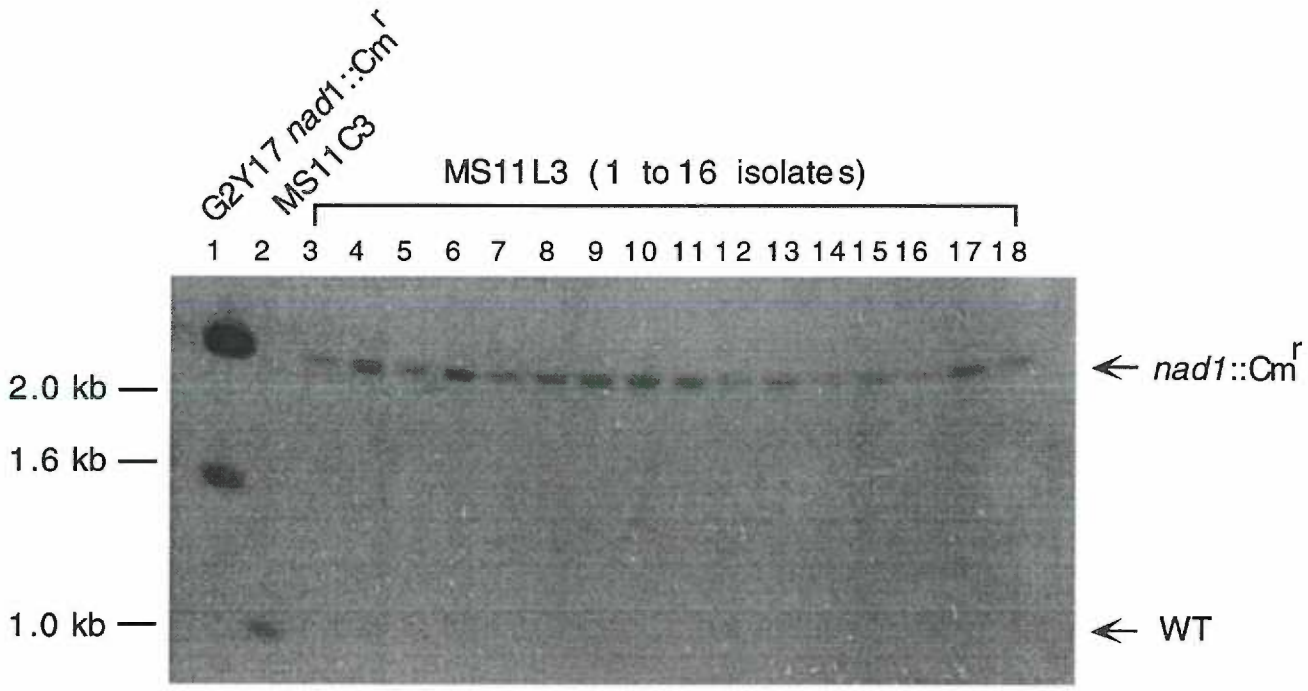


Figure 5. Construction of the P⁻ nad1 MS11A mutant

A: Construction of the *pilE1*::Kan^r mutant. The Bsu36I fragment within the *pilE1* gene in pNG1711 was replaced by the HaeII fragment from pACYC177, containing a Kanamycin resistance gene (kan^r) to generate pGL19 (*pilE1*::Kan^r). MS11C3 (Δ*pilE2*) was transformed with G2Y17 mutant DNA (*nad1*::Cm^r). A transformant was subsequently transformed with pGL19 DNA. The final transformant is P⁻ and *nad1*-. pGL19 DNA was also used to transform MS11C3 to generate MS11K3 (*nad1*+) which was used as a positive control.

B: Southern blot of the DNA from the above transformants digested with ClaI probed with the 1.0 kb ClaI fragment from G2Y17. Lane1: G2Y17 (*nad1*::Cm^r). Lane2: MS11C3. Lane3-18: 16 independent isolates from MS11L3 transformants.

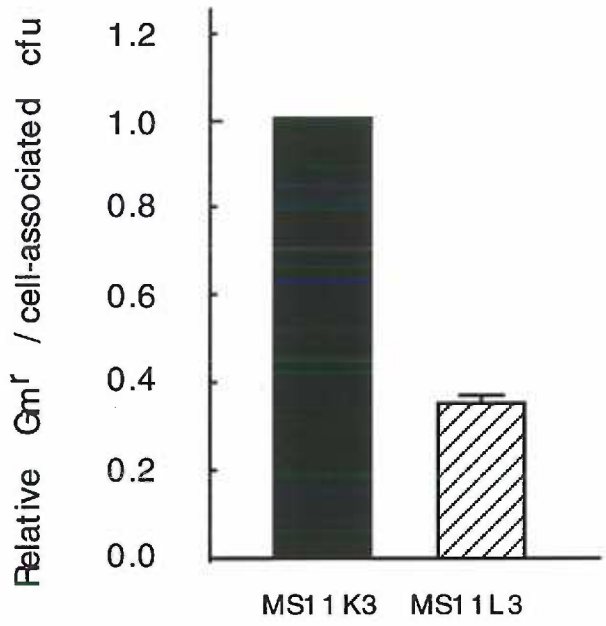


Figure 6. Invasiveness of MS11L3. Hec-1-B cells were infected with the relevant strains for 8 hrs and the Genr cfu/cell associated cfu was determined for each strain. The value for MS11L3 was normalized to the value of the MS11K3 control. Data represent the mean and standard deviation from three independent experiments. All experiments were done in triplicates.

Acknowledgment

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MATERIALS AND METHOD

Strains and plasmids

Cell lines, bacterial strains and plasmids are listed in Table 1.

Cell culture

Hec-1-B cells (ATCC HTB 113) is a human endometrial carcinoma cell line. A431 (ATCC.CRL1555) is a human epidermoid carcinoma cell line. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco) and were used for bacterial infection experiments between the 5th and 20th passages.

Bacterial strains

N. gonorrhoeae strain MS11A is P^+ and Opa^- (Segal et al.,1985); 15063G is a highly invasive P^+ and Opa^+ clinical isolate generously provided by Dr. Charles Davis (UCSD Medical Ceter, San Diego, CA.; Waldbeser et al.,1994). Bacteria were passed every 18-22 hours on GCB plates (Difco) with supplements (Kellogg et al.,1968) as previously described (Segal et al.,1986). All strains used for infection experiments have been passaged fewer than 10 times.

Constructing the CG gene bank in *E. coli*

CG gene bank were produced from partial MboI digests of G15063 chromosome which were size-fractionated on sucrose gradients. The region of the gradient corresponding to 20-35 kb was concentrated and ligated into the phosphatased BamHI cloning site of pCP19. DNA was packaged in vitro and used to infect HB101. The *E. coli* recombinants were selected on LB plates

Table I: Bacterial strains and plasmid

Strain/plasmid	Relavent genotype	Source
Neisseria		
15063G	P^+, Opa^+, clinical isolate	
MS11A	P^+, Opa^-	lab collection
MS11C3	Δ*pilE2*	lab collection
MS11J3	Δ*pilE2*, *nad1*::Cm^r	this study
MS11L3	*pilE1*::Kan^r, Δ*pilE2*, *nad1*::Cm^r	this study
E.coli		
HB101	F- λ–, hsd520 (r-B,m-B),supE44,ara14, galK2,lacZY1,proA2,rpsl20(strR), xy15, leu, mt11,recA13	lab collection
DH5α	endA1, F-, hsdR17 (r-K,m+), supE44, thi-1, λ-, gyr96, recA1, phi80d, *lacZ* Δ m+5	lab collection
plasmid		
pCP19	cosmid, pCP13, ΔKm, Tc^r	lab collection
pNG1711	pMB1, Amp^r	Meyer, 1984
pGL19	derived from pNG1711, *pilE1*:: Kan^r	this study
pBluscript KS	colE1, bla	Stratagen
pACYC177	p15A, Kan^r, Amp^r	NEB
CMXX	PUC19, Amp^r	Tsolis R.

containing 10 ug/ml tetracycline. Shuttle mutagenesis was performed as described by Seifert et al (1986).

Enrichment of adhesive *E. coli* recombinants

Hec-1-B cells were grown in DMEM containing 10% heated inactivated FBS(Gibco), L-glutamine, Na-pyruvate, and without antibiotics. 48 hours before the assay, Hec-1-B cells were seeded in 24-well tissue culture plates 5X104 cells/well. The CG gene bank were grow on LB with 10ug/ml tetracycline at 37°C overnight. The next day, bacteria were resuspended in fresh LB and grown for 1 hour to reach mid-log phase. Hec-1-B cells were infected at MOI of 20 for 1 hour. The infected cells were washed with PBS 15 times and the bacteria remained cell associated were considered adhesive. The cells were lysed in PBS Triton 1% plated on selective LB plates. Colonies were harvested while they were still small and resuspended in LB. The bacteria were incubated at 37°C for 1 hour and infect Hec1-B cells. The cell associated bacteria were cycled twice following the same procedure. Individual colonies were isolated for further characterization.

Adhesion assays

Hec-1-B cells or A431 cells were seeded in 24-well tissue culture plates at a density of 5-8 X 10^4 cells/well 24-36 hours prior to infection. Cells were washed with PBS, pH7.4, incubated in DMEM/5%FBS and infected with bacteria resuspended in the same medium. For adhesion assays, bacteria were added at a multiplicity of infection (MOI) of 10-20 bacteria/cell and incubated for 1 hour for *E. coli* clones and 5-7 hours for CG in 5% CO_2 at 37°C. After that, half of the wells were harvest to determine total cfu. The other half

were washed with PBS(PH 7.4) and lysed with 0.5% Triton-X100 in PBS for *E. coli* and 0.5% saponin (Aldrich) in GCB for CG. The lysates were plated to determine the cell-associated cfu.

Invasion assays

Infections were performed as described for adhesion assays. After infection, the infected cells were washed 6 times with PBS (pH 7.4). Half of the wells were lysed to determine cell-associated cfu. The other half were incubated with DMEM with 100 ug/ml gentamicin for 90 minutes in 5% CO_2 at 37°C to kill extracellular bacteria. The cells were lysed and the lysate were plated to determine intracellular cfu.

Construction of *pilE1* mutant

The plasmid pNG1711 contains two Bsu36I sites in the *pilE1* locus. The vector of pNG1711 is pBR322. pNG1711 was digested with Bsu36I treated with Klenow enzyme and dNTPs to create blunt ends. pACYC177 was digested with HaeII and separated by agarose gel electrophoresis. The fragment containing the Kan^r gene was purified and blunt ended using Klenow and dNTPs. The treated DNA was ethanol precipated, purified and ligated. The ligation mix was transformed into *E. coli* strain HB101 and transformants were selected for Kan^r and Amp^r (pGL19).

Transformation of *Neisseria gonorrhoeae*

Linearized *E. coli* plamid DNA were added to *N. gonorrhoeae* suspended in Difco GCB broth with Kellogg's supplements and 5mM MgCl2. The mixture was incubated at 30C for 30 minutes and then diluted 1:10 in GCB broth with

supplements. The bacteria were incubated in 5% CO_2 at 37C for 4-6 hours and plated on GCB agar containing appropriate antibiotics.

Southern blot analysis

The 1.0kb ClaI fragment of in the G2Y17 insert was used as a template for probes using the Dig-DNA labeling kit (Boehringer) according to the manufacturer. GC chromosomal DNA was digested with restriction enzymes and the fragments were separated in 0.8% argarose gels. DNA from the gels was transferred to Nylon membranes (Biotrans, ICN) according to the manufacturers instructions. The filters were hybridized with probes and washed at high stringency according to manufacturers instructions. Signals were detected using the lumiPhos systems (Boehringer-Mannheim) and autoradiographed.

DNA sequencing

DNA sequences were determined using fluorescence oligonucleotides by the OHSU MMI department core facility.

Discussions and conclusions

Neisseria gonorrhoeae and *Neisseria meningitidis* are two pathogens which only infect humans. Many problems in understanding the biology of these organisms and in the prevention and treatment of the diseases they cause still exist. These include the circumstances which initiate the disease and the mechanisms underlying the carrier state.

In order to cause a successful infection, pathogenic Neisseria must first successfully colonize the host. Many outer membrane components such as pili, Opa, Opc and LOS have been shown to contribute to the interaction of these bacteria with host cells at the early stage of infection. In the first part of this study, we identified a new GC gene, *nad1*, which enhances bacterial invasion into, but not adhesion to, the human epithelial cell line HEC-1-B. This observation further suggests that Neisserial infection is a mutifactorial process and that adhesion and invasion are mechanistically separate events.

After colonization, the bacteria must penetrate the epithelium before initiating disease. It is well established that MC and GC cross the epithelial barrier by entering epithelial cells, trafficking through them, and exiting these cells without damaging them. While inside these cells the bacteria must survive within a rigorous environment and avoid killing by lysosomes. Inside the eukaryotic cells, lysosomes are the final degradative compartment in the endocytic route. This compartment contains hydrolytic enzymes and other microbicidal compounds and plays an important role in defending the body against invading microbes. Most phagosomes are targeted to fuse with lysosomes. Many intracellular pathogens have developed various means to modify their

phagosomal environments in order to avoid lysosome killing. Some of them escape into the cytoplasm by lysing the phagosomal membrane, others avoid lysosomal fusion or attenuate the pH in the phagolysosomes where they reside, and by doing so attenuate the activities of the acid hydrolases in these compartments.

In the second part of this study, we provided evidence that the Neisserial type 2 IgA1 protease plays an important role in helping the bacteria avoid killing by lysosomes. The IgA1 protease, first discovered over twenty years ago, cleaves human IgA1 at its proline-rich hinge region. They have been hypothesized to play an important role in helping colonizing bacteria escape inactivation by mucosal immunoglobulins. Our experiments have revealed a second function of the IgA protease; that of promoting intracellular survival. The IgA1 protease cleaves at the prolin-rich hinge region of LAMP1, a major glycoprotein in the membrane of late endosomes and lysosomes, thereby increasing its turnover rate. As LAMP1 is thought to function in maintaining the stability of these compartments, increased LAMP1 turnover is likely to alter or modify these compartments.

The reduction of three lysosomal markers in infected cells strongly suggest that LAMP1 compartments are modified. Whether the modification is a direct result of the cleavage of LAMP1 is unclear. Lysosomes contain acid hydrolases that function in the digestion of a wide variety of macromolecules and are maintained at low pH by the vacuolar proton-ATP pump. The mildly acidic late endosomes also contain LAMP1 and many hydrolases. LAMP1 is thought to play an important role in maintaining the stability of these compartments. Increased LAMP1 turnover could therefore set into motion a series of events

that ultimately would promote bacterial survival: a decrease in the integrity of the membranes of late endosomes and lysosomes, perhaps enough to lead to their disintegration; leakage of the contents of these compartments into the cytoplasm; an increase in pH in the compartments and attenuation or inactivation of the acid hydrolases or an alteration of the processing of some of the acid hydrolases (eg. Cathepsin D). Modified (weakened) membranes could account for the contradictory reports regarding whether intracellular Neisseriae are surrounded by a membrane. Reduced LAMP1 levels could also result in a decrease in the number of late endosomes and lysosomes in a cell.

Several other mucosal pathogens also produce IgA1 proteases. *Haemophilus influenzae, Ureaplasma urealyticum, Clostridium ramosum, Bacteriodes melaninogenicus* and several Streptococcal species (reviewed by Mulks and Shoberg, 1994). The exact function of these proteases is unknown. It will be interesting to determine whether these bacterial IgA1 proteases play a similar role in the intracellular survival of these pathogens.

In summary, infection of human epithelial cells by pathogenic Neisseriae is a mutifactorial process. Through the studies presented here, we have identified a new GC gene which contributes to the invasion of human epithelial cells. In addition, we have revealed a mechanism by which the pathogenic Neisseriae survive within epithelial cells.

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