An Investigation of Intracellular Iron Acquisition by *Neisseria meningitidis*

By

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"These damn bugs always want to eat iron. Sometimes they use a fork, sometimes a spoon, but they're always hungry"

-Igor Stojiljkovic

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Abstract

Neisseria meningitidis (*meningococcus*, MC) is a causative agent of meningitis and septic shock. Two factors that are most likely to contribute to dissemination of MC within the host are survival within epithelial cells and the acquisition of iron. In this dissertation I investigate the crossroads of these two factors. Because MC can replicate within epithelial cells, I reasoned that they must have an iron source in this location. I have demonstrated that intracellular MC must acquire host cell iron in order to replicate. Further, this acquisition is via a previously uncharacterized meningococcal TonB-dependent receptor. I have ruled out all of the previously identified *neisserial* iron sources, indicating that a novel iron source is utilized within cells. I have also found that cells infected with MC rapidly degrade cytosolic ferritin. Inhibitors that prevent this process also inhibit replication of intracellular MC. Thus, MC appears to acquire an intracellular iron source derived from degraded ferritin.

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Chapter 1 - Introduction

Since the end of the 17th century when Koch and Pastuer developed the germ theory of disease it has become common knowledge that physical contact with pathogenic bacteria can lead to illness. Simple exposure to low doses of a pathogen, however, is rarely sufficient to produce disease. Disease is merely a possible outcome of a complex and dynamic interplay between host and pathogen. This concept is vividly displayed in the study of meningococcal disease. *Neisseria meningitidis* (Meningococci; MC) is the causative agent of several forms of illness, primarily meningitis and septicemia. This microbe colonizes the nasopharynx of approximately 15% of the global population. MC infections are predominantly asymptomatic with MC behaving like a commensal organism, however, the pathogenic capability of MC is underscored by the fact that it is the most frequent cause of bacterial meningitis in infants and young adults (Kline *et al.*, 2004).

This introduction will summarize several of the known factors that contribute to MC pathogenesis. These factors are comprised of environmental influences, the host immune system and bacterial virulence determinants. Later sections will summarize the important roles that iron plays in biology and pathogenesis. Human iron physiology will be summarized followed by bacterial iron acquisition systems. Finally, the iron acquisition systems of MC will be described in order to

set the stage for a novel iron acquisition strategy detailed in the body of this thesis.

Neisseria meningitidis as a global health threat

The gram-negative diploccocus *Neisseria meningitidis* remains a major cause of meningitis and septicemia throughout the world. According to CDC statistics, in 2002 there were over 1,500 cases of meningococcal disease reported in the United States. MC colonizes the nasopharynx of approximately 5-10% of the US population yet disease develops in only about 1 per 200,000 (Wenger *et al.*, 1990). In underdeveloped nations including many in West Africa, MC is responsible for periodic epidemics, claiming as many as 10,000 lives in a single outbreak (Hart and Cuevas, 1997).

MC-related disease typically presents flu-like symptoms with rapid progression to septicemia and fulminant meningitis, causing death in approximately 10% of cases. Sublethal disease produces a multitude of complications such as neurological damage, blindness, organ failure and tissue damage severe enough to warrant amputation (Vermont *et al.*, 2003). The vast majority of MC strains are susceptible to killing by antibiotics such as penicillin and chloramphenicol, however, disease can progress from initial symptoms to death in a matter of hours (Dabernat *et al.*, 1984). Rapid diagnosis and treatment are frequently

impossible, a factor that contributes to the morbidity and mortality of meningococcal disease.

MC is likely to remain a worldwide health threat until a broadly protective vaccine is developed and effectively deployed. Currently, vaccines are available for MC serogroups A, C, Y and W135. These vaccines are based on polysaccharide components of the bacterial outer membrane and are not effective in children under two years of age, who are frequently targets of meningoccal disease. Recent advances in conjugate vaccines consisting of protein-polysaccharide fusions provide hope for this age group (Wildes and Tunkel, 2002). There is currently no effective vaccine for MC serogroup B and several untypeable MC strains. Due to the shortcomings of the current vaccines, they are only used for high-risk populations. An in-depth understanding of the factors that contribute to meningococcal disease should aid in the development of a more effective vaccine.

Environmental factors that contribute to meningococcal disease

The human host is subject to multiple environmental factors that can affect their susceptibility to disease. Outbreaks of meningococcal disease in many geographical locations tend to fluctuate annually with peaks during cold, dry seasons. These outbreaks often follow upper respiratory tract infections by influenza virus. Because of these factors, it is presumed that disruption of the

nasopharyngeal mucosal barrier can promote the invasiveness of MC (Merz and So, 2000).

Another factor that has a profound influence on the development of meningococcal disease is the host social environment. Military recruits and college freshmen living in dormitories show a very high incidence of disease. Both of these environments consist of young adults from diverse locations packed into close quarters. Primary exposure to MC serogroups in this environment is believed to be the major catalyst to meningococcal disease. This is because the adaptive immune response is key in limiting MC invasiveness.

Host factors that influence the development of meningococcal disease

The cornerstone of the human adaptive immune system is the production of pathogen-specific antibodies. In serum, G-class immunoglobulins (IgGs) play two key roles in limiting the invasiveness of MC. First, the deposition of IgGs on the MC surface can activate the classic compliment pathway resulting in lysis of bacteria that breach the mucosal barrier (Jarvis, 1995). The importance of the classical complement pathway in limiting MC invasiveness is underscored by the fact that people with late complement component deficiencies frequently experience MC-related disorders (Vermont *et al.*, 2003).

A second function of IgG's is the opsonization of MC that promotes their phagocytosis by Polymorphonuclear leukocyes (PMNs). The importance of this function is highlighted by the fact that populations carrying a rare allotype of a PMN-expressed Fcg-receptor, which is defective in IgG binding, are more susceptible to meningococcal disease (Vermont *et al.*, 2003).

Colonization by MC or "carriage" results in elevated levels of strain-specific serum IgGs (Robinson *et al.*, 2002) and, therefore, carriage appears to be a two-edge sword. On one hand carriage stimulates the immune system and is likely to protect the carrier from invasive disease. On the other hand, it increases the likelihood of transmission to those who have no protective antibodies.

Humans lacking MC-specific antibodies depend upon their innate immune system to protect them from disease. Both the alternative complement pathway and the mannose-binding lectin (MBL) complement pathway are believed to play key roles in MC immunity (Vermont *et al.*, 2003)(Jack *et al.*, 1998). As with the classical pathway, humans with genetic defects in the alternative and MBL pathways experience a higher incidence of MC-related disorders (Vogel and Frosch, 1999).

Perhaps the most significant function of the immune system in MC infection is the inflammatory response produced by meningococcal endotoxin, or LPS

(lipopolysaccharide). Unfortunately, this response is largely responsible for the damage incurred by the host during septic shock and meningitis (Pathan *et al.*, 2003).

Meningococcal factors that contribute to its pathogenesis

Aside from laboratory settings, MC lives exclusively within its human host. Its long-term survival, therefore, depends upon successful colonization of the human nasopharynx. MC is confronted with several obstacles in doing so. MC must adhere to the nasopharyngeal epithelia, which is coated in mucus that is constantly swept away via ciliated cells. After the host immune response is initiated, secretory A-class immunoglobulins (sIgAs) form a tight bridge between the mucous and MC. In order to diminish the effects of sIgA, MC secretes an IgA protease, which can cleave sIgA and is likely to diminish mucosal sweeping. MC also has cytotoxic activity that can halt ciliary motion and probably further dampens the protective function of mucous (Stephens *et al.*, 1986).

In order to withstand physical stripping from the epithelial surface and also compete with resident microflora for attachment sites, MC must adhere tightly to cells. This is accomplished by several molecular adhesins expressed on the MC surface. There are two major classes of MC adhesins, the type IV pilus, and opacity (opa) proteins.

The neisseial type IV pilus is a filamentous polymer that is 6nm in diameter and up to several microns in length (Merz and So, 2000). Pili can retract and extend, allowing diverse functions such as microcolony formation, DNA uptake, twitching motility, and host cell signaling (Merz and So, 2000). A host cell receptor for the neisserial type IV pilus, CD46, was recently discovered (Kallstrom *et al.*, 1997) and has since been shown to undergo phosphorylation of its cytoplasmic tail in response to pilus binding (Lee *et al.*, 2002). MC adhesion does not increase in correlation to CD46 expression on epithelial surfaces so it is likely that CD46 promotes MC adhesion via its signaling properties and does not function as a traditional receptor (Tobiason and Seifert, 2001). Following pilus-mediated binding, the epithelial cell undergoes morphological changes in which cortical actin is recruited beneath MC microcolonies. In a subsequent phase of adhesion, MC attains an intimate adherence to the plasma membrane with epithelial microvilli extending around the bacteria (Merz *et al.*, 1999).

MC expresses opa proteins on its outer membrane that enhance intimate adhesion. The genome of most MC strains encodes 4-5 different opa proteins that are variably expressed (Dehio *et al.*, 1998). Opa proteins have been demonstrated to bind a number of host-cell surface receptors including CD66, heparin sulfate proteoglycans and integrins (Merz and So, 2000). Adhesion driven by pili and opa proteins can subsequently lead to invasion of epithelial cells. Invasion of cells by MC is thought to lead to dissemination from the

nasopharynx to the bloodstream, but it may also play a major role in promoting long-term colonization. Intracellular MC are resistant to physical removal via mucous, and are also protected from host antibodies (Meyer, 1991).

Upon invading the bloodstream MC must evade the host immune system in order to survive, replicate and produce disease. MC has a variety of protective functions that allow it to subvert innate and adaptive immunity. MC is usually encased within a polysaccharide capsule that protects it from complement-mediated killing and also interferes with phagocytosis by both PMNs and dendritic cells (Kolb-Maurer *et al.*, 2003; Vogel and Frosch, 1999). Some MC serogroups also modify their LPS with sialic acid, generating a structure that mimics host glycosphingolipids. Sialylated LPS, via its similarity to host self-antigens is able to subvert the immune system. Sialylated LPS also reduces complement activation and protects MC from phagocytosis (Moran *et al.*, 1996).

MC are genetically optimized for evading the host immune system by their capacity to alter the expression and amino acid sequence of their surface proteins. The expression of over 30 meningococcal surface-exposed molecules is controlled by phase variation (Richardson and Stojiljkovic, 2001). In this process, repeated nucleotide tracts upstream of genes are frequently altered by slip-strand mispairing during DNA replication, leading to changes in their reading

frame. This introduces or deletes premature termination codons that alter gene expression (Belland *et al.*, 1997; Bucci *et al.*, 1999).

MC also frequently undergoes antigenic variation, which alters the amino acid sequence of surface exposed proteins such as pili. This process occurs via homologous recombination using a template of either multiple silent loci or DNA introduced by transformation. MC undergoes transformation at a high rate because it has over 2,000 copies of a 12 bp neisserial uptake sequence scattered around its chromosome (Goodman and Scocca, 1988; Smith *et al.*, 1999). DNA released from dead MC during autolysis can be taken up by neighboring bacteria via this sequence resulting in a high frequency of transformation (Smith *et al.*, 1999). The combination of high frequency transformation, phase variation, and antigenic variation allows MC to alter its surface epitopes quickly, making host antibodies obsolete.

The host and pathogen factors that contribute to the development of meningococcal disease were presented in two different sections, however, they depict a complicated chess match that takes place within the host. The host has multiple strategies for eliminating bacterial pathogens, while MC has evolved counterattacks for each of them as summarized in Table 1. One additional host strategy that confronts invading bacteria is the limitation of iron availability. The bulk of this introduction will describe the important role that iron plays in human

physiology and bacterial pathogenesis. The end of this introduction will summarize the iron acquisition strategies of MC.

Iron, an essential biological element

In nearly all life forms, iron serves as a cofactor for essential functions. One reason for the prevalence of iron in biological systems is its ability to change valence under physiological conditions. Iron can exist in the ferric [Fe^{+3}] or ferrous [Fe^{+2}] state and its redox potential can be modified by a wide range of organic ligands (Crichton and Ward, 1992; Harrison and Arosio, 1996). Because of this property, iron is often incorporated into electron transport proteins, such as those used in bacterial and mitochondrial respiration. One class of these proteins, cytochromes, incorporates a complex of iron with protoporphyrin IX known as heme (Ponka, 1999b). Two other heme-containing proteins, myoglobin and hemoglobin, bind oxygen and ensure that it is provided to all living cells in the human body.

Iron is also found in the catalytic center of many enzymes common to prokaryotes and eukaryotes. One of these is the most prevalent form of ribonucleotide reductase, an enzyme that catalyzes the synthesis of deoxyribonucleotides (DNA monomers) (Eklund *et al.*, 2001). Another class of iron-containing enzymes, aconitases, participate in the NADH-yielding Kreb's cycle

Table 1

Host Immune functions versus meningococcal virulence determinants

Host Mechanism	Meningococcal counterattacks
Mucins	Adhesion molecules I.e. Pili and Opa
sIgA	IgA protease
Ciliary sweeping of mucus	Toxicity to ciliated cells
Complement	Capsule, LOS
opsinization	Capsule, LOS
IgGs	Antigenic variation, phase variation and transformation
Iron withholding	**TonB-dependent receptors**

(Lieu *et al.*, 2001). An extreme example of the universality of iron in biological systems was identified in a Martian meteorite discovered in Antarctica in 1984. This meteorite contains magnetite (Fe_3O_4), a mineral that is not believed to be generated outside of lifeforms such as magnetotactic bacteria (Thomas-Keprta *et al.*, 2002). Apparently, Martians need iron too. To ensure the function of vital metabolic components, organisms have evolved elaborate means of obtaining sufficient iron.

Iron as a biological paradox

Iron presents two fundamental paradoxes to life forms. The first is the question of availability. Iron is the second most abundant metal in the Earth's crust, however, the vast majority is in the ferric form, which is highly insoluble (Harrison and Arosio, 1996). Ferric iron in solution at nominal pH values (pH 5-9) and under physiological oxygen concentrations will hydrolyze H_2O and form ferric hydroxide [$Fe(OH)_3$] (Ponka, 1999a). Ferric hydroxide in turn can form highly insoluble polymers known commonly as rust (Aisen *et al.*, 2001). In order for organisms to absorb ferric iron they must increase its solubility via chelation or reduce it to the ferrous form prior to its absorption.

The second paradox of iron is that although essential, it is potentially toxic. Within the reducing environment of the cellular cytosol, ferric iron can be reduced to the ferrous form. Ferrous iron reacts with hydrogen peroxide to form hydroxyl

radicals that can cause oxidative damage to biomolecules. The reaction of iron with hydrogen peroxide was first described by Haber and Weiss in the 1930s and named the "Fenton reaction" in honor of the first man to modify organic compounds by adding iron and hydrogen peroxide (Koppenol, 2001). The equation for the Fenton reaction is

$$Fe(II) + H_2O_2 \quad Fe(III) + HO + HO$$

Two other equations were proposed at this time to describe the interactions of these oxidative species in solution. The Haber-Weiss reactions are depicted by the following two equations.

$$HO + H_2O_2 \quad H_2O + O_2 + H$$

$$O_2 + H + H_2O_2 \quad O_2 + HO + H_2O$$

Another set of equations termed the "Fenton catalyzed Haber-Weiss cycle" supposes that superoxide can reduce Fe^{+3} to Fe^{+2} and instigate another round of the Fenton reaction. It has been verified that Fe^{+2} regeneration is instigated by biological reductases such as ascorbate and not by superoxide.

The Haber-Weiss cycle is often cited as a factor contributing to iron-catalyzed oxidative damage, yet it has been proven that the second Haber –Weiss reaction does not occur under physiological conditions (Koppenol, 2001). The reaction of

hydroxyl free radicals with hydrogen peroxide to produce superoxide, however, is likely to contribute to cellular damage initiated via Fenton chemistry.

Free radicals produced via iron catalysis can cause extensive damage to living organisms in the form of DNA strand breakage, lipid peroxidation, and enzyme deactivation (Aisen *et al.*, 2001; Harrison and Arosio, 1996). It should be noted that cells are not completely defenseless against assault from free radicals. Reductases and enzymes such as superoxide dismutase, catalase, and peroxidase provide cells with a level of protection that is sufficient unless iron overload occurs (Hermes-Lima *et al.*, 1998). Because of the paradoxes presented by iron, abundant yet unavailable and vital yet toxic, organisms have evolved tightly regulated mechanisms for iron absorption, transport, and storage (Ponka, 1999a).

Human iron physiology I – absorption

Absorption of dietary iron in humans occurs in the proximal intestine or duodenum (Conrad and Umbreit, 2002). The duodenum has a massive surface area due to small projections called vili that increase the absorptive capabilities of this organ. Enterocytes are specialized cells that are localized near the tips of vili and absorb Fe^{+2} ,Fe^{+3}, and heme. Absorption of iron is a tightly regulated process, because humans have no regulated mechanism of iron excretion (Roy and Enns, 2000). In order to prevent iron overload, its absorption and transport

through enterocytes to the bloodstream is highly coordinated to match the relatively small amount of iron that is lost each day, due mainly to exfoliation of dead cells.

Fe^{+3} is soluble at the low pH of the stomach and it is bound there by mucins and other small molecules that can maintain its solubility in the higher pH of the duodenum. Fe^{+2} and heme, in contrast, are readily soluble in the intestine (Conrad and Umbreit, 2000). Enterocytes have small appendages called microvilli on their apical membrane to further increase the surface area of the duodenum, and upon them express apical iron transporters. One transporter called DMT-1 (Nramp2, DCT1) transports Fe^{+2} as well as several other divalent cations. A ferrireductase, DcytB, increases the Fe^{+2} availability by reducing Fe(III) within the intestinal lumen in a NADPH-dependent manner (Kaplan, 2002). Another receptor complex, B_3 integrin/mobilferrin, transports only Fe^{+3} (Conrad and Umbreit, 2002). A receptor for heme has not yet been identified, but its internalization is believed to occur via endocytosis. Once heme is internalized, iron is liberated from it by the cytoplasmic enzyme heme oxygenase (Conrad and Umbreit, 2000).

Once iron enters the enterocyte, regardless of its source, it most likely enters the labile iron pool (LIP). The labile iron pool is loosely defined as the metabolically active and regulatory forms of chelateable iron within the cytoplasm (Konijn *et al.*,

1999). Once there it can be stored within the cell, utilized for metabolic purposes, or transported to the bloodstream. Iron exits the basolateral membrane of enterocytes via a recently identified transporter called ferroportin1 (Ireg1, MTP1) (Donovan *et al.*, 2000) (McKie *et al.*, 2000) (Abboud and Haile, 2000). Another recently identified molecule, hephaestin, is a membrane-associated multicopper ferroxidase that is believed to facilitate iron export via ferroportin1 and its subsequent loading onto transferrin (Vulpe *et al.*, 1999). Transferrin is an abundant serum glycoprotein that serves to transport iron to cells throughout the body, a role that will be discussed in the next section.

The regulation of intestinal iron uptake is currently under intense focus. Two recently discovered molecules, HFE and hepcidin, play major roles in this process; however, a regulatory mechanism has not yet been defined. HFE is a membrane protein that binds to the cellular transferrin receptor and decreases the uptake of transferrin iron (Roy and Enns, 2000). This protein is frequently mutated in patients who suffer from primary hemochromatosis, an inherited iron-overload disorder (Feder *et al.*, 1996).

Hepcidin is a 25-amino acid peptide secreted by hepatocytes that acts as a negative regulator of intestinal iron uptake (Ganz, 2003). Patients with hemochromatosis due to HFE mutations have lower than normal levels of hepcidin mRNA in the liver, despite hepatic iron overload, indicating that these

molecules may be part of the same regulatory circuit (Ganz, 2003). Hepcidin is also involved in an innate immune response called anemia of infection that will be discussed in a later section.

Human iron physiology II – Iron transport and storage

Transferrin, mentioned previously, provides iron to cells throughout the body via the bloodstream. Transferrin is an 80-kDa bilobed molecule with one high-affinity iron-binding site on each lobe. In plasma where the pH is near neutral, transferrin has a dissociation constant for Fe(III) of approximately 10^{-23}mol/liter, however below pH 4.5, it displays virtually no iron binding (Ponka, 1999a). This property is a key attribute in the ability of transferrin to deliver iron to cells and return to the plasma for another cycle of iron binding. Transferrin molecules typically outnumber the iron atoms in the circulation to an extent that transferrin is only about 30% saturated (Aisen *et al.*, 2001). As a result there is virtually no free iron within the bloodstream.

The majority of iron in the bloodstream is derived from macrophages that are charged with the duty of phagocytosing senescent erythrocytes and degrading the iron-bearing hemoglobin within them (Ponka, 1999a). The export of iron from macrophages is believed to occur via ferroportin with oxidation of Fe^{+2} to Fe^{+3} catalyzed by the serum multicopper ferroxidase ceruloplasmin (Anderson, 2001; Kaplan, 2002).

Iron-requiring human cells express transferrin receptors (TfRs), which act as a dimer of two identical 90kDa membrane proteins (Aisen *et al.*, 2001). TfRs have a high affinity for iron-loaded (holo)transferrin, but a low affinity for iron-free (apo)transferrin at pH 7.2. After the TfR binds holotransferrin the complex is endocytosed in a clathrin-dependent manner. Acidification of the endosome results in the low-pH release of iron from transferrin. The pH of acidified endosomes ranges from 5.5 to 6, and is not low enough to fully account for the rapid release of Fe^{+3} from transferrin. Because of this, it is believed that other factors such as iron-binding molecules or a membrane ferrireductase aid in Fe^{+3} release from transferrin (Aisen *et al.*, 2001; Kaplan, 2002). TfRs with bound apotransferrin are recycled to the surface where the apotransferrin is released and can undergo another round of iron binding and internalization. During its lifetime in the circulation, a single transferrin protein can undergo 100-200 cycles of iron transport (Aisen *et al.*, 2001).

Iron that is released from transferrin within the endosome is believed to be reduced to Fe^{+2}, and transported out of the endosome via DMT-1 (Kaplan, 2002). As mentioned previously, reduction of Fe^{+3} to Fe^{+2} and release from transferrin might be a coupled event. Iron transported out of the endosome is believed to directly enter the labile iron pool (Breuer *et al.*, 1995) although there are reports that it can be directly taken up by cytosolic ferritin (Vyoral and Petrak, 1998) or

mitochondria (Ponka *et al.*, 2002). Iron within the labile pool can have several possible fates. Some is utilized in the synthesis of metalloenzymes, while other atoms are transported into the mitochondria for heme synthesis and subsequent respiration duties. Other iron atoms remain within the labile pool serving a regulatory function. A significant portion is also designated for storage within cytosolic ferritin.

Ferritins are a family of proteins that are used in all biological kingdoms as a means to store vast quantities of iron in a non-toxic form. Human ferritin is a heteropolymer of two types of subunits [heavy (H) and light (L) chain] that form a spherical cage-like structure (Aisen *et al.*, 2001; Harrison and Arosio, 1996). Within the sphere is a cavity approximately 80 angstroms in diameter that is capable of storing up to 4,000 atoms of iron (Theil, 2003). Eight pores within the ferritin shell allow entry and exit of iron and other small molecules. Fe^{+2} enters ferritin where it is oxidized to Fe^{+3} and stored as a ferric oxide polymer similar to rust but complexed with phosphate (Aisen *et al.*, 2001).

Ferritin H chains have an active ferroxidase site that initiates the incorporation of Fe^{+2} and produces H_2O_2 as a bi-product. This H_2O_2 can potentially interact with Fe^{+2} in the labile iron pool to produce toxic free radicals. Because of this, cells with high iron loads usually contain ferritin with a lower H/L chain ratio. Iron accumulation in this type of ferritin is slower, but produces less H_2O_2 (Theil,

2003). It has also been suggested that a multicopper ferroxidase, such as ceruloplasmin, may aid iron deposition into ferritin in vivo without producing free radicals; however, a specific cytosolic ferroxidase dedicated to ferritin loading has not been identified (Welch *et al.*, 2001). By storing Fe^{+3} within ferritin, cells can increase its relative solubility by a factor of 10^{15}; enough to supply adequate iron for metabolic purposes (Theil, 2003).

Release of iron from ferritin has not been given nearly the amount of attention as iron incorporation. Cells can utilize iron stored within ferritin for metabolic purposes, but the mechanism by which iron is redistributed from ferritin to the cytosol is not known. A combination of low molecular weight reductases and Fe^{+2} chelators can effectively remove iron from ferritin in vitro and it is suggested that this can also occur within cells (Harrison and Arosio, 1996) (Radisky and Kaplan, 1998).

Another pathway of iron release from ferritin occurs in iron-starved cells. Upon iron starvation, cytosolic ferritin can be delivered to lysosomes via autophagy where it is degraded (Bridges and Hoffman, 1986; Bridges, 1987; Hoffman *et al.*, 1991; Kakhlon *et al.*, 2001; Ollinger and Roberg, 1997). Ferritin-derived iron can then be redistributed to the cytosol via an undefined mechanism (Radisky and Kaplan, 1998). Iron starvation-induced degradation of ferritin is inhibited by

ascorbic acid or lysosomal protease inhibitors (Hoffman *et al.*, 1991; Ollinger and Roberg, 1997).

Iron-overloaded cells display another iron-containing species that is often found in lysosomes. Hemosiderin is believed to be a degradation product of ferritin that serves as an end point for storage of excess iron. Hemosiderin has been shown to contain denatured ferritin H-chain as well as ferric oxide polymers resembling ferritin but also contains lipids and is highly amorphous (Harrison and Arosio, 1996).

Transferrin receptors and ferritin are inversely regulated by the labile iron pool. Regulation is achieved post-translationally via two different iron regulatory proteins (IRPs) in the cytosol that bind to stem-loop structures on mRNA called iron responsive elements (IREs). When the labile iron pool is low, IRPs bind a single IRE on the 5' untranslated region of ferritin mRNA and prevent its translation. There are several IREs on the 3' end of transferrin receptor mRNA, and binding of IRPs to them when the labile iron pool is low prevents 3' to 5' exonuclease activity thus stabilizing the message and resulting in increased translation.

When the labile iron pool is high, one of the IRPs (IRP1) loses its RNA-binding activity and functions as an aconitase, while the other IRP (IRP2) is degraded.

The loss of IRP binding activity allows translation of ferritin, while transferrin receptor message undergoes rapid degradation reducing translation (Aisen *et al.*, 2001; Torti and Torti, 2002). Several other iron-related proteins are regulated by the IRP/IRE system including the heme biosynthetic enzyme 5-aminolevulinic acid synthase and DMT-1 (Theil and Eisenstein, 2000). Expression of DMT-1, like the transferrin receptor, is positively regulated by IRP binding activity, ensuring that transferrin uptake can proceed efficiently (Anderson, 2001).

Although transferrin is the major mediator of iron transport in humans, there is strong evidence for a transferrin-independent system. Most of this evidence comes from developmental studies, which demonstrate that early tissue development can proceed in the absence of cellular transferrin uptake. A recent advance in this field was the demonstration that a lipocalin, NGAL, mediates iron transport during the differentiation of mesenchhymal tissue into epithelia (Yang *et al.*, 2002). NGAL was shown to bind catecholate siderophores, predicting the existence of a human siderophore involved in transferrin independent iron transport (Kaplan, 2002). NGAL is also secreted by neutrophils and has bacteriostatic action by virtue of its siderophore-binding activity (Goetz *et al.*, 2002). The possible role of lipocalin in the innate immune system will be discussed in the next section.

Human iron metabolism III - Iron withholding in the innate immune system

The innate immune system serves as a first line of defense against invading bacteria. Many of the innate immune components were described in the beginning of this introduction. One additional and important function of the innate immune system is the withholding of iron from microbes. As described earlier, iron serves many critical functions in metabolism and this is as true in bacteria as it is in humans. It has been suggested that iron acquisition is the key determinant in the ability of bacteria to sustain themselves in the host and produce disease (Ratledge and Dover, 2000). This final section on human iron metabolism will describe the lengths that the human body goes to in order to prevent bacteria from stealing this valuable element.

The distribution of iron in the body of an adult male is summarized in Table 2. Of approximately 4 grams, only about 3mg or .1% is found outside of cells in the bloodstream bound to transferrin. This does not take into account the amount of iron that is in the digestive tract including the mouth and nasopharynx, because these areas are subject to dietary fluxes. The intracellular localization of iron presents a barrier to most bacteria because relatively few have invasive capabilities. The innate immune system serves two basic functions in iron withholding; protein-mediated binding of iron in extracellular fluids and reduction of the serum iron pool in response to infection (anemia of infection).

Table 2

The Distribution of Iron Within the Human Body (Average Adult Male)

Molecules	Iron (grams)	Percent of Total
Hemoglobin	2.7	66
Myoglobin	0.2	3
Heme Enzymes	0.008	0.1
Intracellular Ferritin	1.0	30
Non-heme Enzymes	0.0001	-
Intracellular Labile Iron	.07	1
Transferrin	.003	-
Total Intracellular Iron	3.997	99.9

Information obtained from Harvard University Website
http://sickle.bwh.harvard.edu/iron_transport.html

As described earlier iron within the serum is bound by transferrin and as a result there is only about 10^{-18}M free iron in this pool. Most bacteria require approximately 10^{-6}M iron for optimum growth and this quality of serum is, therefore, bacteriostatic (Rohde and Dyer, 2003). Bacteria that can replicate in serum do so because they have evolved iron-acquisition strategies such as those that will be described in the next section.

Mucosal secretions contain high concentrations (6-13μM) of lactoferrin, a transferrin homologue, that is able to bind iron in mucous and prevent bacterial access to it. Unlike transferrin, lactoferrin retains its iron-binding affinity at low pH (Rohde and Dyer, 2003). Lactoferrin is also secreted by PMNs and has additional bactericidal activities against gram-negative pathogens that are not related to iron binding (Ellison, 1994).

A third protein, NGAL, is believed to aid in iron withholding on epithelial surfaces. As mentioned previously, NGAL has the ability to bind siderophores (Goetz *et al.*, 2002). Many bacteria secrete siderophores, which are soluble iron-binding molecules that can bind free iron or remove it from other proteins. Bacteria take up siderophores via outer membrane receptors (Crosa, 1989). Siderophore-based iron utilization will be covered in more detail in the next section. Lipocalin may serve to deny bacterial access to iron-loaded siderophores thus providing a backup for lactoferrin on epithelial surfaces (Goetz *et al.*, 2002).

During a productive infection the host inflammatory response can initiate a decrease of iron levels in serum. This effect, called anemia of infection, is a result of both decreased iron absorption and sequestration of iron in macrophages. The soluble signal that initiates anemia of infection was recently identified. Hepcidin is produced at high levels by the liver in response to LPS or interleukin-6 and signals enterocytes and macrophages to stop exporting iron. Like lactoferrin, hepcidin has additional antimicrobial properties unrelated to iron (Ganz, 2003).

The previous three sections summarized the mechanisms that humans use to absorb, transport, store, and withhold iron. The next section will summarize mechanisms of iron acquisition used by pathogenic bacteria, focusing on strategies for iron acquisition within the human host.

Iron and bacterial pathogenesis

Iron availability has a profound influence on the ability of bacteria to produce disease in humans. A Parisian professor of clinical medicine, Dr. Armand Trousseau, was one of the first to make the connection between iron and infection in the mid 19th century. Trousseau warned his students against injecting iron into patients suffering from tuberculosis (caused by *Mycobacterium tuberculosis*), because he knew it would worsen their condition (Raymond *et al.*,

2003). Iron is now known to contribute to the pathogenesis of myriad other bacteria including *Escherichia, Klebsiella, Listeria, Pasteurella, Shigella, Salmonella, Vibrio, Neisseria, Streptococci, Haemophilus and Yersinia* species. (Raymond *et al.*, 2003). Most pathogenic bacteria have been proven to have multiple strategies for iron acquisition within the human host.

Siderophore-mediated iron acquisition

By far the most common means of bacterial iron acquisition is the secretion and uptake of siderophores. Siderophores are small molecules that chelate Fe(III) with high affinity and, hopefully, return it to the bacteria (Ratledge and Dover, 2000). Many siderophores are peptide derivatives that are generated on nonribosomal peptide synthetases (Cane and Walsh, 1999). Bacteria take up siderophores via membrane receptors. In gram-negative bacteria these receptors are integrated into the outer membrane and are powered by the TonB complex, which will be described later. Most siderophores and their receptors are only expressed under iron-limiting conditions.

There are currently over 500 siderophores identified and there are certainly exceptions to every rule as to their synthesis, activity, and uptake. The most frequently studied siderophore systems is the enterobactin system of *Escherichia coli* and, for simplicity, only this system will be described in detail. Some of the many pathogenic bacteria that depend upon siderophore-mediated iron uptake

are uropathogenic *E. coli*, enterohemorrhagic *E. coli*, *Salmonella typhimurium*, *Yersinea pestis, Yersinia enterocolitica*, *Mycobacteria tuberculosis*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Shigella flexneri (Ratledge and Dover, 2000).*

Enterobactin (also known as enterochelin) was first isolated in 1970. This molecule is synthesized via a complex sequence of reactions that are catalyzed by nonribosomal peptide synthetases. Chorismic acid, an aromatic amino acid precursor, is converted to 2,3 dihydroxybenzoic acid (DHB) and then linked to L-serine. Three molecules of DHB-Serine are then cyclized to form enterobactin. Three catecholate functionalities (a catacholate has two adjacent hydroxyl groups on a benzene ring) bind to Fe^{+3} fulfilling iron's hexadentate binding capabilities (Raymond *et al.*, 2003). Enterobactin undergoes a conformational change upon iron binding that buries iron within its structure and limits chemical reactivity and as a result, enterobactin has the highest affinity for iron of any characterized siderophore (Ratledge and Dover, 2000).

The outer membrane receptor FepA allows uptake of Fe^{+3}-enterobactin in a TonB-dependent manner. FepA forms a transmembrane pore via a 22-stranded B-barrel and also has an N-terminal plug domain. During uptake of enterobactin, the plug domain is thought to undergo a coformational change within the barrel in order to allow Fe^{+3}-enterobactin to enter the periplasm. Transport of Fe^{+3}-

enterobactin through the cytoplasmic membrane is mediated by an ATP binding cassette (ABC) transporter. This transporter is composed of a periplasmic binding protein (FepB), an inner membrane permease, FepD/FepG, and a third membrane protein that hydrolyzes ATP (FepC), powering transport. Once in the cytosol, Fe(III)-enterobactin is hydrolyzed by an esterase that allows iron removal and utilization (Raymond *et al.*, 2003).

Uptake of enterobactin by FepA requires the TonB complex (TBC). Many outer membrane receptors in gram-negative bacteria depend upon this complex to provide energy for transport of their ligands. The gram-negative outer membrane has porins that allow translocation of molecules smaller that 600 Daltons. As a result there is no proton gradient across this membrane. There is also a lack of energy-supplying molecules such as ATP and GTP. The TBC evolved to translocate energy to outer membrane receptors. This energy is derived from the proton motive force that is generated across the inner membrane (Postle and Kadner, 2003).

The TBC is composed of three proteins, TonB, ExbB and ExbD that are believed to function in a 1:7:2 stoichiometry (Braun and Braun, 2002b). Most of the TonB protein is located in the periplasm, except for the N-terminus, which contains one transmembrane domain that inserts into the cytoplasmic membrane. A histidine residue in this transmembrane domain is believed to respond to the proton

motive force by acquiring a cytoplasmic proton and transferring it to the transmembrane regions of ExbB and/or ExbD. This proton cycling is believed to power comformational changes in both TonB and the receptors that interact with it (Braun and Braun, 2002b).

The mechanism by which the TBC provides energy to outer membrane receptors has not yet been elucidated, but there are many characteristics of the interaction that have been defined. The C-terminus of TonB contains a rigid extension in a region that is rich in proline residues. This domain interacts with the plug of TonB-dependent receptors at a motif called the "TonB box". A general model for receptor function is that the barrel itself is sufficient for uptake of the ligand but the plug seals the pore when empty and helps pull the ligand into the periplasm thus clearing the receptor for a new ligand (Braun and Braun, 2002a). In this "gated pore" model, extracellular loops of FepA close around the ligand before the plug is opened. Thus the pore of FepA is never fully exposed, which would prevent the movement of non-ligand molecules through FepA (Rohde and Dyer, 2003).

Several models for TonB function have been proposed including one in which TonB acts like a propeller, revolving and interacting with the receptor in a sequence of binding and release (Cascales *et al.*, 2001). A more viable model depicts TonB shuttling between the cytoplasmic and outer membranes, thus

delivering stored energy to the receptor (Larsen *et al.*, 2003; Postle and Kadner, 2003). Evidence for this model was provided by experiments that separated the cytoplasmic and outer membrane using a sucrose gradient. Wild type TonB was found to associate almost equally with both membranes (Letain and Postle, 1997). Mutant forms of TonB lacking N-terminal membrane anchors localized to only the outer membrane, while C-terminal deletions localized to only the cytoplasmic membrane. Interestingly, in the absence of ExbB/ExbD, localization was also confined to the outer membrane. It should be noted that this data could also be explained by the fact that Tonb simultaneously interacts with both membranes. The separation could be an artifact of the procedure.

Evidence for the shuttling model was recently obtained by use of a cross-linked label that is only accessible to periplasmically exposed domains. It was verified that the N-terminus of TonB, which would be inaccessible to the periplasm in the absence of shuttling, acquired label, but only when the C-terminus of TonB was present (Larsen *et al.*, 2003). Although the shuttling model of TonB is the most viable, more evidence will be required to rule out other possibilities.

Bacterial transport of Fe^{+2}

Most of the iron encountered by bacteria within the human body is in the Fe^{+3} form, which can be taken up via siderophores or other mechanisms to be examined later. Some bacteria, however, thrive in anaerobic environments

where Fe(II) is likely to be more available (Kammler *et al.*, 1993). Host or bacterial reductases may increase the availability of Fe^{+2} to bacteria. A ferrous iron transport system consisting of FeoA and FeoB has been isolated in *E. coli* and was shown to be important for growth within the intestine (Kammler *et al.*, 1993). FeoB-dependent Fe^{+2} transport has also been demonstrated to serve a role in infection by *Heliobacter pylori, Shigella flexneri, Salmonella typhimurium* and *Legionella pneumophila* (Boyer *et al.*, 2002; Robey and Cianciotto, 2002; Runyen-Janecky *et al.*, 2003; Velayudhan *et al.*, 2000).

Heme as a bacterial iron source

Many bacteria are able to utilize heme as an iron source. While heme is complexed to myriad proteins that can be free within the body due to lysis of cells, the majority of heme encountered is derived from hemoglobin. Hemoglobin can be found on mucosal surfaces as a result of menses or upon tissue damage such as that resulting from the gum disease gingivitis. Spontaneous lysis of red blood cells also results in low levels of heme and hemoglobin in the bloodstream. Heme in the bloodstream is usually complexed to serum albumin or hemopexin, while hemoglobin is bound by the serum glycoprotein haptoglobin. Many bacteria have receptors that enable the uptake of free heme, hemoglobin, heme/hemopexin or hemoglobin/haptoglobin. Most of these receptors are TonB-dependent. Some bacteria also secrete proteases that liberate heme from carrier proteins. Following uptake by bacteria heme is hydrolyzed in the cytosol

by heme oxygenase after which the liberated iron is utilized for metabolic purposes (Stojiljkovic and Perkins-Balding, 2002).

Transferrin, lactoferrin and ferritin as bacterial iron sources

Many bacteria can utilize transferrin or lactoferrin as an iron source. Most of these bacteria do so via siderophore secretion. Several siderophores have an affinity for iron that is greater than that of transferrin or lactoferrin and can successfully compete with these proteins for iron within the host. Some bacteria, such as *Neisseria, Pasteurella*, and *Moraxellaceae* Spp. have outer membrane receptors that are able to bind transferrin or lactoferrin directly and remove and transport iron. Transferrin and lactoferrin iron uptake via specific receptors has been studied mostly in *Neisseria*, so it will be discussed further the next section.

It has frequently been suggested that ferritin can serve as a bacterial iron source. Despite common speculation, there is no definitive evidence that ferritin iron acquisition occurs during infection. Many siderophores can remove ferritin iron in the presence of a reductase, which has led to most of this speculation. In one study researchers found that *Salmonella typhimurium* in a culture system appeared to take up transferrin iron via enterobactin, while aerobactin acquired iron from the cultured cells. While the authors suggest that ferritin was the source of cellular iron, they did not clearly demonstrate this assumption (Brock *et al.*, 1991).

Iron acquisition by Neisseria meningitidis

The importance of iron acquisition in the establishment of meningococcal disease has been highlighted by several experiments using animal models. MC does not colonize animals and cannot produce disease in them without some manipulation. These models rely upon injection of MC directly into the bloodstream or cerebrospinal fluid. Using these models, researchers have shown that administration of a readily usable iron source within the animals produced a dramatic increase in virulence (Holbein *et al.*, 1979; Holbein, 1980, 1981).

Unlike most pathogenic bacteria, MC does not produce siderophores. The reasons for this are unknown but one logical assumption is that it is a product of living exclusively within the human host. Siderophores have the advantage of being able to remove iron from anything that does not sterically block access and has a low enough iron affinity. The disadvantage of a siderophore is that there is no guarantee it will return to the species of origin. Siderophore production is, therefore, less efficient that the direct, receptor-mediated removal of iron from host iron proteins.

MC is able to obtain iron from transferrin, lactoferrin, hemoglobin, hemoglobin/haptoglobin, and the xenosiderophore enterobactin. All of these

molecules are bound on the MC surface by specific TonB-dependent receptors. Uptake of all of these iron sources except enterobactin, which was not examined, was shown to be defective in a MC strain lacking TonB (Stojiljkovic and Srinivasan, 1997). Three of these receptors differ from siderophore receptors in that they have an associated lipoprotein that plays a role in ligand binding (Schryvers and Stojiljkovic, 1999).

MC take up transferrin via the TbpA/B receptor complex. TbpA forms a 22-stranded B-barrel pore with an N-terminal plug similar to FepA, while TbpB is a globular protein expressed on the external face of the outer membrane where it is anchored by palmitic acid. Both proteins have transferrin-binding activity and both are required for transferrin iron utilization by MC (Rohde and Dyer, 2003).

Lactoferrin iron utilization is achieved via a similar two-component receptor, LbpA/B. This receptor is believed to be functionally and structurally similar to TbpA/B, with the exception that the LbpB lipoprotein is not essential for lactoferrin iron utilization (Rohde and Dyer, 2003).

Once iron is released from either lactoferrin or transferrin it is taken up into the MC periplasm where it is bound by FbpA. FbpA is part of a meningococcal ABC transporter, FbpA/B/C, which is required for transferrin and lactoferrin utilization. This receptor complex transports ferric iron though the cytoplasmic membrane.

FbpA is also required for high-affinity uptake of Fe^{+3} salts such as ferric nitrate (Khun *et al.*, 1998).

MC can remove heme from hemoglobin via two different receptors. HpuA/B is a third two-component receptor that has similarities to TbpA/B. In this case the A component is the lipoprotein, while the B component forms the pore. HpuA/B can remove heme from hemoglobin or hemoglobin bound to haptoglobin. The second hemoglobin receptor, HmbR, has no lipoprotein component (Rohde and Dyer, 2003). HmbR forms another 22-stranded B-barrel pore that can transport heme from hemoglobin, but not hemoglobin bound to haptoglobin. Both of these receptors are also capable of transporting free heme, although this function in not TonB-dependent. Strains lacking both of these receptors can still transport free heme, indicating the presence of another transporter that is also not TonB-dependent (Schryvers and Stojiljkovic, 1999).

The expression of both HmbR and HpuA/B are controlled by phase variation such that most strains express only one type of hemoglobin receptor at a time. Both receptors remove heme from hemoglobin and transport it into the periplasm. Heme is then transported into the cytosol via an unidentified cytoplasmic membrane transporter. Heme oxygenase within the cytosol is required to hydrolyze heme and allow the removal and utilization of iron (Schryvers and Stojiljkovic, 1999).

A second single component TonB-dependent receptor, FetA, allows MC to utilize the xenosiderophore enterobactin. The affinity of FetA for enterobactin is much lower than that of its natural receptor, FepA, indicating that FetA may have a preferred ligand that is similar in structure to enterobactin (Rohde and Dyer, 2003). Enterobactin is mostly found in the intestines where MC cannot survive.

To summarize, the uptake of iron from transferrin, lactoferrin and hemoglobin requires both a functional TonB and their respective outer membrane receptors. Uptake of enterobactin is dependent upon a putative TonB-dependent receptor but a role for TonB has not been experimentally determined. Transport of iron from both transferrin and lactoferrin from the periplasm to the cytosol requires the FbpA/B/C transport system.

Utilization of ferric salts such as ferric nitrate does not require TonB or a specific outer membrane receptor, but at low concentrations, FbpA/B/C is required. Uptake of free heme is also independent of TonB and can cross the outer membrane via Hmbr, HpuA/B, or an unidentified pore. Both free heme and Fe(III) may be small enough to enter the outer membrane through a channel such as porin. MC does not have a FeoA/B system, although Fe^{+2} iron may get in via another route.

All of the MC iron sources characterized prior to this thesis share one trait. They are all found predominantly in the extracellular environment. As pointed out earlier in this introduction, invasion of epithelial cells and replication within them is likely to play a major role in the pathogenesis of MC. The manuscripts presented in this thesis describe an investigation of intracellular iron acquisition by MC.

The present work

At the initiation of this project the meningococcal TonB gene had just been cloned by Dr. Igor Stojiljkovic's lab and through collaborating with him we obtained a set of isogenic mutants in TonB and several receptors. Most of the *Neisseria* iron field was working on refining the mechanisms of iron uptake by the known receptors as well as testing the feasibility of TonB-dependent receptors as vaccine candidates. We had dabbled in this field ourselves with studies on HmbR. We found that although HmbR is highly immunogenic in rabbits, antibodies to HmbR lacked bactericidal activity, so the project was abandoned.

The focus in many *Neisseria* labs was gravitating toward bacterial interactions with cultured cells, mostly epithelial. Many of the important interactions that enable MC to adhere to and invade epithelial cells were beginning to unfold. Preferring to jump ahead of the pack, I began asking questions about what happens to MC after they enter cells. Our laboratory had recently shown that

pathogenic *Neisseria* could invade and traffic through polarized T-84 cells. Dr. Sylvia Hopper had isolated a mutant strain of *Neisseria gonorrhoeae* that replicated at a high rate inside of cells and also trafficked much faster than wildtype bacteria. This suggested that replication might be part of the transcytosis mechanism.

It was clear that without a good supply of iron, MC grow very poorly. In some preliminary experiments, doubling times for intracellular MC were found to be 30-40 minutes, which was on par with their maximum growth-rate in media supplemented with excess hemoglobin.

Because of this, it was hypothesized that MC must have an iron source within cells. The experiments presented in Manuscript 1 were intended to first, verify that intracellular MC must acquire iron in order to replicate. Secondly, we attempted to show that the iron was coming from the host cells and not the culture media. Lastly, we hoped to learn as much as possible about this process by testing the phenotype of the isogenic mutant strains.

The strategy proved to be a success, as we were able to show that in order to replicate inside of epithelial cells, MC has to acquire iron from the host cell and not the media. In addition we showed that this process was TonB-dependent but

did not rely upon any of the previously characterized iron sources. This implied both a novel receptor and novel iron source for MC.

The next obvious step was to identify both the receptor and iron source involved. At this time the genomes of several MC strains had been published and were available on the internet for examination. There were six putative TonB-dependent receptors that had been annotated. They were defined as having homology to known TonB-dependent receptors including putative B-barrel domains as well as N-terminal plugs with putative "TonB-boxes". At this point I was faced with either generating six isogenic mutants or performing cell biology experiments, so I decided to take the next natural step.....I checked the literature for some ideas.

Unfortunately, despite the great emphasis on iron acquisition mechanisms of pathogenic bacteria, there is a relative scarcity of information on intracellular iron acquisition. Shelly Payne's lab had shown that TonB is required for intracellular growth of *Shigella dysenteriae.* The *tonB* mutant, however, did not appear to be iron starved in this environment as shown with a promoter fusion assay. The replication defect of this mutant could not be rescued by iron supplementation. One possible explanation is that the TonB mutant over-expressed siderophores, which chelated the iron supplement and could not be taken up by the mutant bacteria (Reeves *et al.*, 2000). The Payne lab had also shown that the

siderophore aerobactin was not required for intracellular iron acquisition by *Shigella flexnei* (Headley *et al.*, 1997). They have recently found that intracellular iron uptake by this organisms can occur via three transport systems, FeoA/B, aerobactin, or an uncharacterized iron transporter called SitA (Runyen-Janecky *et al.*, 2003). They have not determined the source of the iron taken up by intracellular *Shigella*.

A study by Marcus Horwitz's lab had shown that the phagosome formed by *Mycobacterum tuberculosis* is accessible to transferrin (Clemens and Horwitz, 1996). They did not, however, determine if the transferrin delivered iron to these bacteria. They were more interested in endosome fusion with the phagosome. Recently Olakami and Colleagues demonstrated that *M. tuberculosis* within phagosomes can acquire iron from both transferrin and an unidentified intracellular pool. Interestingly, they showed that iron acquisition from both routes was impaired in macrophages from patients with mutations in the hemochromatosis protein HFE (Olakanmi *et al.*, 2002).

The most elegant studies on iron acquisition by an intracellular bacterium at this point was performed by Thomas Byrd. He was able to show that *Legionella pneumophila* within monocytes can acquire iron from transferrin. He also showed that activation of the monocytes with interferon gamma limited transferrin iron availability to the bacteria (Byrd and Horwitz, 1989). In a later study, he

showed that neutralization of endosomal pH with chloroquine prevented iron uptake by intracellular *Legionella* (Byrd and Horwitz, 1991). If there was a consensus on intracellular iron sources for bacteria at this time, it would have been transferrin. These reports fortuitously caused me to take a second look at transferrin as a possible iron source for intracellular MC.

Having learned relatively little from the literature I set about making the isogenic mutants. I found that only four of six receptors had full-length genes in our serogroup C strain. For several reasons, this end of the project did not prove overly fruitful. My findings on the putative TonB-dependent receptors will be summarized in the discussion section.

The biggest breakthrough came when I took a second look at the possibility that transferrin was serving as an intracellular iron source for MC. It became apparent that the more transferrin iron I gave the cells, the less the bacteria replicated. I also found the converse to be true. Iron starvation of the cells caused MC to replicate like mad. Having shown that iron starved cells are a more conducive environment for MC replication, I began a literature search on cellular responses to iron starvation. This pointed me towards a set of experiments presented in Manuscript 2.

The findings in the two manuscripts predict the existence of an additional iron acquisition mechanism used by MC to access the vast reserves of iron within cells. Importantly, our lab has increased the knowledge of intracellular iron acquisition by MC to a level that at least equals that of any other bacterial pathogen. In the discussion section I will elaborate upon the significance of these manuscripts as well as present some of the possible scenarios that have emerged from this work

Chapter 1 References

Aisen, P., Enns, C., and Wessling-Resnick, M. (2001) Chemistry and biology of eukaryotic iron metabolism. *Int J Biochem Cell Biol* **33**: 940-959.

Anderson, G.J. (2001) Ironing out disease: inherited disorders of iron homeostasis. *IUBMB Life* **51**: 11-17.

Belland, R.J., Morrison, S.G., Carlson, J.H., and Hogan, D.M. (1997) Promoter strength influences phase variation of neisserial opa genes. *Mol Microbiol* **23**: 123-135.

Boyer, E., Bergevin, I., Malo, D., Gros, P., and Cellier, M.F. (2002) Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. *Infect Immun* **70**: 6032-6042.

Braun, M., Killmann, H., and Braun, V. (1999) The beta-barrel domain of FhuADelta5-160 is sufficient for TonB-dependent FhuA activities of Escherichia coli. *Mol Microbiol* **33**: 1037-1049.

Braun, V., and Braun, M. (2002a) Active transport of iron and siderophore antibiotics. *Curr Opin Microbiol* **5**: 194-201.

Braun, V., and Braun, M. (2002b) Iron transport and signaling in Escherichia coli. *FEBS Lett* **529**: 78-85.

Breuer, W., Epsztejn, S., and Cabantchik, Z.I. (1995) Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II). *J Biol Chem* **270**: 24209-24215.

Bridges, K.R., and Hoffman, K.E. (1986) The effects of ascorbic acid on the intracellular metabolism of iron and ferritin. *J Biol Chem* **261**: 14273-14277.

Bridges, K.R. (1987) Ascorbic acid inhibits lysosomal autophagy of ferritin. *J Biol Chem* **262**: 14773-14778.

Brock, J.H., Williams, P.H., Liceaga, J., and Wooldridge, K.G. (1991) Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of Escherichia coli and to other microorganisms. *Infect Immun* **59**: 3185-3190.

Bucci, C., Lavitola, A., Salvatore, P., Del Giudice, L., Massardo, D.R., Bruni, C.B., and Alifano, P. (1999) Hypermutation in pathogenic bacteria: frequent phase variation in meningococci is a phenotypic trait of a specialized mutator biotype. *Mol Cell* **3**: 435-445.

Byrd, T.F., and Horwitz, M.A. (1989) Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of Legionella pneumophila by limiting the availability of iron. *J Clin Invest* **83**: 1457-1465.

Byrd, T.F., and Horwitz, M.A. (1991) Chloroquine inhibits the intracellular multiplication of Legionella pneumophila by limiting the availability of iron. A potential new mechanism for the therapeutic effect of chloroquine against intracellular pathogens. *J Clin Invest* **88**: 351-357.

Cane, D.E., and Walsh, C.T. (1999) The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases. *Chem Biol* **6**: R319-325.

Cascales, E., Lloubes, R., and Sturgis, J.N. (2001) The TolQ-TolR proteins energize TolA and share homologies with the flagellar motor proteins MotA-MotB. *Mol Microbiol* **42**: 795-807.

Clemens, D.L., and Horwitz, M.A. (1996) The Mycobacterium tuberculosis phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. *J Exp Med* **184**: 1349-1355.

Conrad, M.E., and Umbreit, J.N. (2000) Iron absorption and transport-an update. *Am J Hematol* **64**: 287-298.

Crichton, R.R., and Ward, R.J. (1992) Iron metabolism--new perspectives in view. *Biochemistry* **31**: 11255-11264.

Crosa, J.H. (1989) Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiol Rev* **53**: 517-530.

Dabernat, H., Delmas, C., and Lareng, M.B. (1984) [Antibiotic sensitivity of meningococci isolated from patients and carriers]. *Pathol Biol (Paris)* **32**: 532-535.

Dehio, C., Gray-Owen, S.D., and Meyer, T.F. (1998) The role of neisserial Opa proteins in interactions with host cells. *Trends Microbiol.* **6**: 489-495.

Eklund, H., Uhlin, U., Farnegardh, M., Logan, D.T., and Nordlund, P. (2001) Structure and function of the radical enzyme ribonucleotide reductase. *Prog Biophys Mol Biol* **77**: 177-268.

Ellison, R.T., 3rd (1994) The effects of lactoferrin on gram-negative bacteria. *Adv Exp Med Biol* **357**: 71-90.

Ganz, T. (2003) Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* **102**: 783-788.

Goetz, D.H., Holmes, M.A., Borregaard, N., Bluhm, M.E., Raymond, K.N., and Strong, R.K. (2002) The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell* **10**: 1033-1043.

Goodman, S.D., and Scocca, J.J. (1988) Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. USA* **85**: 6982-6986.

Harrison, P.M., and Arosio, P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* **1275**: 161-203.

Hart, C.A., and Cuevas, L.E. (1997) Meningococcal disease in Africa. *Ann Trop Med Parasitol* **91**: 777-785.

Headley, V., Hong, M., Galko, M., and Payne, S.M. (1997) Expression of aerobactin genes by Shigella flexneri during extracellular and intracellular growth. *Infect Immun* **65**: 818-821.

Hermes-Lima, M., Storey, J.M., and Storey, K.B. (1998) Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. *Comp Biochem Physiol B Biochem Mol Biol* **120**: 437-448.

Hoffman, K.E., Yanelli, K., and Bridges, K.R. (1991) Ascorbic acid and iron metabolism: alterations in lysosomal function. *Am J Clin Nutr* **54**: 1188S-1192S.

Holbein, B.E., Jericho, K.W., and Likes, G.C. (1979) Neisseria meningitidis infection in mice: influence of iron, variations in virulence among strains, and pathology. *Infect Immun* **24**: 545-551.

Holbein, B.E. (1980) Iron-controlled infection with Neisseria meningitidis in mice. *Infect Immun* **29**: 886-891.

Holbein, B.E. (1981) Enhancement of Neisseria meningitidis infection in mice by addition of iron bound to transferrin. *Infect Immun* **34**: 120-125.

Jack, D.L., Dodds, A.W., Anwar, N., Ison, C.A., Law, A., Frosch, M., Turner, M.W., and Klein, N.J. (1998) Activation of complement by mannose-binding lectin on isogenic mutants of Neisseria meningitidis serogroup B. *J Immunol* **160**: 1346-1353.

Jarvis, G.A. (1995) Recognition and control of neisserial infection by antibody and complement. *Trends Microbiol* **3**: 198-201.

Kakhlon, O., Gruenbaum, Y., and Cabantchik, Z.I. (2001) Repression of ferritin expression increases the labile iron pool, oxidative stress, and short-term growth of human erythroleukemia cells. *Blood* **97**: 2863-2871.

Kallstrom, H., Liszewski, M.K., Atkinson, J.P., and Jonsson, A.B. (1997) Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. *Mol Microbiol* **25**: 639-647.

Kammler, M., Schon, C., and Hantke, K. (1993) Characterization of the ferrous iron uptake system of Escherichia coli. *J Bacteriol* **175**: 6212-6219.

Kaplan, J. (2002) Mechanisms of cellular iron acquisition: another iron in the fire. *Cell* **111**: 603-606.

Khun, H.H., Kirby, S.D., and Lee, B.C. (1998) A Neisseria meningitidis fbpABC mutant is incapable of using nonheme iron for growth. *Infect Immun* **66**: 2330-2336.

Kline, K.A., Sechman, E.V., Skaar, E.P., and Seifert, H.S. (2004) Recombination, repair and replication in the pathogenic Neisseriae: the 3 R's of molecular genetics of two human-specific bacterial pathogens. *Mol Microbiol* **51**: 297.

Kolb-Maurer, A., Kurzai, O., Goebel, W., and Frosch, M. (2003) The role of human dendritic cells in meningococcal and listerial meningitis. *Int J Med Microbiol* **293**: 241-249.

Konijn, A.M., Glickstein, H., Vaisman, B., Meyron-Holtz, E.G., Slotki, I.N., and Cabantchik, Z.I. (1999) The cellular labile iron pool and intracellular ferritin in K562 cells. *Blood* **94**: 2128-2134.

Koppenol, W.H. (2001) The Haber-Weiss cycle--70 years later. *Redox Rep* **6**: 229-234.

Larsen, R.A., Wood, G.E., and Postle, K. (1994) The conserved proline-rich motif is not essential for energy transduction by Escherichia coli TonB protein. *Mol Microbiol* **12**: 857.

Larsen, R.A., Letain, T.E., and Postle, K. (2003) In vivo evidence of TonB shuttling between the cytoplasmic and outer membrane in Escherichia coli. *Mol Microbiol* **49**: 211-218.

Lee, S.W., Bonnah, R.A., Higashi, D.L., Atkinson, J.P., Milgram, S.L., and So, M. (2002) CD46 is phosphorylated at tyrosine 354 upon infection of epithelial cells by Neisseria gonorrhoeae. *J Cell Biol* **156**: 951-957.

Lieu, P.T., Heiskala, M., Peterson, P.A., and Yang, Y. (2001) The roles of iron in health and disease. *Mol Aspects Med* **22**: 1-87.

Merz, A.J., Enns, C.A., and So, M. (1999) Type IV pili of pathogenic neisseriae elicit cortical plaque formation in epithelial cells. *Mol Microbiol* **32**: 1316-1332.

Merz, A.J., and So, M. (2000) Interactions of pathogenic neisseriae with epithelial cell membranes. *Annual reviews in Cell and Developmental Biology* **16**: 423-457.

Meyer, T.F. (1991) Evasion mechanisms of pathogenic Neisseriae. *Behring Inst Mitt*: 194-199.

Moran, A.P., Prendergast, M.M., and Appelmelk, B.J. (1996) Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol Med Microbiol* **16**: 105-115.

Olakanmi, O., Schlesinger, L.S., Ahmed, A., and Britigan, B.E. (2002) Intraphagosomal Mycobacterium tuberculosis acquires iron from both extracellular transferrin and intracellular iron pools. Impact of interferon-gamma and hemochromatosis. *J Biol Chem* **277**: 49727-49734.

Ollinger, K., and Roberg, K. (1997) Nutrient deprivation of cultured rat hepatocytes increases the desferrioxamine-available iron pool and augments the sensitivity to hydrogen peroxide. *J Biol Chem* **272**: 23707-23711.

Pathan, N., Faust, S.N., and Levin, M. (2003) Pathophysiology of meningococcal meningitis and septicaemia. *Arch Dis Child* **88**: 601-607.

Ponka, P. (1999a) Cellular iron metabolism. *Kidney Int Suppl* **69**: S2-11.

Ponka, P. (1999b) Cell biology of heme. *Am J Med Sci* **318**: 241-256.

Postle, K., and Kadner, R.J. (2003) Touch and go: tying TonB to transport. *Mol Microbiol* **49**: 869-882.

Radisky, D.C., and Kaplan, J. (1998) Iron in cytosolic ferritin can be recycled through lysosomal degradation in human fibroblasts. *Biochem J* **336**: 201-205.

Ratledge, C., and Dover, L.G. (2000) Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol* **54**: 881-941.

Raymond, K.N., Dertz, E.A., and Kim, S.S. (2003) Enterobactin: an archetype for microbial iron transport. *Proc Natl Acad Sci U S A* **100**: 3584-3588.

Reeves, S.A., Torres, A.G., and Payne, S.M. (2000) TonB is required for intracellular growth and virulence of Shigella dysenteriae. *Infect Immun* **68**: 6329-6336.

Richardson, A.R., and Stojiljkovic, I. (2001) Mismatch repair and the regulation of phase variation in Neisseria meningitidis. *Mol Microbiol* **40**: 645-655.

Robey, M., and Cianciotto, N.P. (2002) Legionella pneumophila feoAB promotes ferrous iron uptake and intracellular infection. *Infect Immun* **70**: 5659-5669.

Robinson, K., Neal, K.R., Howard, C., Stockton, J., Atkinson, K., Scarth, E., Moran, J., Robins, A., Todd, I., Kaczmarski, E., Gray, S., Muscat, I., Slack, R., and Ala'Aldeen, D.A. (2002) Characterization of humoral and cellular immune responses elicited by meningococcal carriage. *Infect Immun* **70**: 1301-1309.

Rohde, K.H., and Dyer, D.W. (2003) Mechanisms of iron acquisition by the human pathogens Neisseria meningitidis and Neisseria gonorrhoeae. *Front Biosci* **8**: d1186-1218.

Roy, C.N., and Enns, C.A. (2000) Iron homeostasis: new tales from the crypt. *Blood* **96**: 4020-4027.

Runyen-Janecky, L.J., Reeves, S.A., Gonzales, E.G., and Payne, S.M. (2003) Contribution of the Shigella flexneri Sit, Iuc, and Feo iron acquisition systems to iron acquisition in vitro and in cultured cells. *Infect Immun* **71**: 1919-1928.

Schryvers, A.B., and Stojiljkovic, I. (1999) Iron acquisition systems in the pathogenic Neisseria. *Mol Microbiol* **32**: 1117-1123.

Smith, H.O., Gwinn, M.L., and Salzberg, S.L. (1999) DNA uptake signal sequences in naturally transformable bacteria. *Res Microbiol* **150**: 603-616.

Stephens, D.S., Whitney, A.M., Melly, M.A., Hoffman, L.H., Farley, M.M., and Frasch, C.E. (1986) Analysis of damage to human ciliated nasopharyngeal epithelium by Neisseria meningitidis. *Infect Immun* **51**: 579-585.

Stojiljkovic, I., and Srinivasan, N. (1997) Neisseria meningitidis tonB, exbB, and exbD genes: Ton-dependent utilization of protein-bound iron in Neisseriae. *J Bacteriol* **179**: 805-812.

Stojiljkovic, I., and Perkins-Balding, D. (2002) Processing of heme and heme-containing proteins by bacteria. *DNA Cell Biol* **21**: 281-295.

Theil, E.C. (2003) Ferritin: at the crossroads of iron and oxygen metabolism. *J Nutr* **133**: 1549S-1553S.

Thomas-Keprta, K.L., Clemett, S.J., Bazylinski, D.A., Kirschvink, J.L., McKay, D.S., Wentworth, S.J., Vali, H., Gibson Jr, E.K., Jr., and Romanek, C.S. (2002) Magnetofossils from ancient Mars: a robust biosignature in the martian meteorite ALH84001. *Appl Environ Microbiol* **68**: 3663-3672.

Tobiason, D.M., and Seifert, H.S. (2001) Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. *Microbiology* **147**: 2333-2340.

Torti, F.M., and Torti, S.V. (2002) Regulation of ferritin genes and protein. *Blood* **99**: 3505-3516.

Velayudhan, J., Hughes, N.J., McColm, A.A., Bagshaw, J., Clayton, C.L., Andrews, S.C., and Kelly, D.J. (2000) Iron acquisition and virulence in Helicobacter pylori: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol Microbiol* **37**: 274-286.

Vogel, U., and Frosch, M. (1999) Mechanisms of neisserial serum resistance. *Mol Microbiol* **32**: 1133-1139.

Vyoral, D., and Petrak, J. (1998) Iron transport in K562 cells: a kinetic study using native gel electrophoresis and 59Fe autoradiography. *Biochim Biophys Acta* **1403**: 179-188.

Welch, K.D., Van Eden, M.E., and Aust, S.D. (2001) Modification of ferritin during iron loading. *Free Radic Biol Med* **31**: 999-1006.

Wenger, J.D., Hightower, A.W., Facklam, R.R., Gaventa, S., and Broome, C.V. (1990) Bacterial meningitis in the United States, 1986: report of a multistate surveillance study. The Bacterial Meningitis Study Group. *J Infect Dis* **162**: 1316-1323.

Wildes, S.S., and Tunkel, A.R. (2002) Meningococcal vaccines: a progress report. *BioDrugs* **16**: 321-329.

Yang, J., Goetz, D., Li, J.Y., Wang, W., Mori, K., Setlik, D., Du, T., Erdjument-Bromage, H., Tempst, P., Strong, R., and Barasch, J. (2002) An iron delivery pathway mediated by a lipocalin. *Mol Cell* **10**: 1045-1056.

Chapter 2: Manuscript 1

Replication of *Neisseria meningitidis* Within Epithelial Cells Requires TonB-Dependent Acquisition of Host-Cell Iron

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Abstract

Neisseria meningitidis (meningococcus, MC) is able to enter and replicate within epithelial cells. Iron, an essential nutrient for nearly all organisms, is an important determinant in the ability of MC to cause disease, however, its role in MC intracellular replication has not been investigated. We have analyzed the growth of MC within the A431 human epithelial cell line and the dependence of this growth on iron uptake. We present evidence that chelation of iron from infected tissue culture cells with Desferal strongly inhibited intracellular replication of wild-type (wt) MC. We also provide genetic evidence that iron must be acquired by MC from the host cell in order for it to replicate. An *hmbR* mutant that is unable to use hemoglobin iron and could not grow in tissue culture media without iron supplementation replicated more rapidly within epithelial cells than its wt parent strain. An *fbpA* mutant that is unable to utilize human transferrin iron or lactoferrin iron replicated normally within cells. In contrast, a *tonB* mutant could not replicate intracellularly unless infected cultures were supplemented with ferric nitrate. Taken together, these findings strongly suggest that MC intracellular replication requires TonB-dependent uptake of a novel host-cell iron source.

Introduction

The gram-negative diplococcus *Neisseria meningitidis* remains a significant cause of morbidity and mortality in developed and developing nations. The meningococcus (MC) is a common inhabitant of the human nasopharynx that can cross the epithelial barrier to enter the bloodstream. Within the bloodstream, MC can replicate, causing septicemia. From this site, it can also cross the blood-brain barrier to enter the cerebrospinal fluid, where it causes meningitis. The mechanisms by which MC crosses the epithelial and blood-brain barriers are poorly understood.

Humans are the only reservoir for MC, and this strict host tropism has prevented the development of a suitable animal model for studying the mechanisms by which MC colonizes and crosses the epithelial barrier. Most of the knowledge of MC infection has been derived from studies of its interactions with organ cultures, primary cell cultures and immortalized cell lines. These studies reveal that MC is able to adhere to, enter and traffic through human epithelial cells **(Merz and So, 2000)**. MC can also replicate within cultured epithelial cell lines **(Lin *et al.*, 1997)**. Transmission electron micrographs of infected nasopharyngeal organ cultures reveal MC in large numbers, apparently within phagosomes, suggesting that intracellular replication also occurs in this *ex vivo* setting **(Stephens *et al.*, 1983)**. The significance of intracellular MC replication is unclear although it is likely to

play a role in promoting disease. A mutant was recently identified in a closely related species, *Neisseria gonorhoeae*, which trafficked through polarized epithelial cells at an enhanced rate **(Hopper *et al.*, 2000)**. This fast intracellular trafficking mutant, *fitA*, also had an accelerated intracellular replication phenotype, suggesting that replication may be part of the transcytotic process for pathogenic *Neisseria* spp.

The requirements for MC replication within cultured epithelial cells have not been addressed, but iron acquisition is likely to be a key attribute. Iron is an essential element for nearly all organisms, facilitating fundamental processes such as nucleotide biosynthesis and electron transport **(Conrad and Umbreit, 2000)**. The importance of iron for MC pathogenesis has been well documented, beginning over two decades ago when inorganic iron was found to greatly enhance the lethality of MC injected into mice **(Calver *et al.*, 1976)**. Mammals withhold iron from invading microbes as a non-specific means of defense. Thus, unbound or free iron is in low concentration in the body. The glycoproteins transferrin and lactoferrin effectively chelate iron on mucosal surfaces and in the bloodstream. Iron availability in the blood is further decreased during hypoferremia, a mammalian response to infection in which the reticuloendothelial system removes transferrin from the circulation **(Weinberg, 1985)**.

MC is able to circumvent the iron-withholding tactics of its human host **(Schryvers and Stojiljkovic, 1999)**. It can assimilate iron *via* outer membrane receptors for human transferrin and lactoferrin. The MC transferrin receptor, TbpA/B, and lactoferrin receptor, LbpA/B, bind their iron-loaded ligands at the bacterial outer membrane. They next facilitate the removal of iron from these carriers and its subsequent internalization into the bacterial periplasm **(Gray-Owen and Schryvers, 1996)**. Utilization of transferrin and lactoferrin iron by MC also requires FbpA, a 37-kDa ferric binding protein that is thought to deliver iron from these receptors to the inner membrane transporter FbpB/C **(Khun *et al.*, 1998)**. Heme, either free or as a component of hemoglobin, is also an iron source for MC. Two outer membrane receptors, HmbR and/or HpuA/B, allow MC to bind hemoglobin and remove and internalize heme **(Lewis *et al.*, 1997; Lewis *et al.*, 1998; Stojiljkovic *et al.*, 1996)**. Uptake of free heme by MC is poorly understood. Numerous attempts to identify MC mutants that cannot utilize free heme-iron have been unsuccessful, suggesting that redundant heme-iron uptake systems may exist.

MC differs from most pathogenic bacteria in that it does not produce siderophores, small secreted ferric iron chelators that extract iron from host molecules and deliver it to the pathogen *via* membrane-spanning siderophore receptors. MC does, however, produce FetA, an outer membrane receptor that is capable of binding enterobactin, a siderophore secreted by *E. coli*. FetA has

been postulated to allow MC to scavenge iron by binding siderophores secreted by other bacteria colonizing the same mucosal surfaces **(Carson *et al.*, 1999)**.

Uptake of iron by all of the aforementioned outer membrane receptors requires the TonB complex, a highly conserved macromolecule located in the inner membrane and periplasm of Gram-negative bacteria. Proteins in this complex, TonB, ExbB, and ExbD, cooperate in translating the proton-motive force across the inner membrane into conformational changes in TonB-dependent outer membrane receptors **(Moeck and Coulton, 1998)**. MC lacking TonB are unable to grow in media with transferrin, lactoferrin, or hemoglobin as a sole iron source, but retain their ability to grow in media supplemented with free heme or ferric nitrate.

The diverse array of systems utilized by MC to take up iron is evidence of the important role this element plays in promoting its survival within the host. The importance of iron in MC pathogenicity was demonstrated in the infant rat model for MC septicemia. An *hmbR* mutant that could not assimilate hemoglobin iron was injected intraperitoneally into infant rats. This mutant was cleared from the bloodstream of the animal much more efficiently that its wt parent strain, indicating that iron acquisition strongly influences the *in vivo* survival of MC in the bloodstream **(Stojiljkovic *et al.*, 1995)**.

The extracellular environments exploited by MC, such as the nasopharyngeal mucosa, bloodstream and cerebrospinal fluid are supplied with well-characterized iron-containing compounds that are likely to meet the growth requirements of the bacteria **(Schryvers and Stojiljkovic, 1999)**. In contrast, the iron requirements of intracellular MC and the iron sources available to them in this environment are unknown. Many intracellular pathogens, such as *Chlamydia pneumonia, Legionella pneumophila* and *Mycobacterium tuberculosis* must acquire iron from their host cell in order to replicate **(Al-Younes *et al.*, 2001; Olakanmi *et al.*, 2000; Pope *et al.*, 1996)**. We hypothesized that MC replication in the intracellular niche would also require acquisition of iron from the host cell. In this report we present evidence that replication of MC within cultured A431 human endocervical epithelial cells requires iron uptake. We demonstrate that iron is derived from the host cell and not the media. We show that mutants unable to utilize hemoglobin, transferrin or lactoferrin replicate normally within cells. In contrast, a *tonB* mutant is deficient in intracellular growth. These data strongly suggest that MC replication within epithelial cells requires a novel iron source and that uptake of this iron source is TonB-dependent.

Materials and Methods

Cell Culture

The A431 human endocervical epithelial cell line, obtained from S. Schmid, was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco BRL). According to manufacturers specifications, DMEM contains only picomolar quantities of iron, while FCS contains approximately 15μM of both bovine transferrin and bovine hemoglobin. Cells were used for bacterial infection experiments between the 8th and 15th passages.

Bacterial strains

Information for all strains used in this study is shown in Table 1. MC strain 8013.6 is a serogroup C isolate from a patient at the Pasteur Hospital **(Nassif *et al.*, 1993)**. 8013.6 is a capsulated, piliated strain producing high-adhesive pilin. All mutants constructed for this study were obtained by transformation of 8013.6 with chromosomal DNA from published mutants (Table 1). All mutants were piliated as judged by colony morphology and immunoblotting with anti-pilin antisera. All strains were Opa negative as judged by western immunoblotting and their iron-uptake phenotypes were verified by growth on Desferal plates spotted with the appropriate iron source. All strains were phase off for HpuA/B, the neisserial hemoglobin-haptoglobin receptor. Inactivation of the relevant

genes was verified by PCR. All MC strains were maintained on GCB agar plates with Kellog's Supplement I and II. Strains were passed no more than twice before assays and bacterial inocula were harvested from plates 12-16 hours after passage.

Intracellular growth-rate assay

A431 cells were seeded into 12-well plates and grown to 80% confluency before infection. WT 8013.6 or its mutant derivatives were swabbed from GCB plates, directly suspended in DMEM+10% FCS and diluted in the same medium to allow for infection at a multiplicity of infection (MOI) of 2 (2 bacteria per cell). Infected cultures were incubated for 12 hrs, then rinsed 3X in Phosphate Buffered Saline (PBS, Gibco BRL) to remove non-adherent bacteria. Cultures were then incubated for 1 hr in fresh pre-warmed media containing 50 μg/ml Gentamicin (Gibco BRL) to kill extracellular bacteria. Gentamicin was then removed and the cells gently rinsed 3X in PBS. Epithelial cells were lifted and disrupted by incubating them for 3 min in PBS containing 2mM EDTA and 0.05% Saponin (Sigma), followed by transfer to Eppendorf tubes and vortexing at high speed for 30 sec. The lysed host cells were plated at appropriate dilutions on GCB agar to determine MC colony forming units (CFUs). Ferric nitrate (10μM, Sigma) or Desferal (100μM, Ciba-Geigy) were added to selected assays as described in the text and Figure 1. Values for each time point were derived from 3-5 infected

cultures. Error bars represent standard deviation of the mean. All assays were repeated at least 3X with similar results.

MC growth rate in liquid media

To monitor the growth of MC 8013.6 and MC 8013.6 *hmbR*::Kan in tissue culture media (in the absence of epithelial cells), bacteria were swabbed from GCB plates and suspended in DMEM+10% FCS (without phenol red) to an initial OD_{600} of 0.05 as measured with a Beckman 600 series spectrophotometer and the OD_{600} of the cultures was monitored at various time points over 8 hrs. CFUs were also determined at the beginning and ending of each assay to verify that the OD_{600} readings corresponded to viable bacteria. In order to examine the effect of Desferal treatment on the replication of MC 8013.6 in the absence of cells, the bacteria were pre-incubated in DMEM+10% FCS for 12 hours. Bacteria were then isolated by centrifugation and resuspended in fresh DMEM + 10% FCS lacking phenol red. OD_{600} was monitored with or without 100μM Desferal.

Results

Iron chelation inhibits MC replication within epithelial cells

In order to study the role of iron in MC intracellular replication, we first examined its replication within cells. A431 epithelial cells, which have been shown previously to support MC cell entry and subsequent intracellular replication **(Lin *et al.*, 1997)**, were infected with MC strain 8013.6, a Serogroup C isolate **(Nassif *et al.*, 1993)**. Intracellular growth was quantified by means of the Gentamicin protection assay **((Isberg and Falkow, 1985)**; Figure 1). Briefly, A431cells were infected with MC at an MOI of two. After 12 hrs, extracellular bacteria were killed by incubating the cultures with Gentamicin for 1 hr. The termination of the Gentamicin treatment was designated 0 hr. At various times after removal of Gentamicin, intracellular CFUs were quantified. Under such conditions, MC 8013.6 replicated within A431 cells, their intracellular numbers increasing 5-fold over the six hrs following Gentamicin treatment (Figure 2). These results are similar to earlier reports **(Lin *et al.*, 1997)**. Examination of MC-infected cells by immunofluorescence microscopy verified the intracellular location of MC as well as the increase in intracellular cell counts (data not shown).

We next determined whether MC intracellular growth required iron acquisition. Iron uptake of infected cultures was blocked pharmacologically by incubating the cultures with the iron-specific chelator Desferal (deferoxamine mesylate).

Desferal forms stable chelates with ferric iron and the chelated iron cannot be assimilated by MC **(Yancey and Finkelstein, 1981)**. Desferal is a hydrophobic molecule, which enters cells through fluid-phase endocytosis where it can chelate iron in the endosomal and lysosomal compartments. Desferal diminishes cytosolic iron levels via an indirect mechanism **(6).** This treatment has been shown to inhibit intracellular replication of several bacterial pathogens, including *Chlamydia pneumoniae* and *Legionella pneumophila* **(Al-Younes *et al.*, 2001; Pope *et al.*, 1996)**. Desferal is routinely used to treat human iron-overload disorders and is not toxic to tissue culture cells at the concentrations used in our assays **(Cooper *et al.*, 1996)**. Desferal was added to infected cultures after Gentamicin removal and at various times intracellular CFUs were determined. Results show that growth of 8013.6 within Desferal-treated epithelial cells was significantly decreased, compared to growth in control untreated cells (Figure 2). This effect was reversed when Desferal treated cells were incubated with an equimolar amount of ferric nitrate (data not shown).

One possible explanation for the negative effect of Desferal on MC intracellular growth is toxicity of the chelator to the infecting bacteria. However, Desferal did not affect bacterial growth in tissue culture media alone (data not shown). Taken together, these results suggest that iron is necessary for MC replication within epithelial cells, and that Desferal chelates iron from an intracellular source.

Our results additionally suggest that iron stored within the bacteria during the extracellular growth period cannot fulfill the iron requirements of intracellular growth. MC can take up iron from bovine hemoglobin via its hemoglobin receptor, HmbR **(Stojiljkovic *et al.*, 1996)**. The infection medium, DMEM+10% FCS (Fetal Calf Serum), contains ~15μM bovine hemoglobin, a concentration that is capable of fulfilling the iron requirements of MC 8013.6 via HmbR **(28)**. The bacteria in these experiments were, therefore, in iron-replete media prior to entering the epithelial cells.

Iron for intracellular replication of MC is not derived from tissue culture media

DMEM contains only nanomolar amounts of iron, while FCS contains significant amounts of iron, mostly in the form of bovine transferrin and bovine hemoglobin, each at ~15 μM. Bovine transferrin is not an iron source for MC due to the specificity of the Neisseria transferrin receptor, TbpA/B, for human transferrin **(Schryvers and Gonzalez, 1990)**. On the other hand, bovine hemoglobin may

provide the necessary iron, as the Neisserial hemoglobin receptor, HmbR, permits the utilization of iron from bovine and other animal hemoglobin **(28)**. Desferal does not chelate hemoglobin iron, which led us to hypothesize that the inability of this chemical to affect MC replication in tissue culture media was due the fact that hemoglobin is the only significant MC iron source in DMEM+FCS.

To test our hypothesis that bovine hemoglobin provides the only significant iron source for MC in tissue culture media, we examined the replication rate of a hemoglobin uptake mutant in DMEM+10% FCS. MC 8013.6 *hmbR*::Kan lacks a functional hemoglobin receptor and is unable to grow in media in which hemoglobin is the sole iron source **(Stojiljkovic *et al.*, 1995)**. The 8013.6 *hmbR*::Kan mutant replicated very slowly in DMEM+FCS, compared to MC 8013.6, its parent strain with a functional HmbR (Figure 3). Addition of ferric nitrate to the medium rescued the growth defect of 8013.6 *hmbR*::Kan, allowing the mutant to grow at nearly the wt rate. These results strongly suggest that bovine hemoglobin is the major iron source for MC in the infection assay provided by the media. Since hemoglobin iron is not chelatable by Desferal(Rouault *et al.*, 1985), the effect of this compound on intracellular replication strongly suggests that the iron required for intracellular replication by MC is derived from the host cell, not from the cell culture medium.

An MC mutant unable to acquire iron from tissue culture medium replicates faster than the wt parent strain within epithelial cells

To provide further evidence that iron acquired from the host cell is required for intracellular replication of MC, we quantified the intracellular replication rate of MC 8013.6 *hmbR*::Kan using the Gentamicin protection assay. This mutant replicated poorly in media without iron supplementation, therefore, it should not replicate well within cultured A431 cells unless an additional iron source is derived from the host cell. As shown in the previous experiment, this mutant cannot replicate effectively in the DMEM+FCS. It therefore does not grow well during the infection stage, prior to entering the epithelial cells, compared to the wt strain. Our intracellular growth protocol was therefore modified to increase the invasion frequency of the mutant, by addition of ferric nitrate to the infection medium. Ferric nitrate was removed by extensive rinsing of the infected cultures prior to Gentamicin treatment and withheld during the subsequent 6 hours of the intracellular growth phase of the assay (Figure 1). Under these conditions, 8013.6 *hmbR*::Kan replicated faster that its wt parent strain within cells (Figure 4). The significant increase in the rate of replication of this mutant within cells compared to its growth rate in tissue culture medium (compare Figures 3 and 4) lends further support to our hypothesis that a host-cell iron source is required for intracellular replication of MC. As the mutant cannot utilize hemoglobin iron, this experiment also rules out the possibility that bovine hemoglobin can reach the Gentamicin-protected bacteria to serve as an intracellular iron source. The

enhanced replication displayed by this mutant may be due to the fact that unlike wildtype MC, it is not conditioned to utilize heme prior to entering cells. The transition to an intracellular iron source may be more efficient for this mutant.

Transferrin is not the intracellular MC iron source

Transferrin has been shown to serve as an intracellular iron source for several pathogenic bacteria including *Ehrlichia chaffeensis, Mycobacterium tuburculosis, Mycobacterium avium* complex, and *Chlamydia pneumoniae* **(Al-Younes *et al.*, 2001; Barnewall and Rikihisa, 1994; Olakanmi *et al.*, 2000)**. As stated above, the transferrin in our assays is bovine in origin and, therefore, cannot support MC growth in liquid media **(Schryvers and Gonzalez, 1990)**. The formal possibility, however, remained that bovine transferrin may serve as an intracellular iron source for MC. Transferrin enters cells *via* endocytosis. During endosomal acidification, transferrin undergoes conformational changes and releases its cargo of iron **(Dautry-Varsat *et al.*, 1983; Ponka and Lok, 1999)**. It was possible that bovine transferrin in a lower pH compartment could undergo a conformational change that would enable its binding to the MC transferrin receptor, TbpA/B. To explore this possibility, we studied the intracellular replication of the MC 8013.6 mutant *fbpA*::Ω. This mutant is unable to utilize transferrin or lactoferrin as an iron source because it lacks the periplasmic iron binding-protein FbpA, which is thought to transfer iron to the inner membrane

transporter, FbpB/C **(Khun *et al.*, 1998)**. In the Gentamicin protection assay, *fbpA*::Ω replicated as well as its wt parent strain within A431 cells (Figure 5). These results lend further support to the argument that transferrin is not the intracellular iron source for MC.

The MC tonB mutant is defective for intracellular replication, but the defect can be rescued by iron supplementation

Having ruled out both hemoglobin and transferrin as intracellular iron sources for MC, we next examined the replication of an MC mutant that is more broadly deficient in iron acquisition. MC 8013.6 *tonB*::Kan is unable to utilize hemoglobin, transferrin, or lactoferrin iron **(Stojiljkovic and Srinivasan, 1997)**. This mutant is, however, able to grow in media supplemented with free heme or inorganic iron such as ferric nitrate. The intracellular growth of 8013.6 *tonB*::Kan was monitored using the modified Gentamicin protection assay as described above. Like the *hmbR* mutant, the *tonB* mutant is unable to use hemoglobin iron. To maintain bacterial viability, it was necessary to supplement the medium with ferric nitrate in the first 12 hrs of infection, prior to addition of Gentamicin. Under these conditions, the growth rate of the mutant reached wt levels at the beginning of the intracellular growth phase, indicating that iron supplementation during the infection process permitted vigorous extracellular growth and cell invasion. However, the ability of the mutant to replicate within cells after Gentamicin

protection was impaired, and its intracellular CFUs declined significantly over time (Figure 6). This result suggests that at least one TonB-dependent receptor is necessary for MC to replicate within A431 cells.

In other Gram-negative bacteria, TonB energizes outer membrane receptors that are not involved in iron uptake **(Moeck and Coulton, 1998)**. Although the Neisserial TonB has not been demonstrated to function in uptake of non-iron compounds, the possibility remained that the intracellular growth defect of the *tonB* mutant reflected a defect in the uptake of other compounds besides iron. To demonstrate that the inability of the *tonB* mutant to replicate intracellularly was indeed due to an iron-uptake defect, we attempted to rescue replication of the *tonB* mutant by supplementing the infected cultures with ferric nitrate after Gentamicin removal. Iron supplementation had a marked effect, partially restoring the intracellular growth lesion of the mutant. (Figure 7). The inability of the *tonB* mutant to replicate intracellularly is therefore at least partially due to an iron uptake defect within cells. The inability of ferric nitrate to fully rescue the intracellular replication defect may indicate that TonB serves an additional function aside from iron uptake. Alternately, it may mean that ferric nitrate is not delivered efficiently to intracellular MC.

Discussion

Neisseria meningitidis is a successful pathogen in part because it can exploit multiple environments within the human host, acquiring nutrients at each site for its persistence, propagation and dissemination. Iron is a key nutrient in determining bacterial pathogenicity (Ratledge and Dover, 2000). Multiple iron-uptake systems – hemoglobin, transferrin, and lactoferrin – have been shown to perform essential functions for MC replication at extracellular sites within the human host (Schryvers and Stojiljkovic, 1999). In this study we provided both pharmacological and genetic evidence for a novel iron uptake system that plays a crucial role in MC replication within cultured epithelial cells. The iron chelator Desferal inhibited intracellular replication of MC while leaving its extracellular growth unaffected. An *hmbR* mutant, which was defective in growth in liquid media, replicated more rapidly than its wt parent strain within epithelial cells. These two results suggest that iron uptake is essential for intracellular growth of MC, and that the element is derived from a host-cell source. Most importantly, a *tonB* mutant was unable to replicate within epithelial cells without ferric nitrate supplementation. This result implicates a TonB-dependent receptor in the acquisition of this intracellular iron.

The intracellular iron source remains unidentified, however, transferrin, lactoferrin, hemoglobin, free heme, and free iron are unlikely candidates (see Table 1). An *fbpA* mutant that cannot utilize transferrin or lactoferrin iron replicated normally within cells, thus ruling out these iron-containing compounds as intracellular iron sources. An *hmbR* mutant replicated faster than its wt parent within cells, thus ruling out hemoglobin. A *tonB* mutant that grows well on free heme or free iron sources was unable to grow within epithelial cells without iron supplementation, indicating that these iron sources may not be available to intracellular MC. Taken together, our results predict that MC obtains its intracellular iron from a novel source.

A heme-based iron source appears to be an unlikely candidate for two reasons. First, it has been demonstrated that a *Neisseria* mutant that requires exogenous heme for growth is unable to replicate within epithelial cells (Turner *et al.*, 1998). Second, Desferal cannot chelate heme-iron and therefore should not interfere with heme-iron uptake. Yet, it strongly inhibits MC intracellular growth. While the identity of the intracellular iron source is unknown, clues to its identify may lie in the recently published MC genome sequences. An examination of these databases revealed several candidates for TonB-dependent receptors whose functions are unknown. Their role in MC intracellular growth is being examined.

In summary, we have provided evidence of a novel iron uptake system in *N. meningitidis* that utilizes a host cell iron source for its growth within human epithelial cells in culture. Intracellular replication may promote its crossing of the epithelial barrier and the development of septicemia and/or meningitis. The identification of this iron uptake system in MC may therefore provide an additional target for anti-meningococcal therapy. In addition, identification of the host cell iron source may shed new light on mammalian iron metabolism.

Acknowledgements

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References

1. **Al-Younes, H. M., T. Rudel, V. Brinkmann, A. J. Szczepek, and T. F. Meyer.** 2001. Low iron availability modulates the course of Chlamydia pneumoniae infection. Cell Microbiol. **3:**427-37.

2. **Barnewall, R. E., and Y. Rikihisa.** 1994. Abrogation of gamma interferon-induced inhibition of Ehrlichia chaffeensis infection in human monocytes with iron-transferrin. Infect Immun. **62:**4804-10.

3. **Calver, G. A., C. P. Kenny, and G. Lavergne.** 1976. Iron as a replacement for mucin in the establishment of meningococcal infection in mice. Can J Microbiol. **22:**832-8.

4. **Carson, S. D., P. E. Klebba, S. M. Newton, and P. F. Sparling.** 1999. Ferric enterobactin binding and utilization by Neisseria gonorrhoeae. J Bacteriol. **181:**2895-901.

5. **Conrad, M. E., and J. N. Umbreit.** 2000. Iron absorption and transport-an update. Am J Hematol. **64:**287-98.

6. **Cooper, C. E., G. R. Lynagh, K. P. Hoyes, R. C. Hider, R. Cammack, and J. B. Porter.** 1996. The relationship of intracellular iron chelation to the

inhibition and regeneration of human ribonucleotide reductase. J Biol Chem. **271:**20291-9.

7. **Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish.** 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. Proc Natl Acad Sci U S A. **80:**2258-62.

8. **Gray-Owen, S. D., and A. B. Schryvers.** 1996. Bacterial transferrin and lactoferrin receptors. Trends Microbiol. **4:**185-91.

9. **Hopper, S., J. S. Wilbur, B. L. Vasquez, J. Larson, S. Clary, I. J. Mehr, H. S. Seifert, and M. So.** 2000. Isolation of Neisseria gonorrhoeae mutants that show enhanced trafficking across polarized T84 epithelial monolayers. Infect Immun. **68:**896-905.

10. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by Yersinia pseudotuberculosis permits invasion of cultured cells by Escherichia coli K-12. Nature. **317:**262-264.

11. **Khun, H. H., S. D. Kirby, and B. C. Lee.** 1998. A Neisseria meningitidis fbpABC mutant is incapable of using nonheme iron for growth. Infect Immun. **66:**2330-6.

12. **Lewis, L. A., E. Gray, Y. P. Wang, B. A. Roe, and D. W. Dyer.** 1997. Molecular characterization of hpuAB, the haemoglobin-haptoglobin- utilization operon of Neisseria meningitidis. Mol Microbiol. **23:**737-49.

13. **Lewis, L. A., M. H. Sung, M. Gipson, K. Hartman, and D. W. Dyer.** 1998. Transport of intact porphyrin by HpuAB, the hemoglobin-haptoglobin utilization system of Neisseria meningitidis. J Bacteriol. **180:**6043-7.

14. **Lin, L., P. Ayala, J. Larson, M. Mulks, M. Fukuda, S. R. Carlsson, C. Enns, and M. So.** 1997. The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. Mol Microbiol. **24:**1083-94.

15. **Merz, A. J., and M. So.** 2000. Interactions of pathogenic neisseriae with epithelial cell membranes. Annual reviews in Cell and Developmental Biology. **16:**423-57.

16. **Moeck, G. S., and J. W. Coulton.** 1998. TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. Mol Microbiol. **28:**675-81.

17. **Nassif, X., J. Lowy, P. Stenberg, P. O'Gaora, A. Ganji, and M. So.** 1993. Antigenic variation of pilin regulates adhesion of Neisseria meningitidis to human epithelial cells. Mol Microbiol. **8:**719-25.

18. **Olakanmi, O., B. E. Britigan, and L. S. Schlesinger.** 2000. Gallium disrupts iron metabolism of mycobacteria residing within human macrophages. Infect Immun. **68:**5619-27.

19. **Ponka, P., and C. N. Lok.** 1999. The transferrin receptor: role in health and disease. Int J Biochem Cell Biol. **31:**1111-37.

20. **Pope, C. D., W. O'Connell, and N. P. Cianciotto.** 1996. Legionella pneumophila mutants that are defective for iron acquisition and assimilation and intracellular infection. Infect Immun. **64:**629-36.

21. **Ratledge, C., and L. G. Dover.** 2000. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol. **54:**881-941.

22. **Raulston, J. E.** 1997. Response of Chlamydia trachomatis serovar E to iron restriction in vitro and evidence for iron-regulated chlamydial proteins. Infect Immun. **65:**4539-47.

23. **Rouault, T., K. Rao, J. Harford, E. Mattia, and R. D. Klausner.** 1985. Hemin, chelatable iron, and the regulation of transferrin receptor biosynthesis. J Biol Chem. **260:**14862-6.

24. **Schryvers, A. B., and G. C. Gonzalez.** 1990. Receptors for transferrin in pathogenic bacteria are specific for the host's protein. Can J Microbiol. **36:**145-7.

25. **Schryvers, A. B., and I. Stojiljkovic.** 1999. Iron acquisition systems in the pathogenic Neisseria. Mol Microbiol. **32:**1117-23.

26. **Stephens, D. S., L. H. Hoffman, and Z. A. McGee.** 1983. Interaction of *Neisseria meningitidis* with human nasopharyngeal mucosa: attachment and entry into columnar epithelial cells. . **148:**369-376.

27. **Stojiljkovic, I., V. Hwa, L. de Saint Martin, P. O'Gaora, X. Nassif, F. Heffron, and M. So.** 1995. The Neisseria meningitidis haemoglobin receptor: its role in iron utilization and virulence. Mol Microbiol. **15:**531-41.

28. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic Neisseria spp.: iron- regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J Bacteriol. **178:**4670-8.

29. **Stojiljkovic, I., and N. Srinivasan.** 1997. Neisseria meningitidis tonB, exbB, and exbD genes: Ton-dependent utilization of protein-bound iron in Neisseriae. J Bacteriol. **179:**805-12.

30. **Turner, P. C., C. E. Thomas, C. Elkins, S. Clary, and P. F. Sparling.** 1998. Neisseria gonorrhoeae heme biosynthetic mutants utilize heme and hemoglobin as a heme source but fail to grow within epithelial cells. Infect Immun. **66:**5215-23.

31. **Weinberg, E. D.** 1985. Roles of iron in infection and neoplasia. J Pharmacol. **16:**358-64.

32. **Yancey, R. J., and R. A. Finkelstein.** 1981. Assmilation of iron by pathogenic Neisseria spp. Infect Immun. **32:**592-9.

TABLE 1. MC Strains used in this study and relevant phenotypes

Strain	Replication in DMEM with iron source[a]							Intracellular replication	Reference
	Hb	hTf	bTf	hLf	Fe	Heme	FCS		
MC 8013.6	+	+	−	+	+	+	+	+	17
MC 8013.6 *hmbR*::Kan	−	+	−	+	+	+	−	+	27
MC 8013.6 *tonB*::Kan	−	−	−	−	+	+	−	−	29
MC 8013.6 *fbpA*::Ω	+	−	−	−	+	+	+	+	11

[a] Hb, hemoglobin; hTf, human transferrin; bTf, bovine transferrin; hLf, human lactoferrin; Fe, ferric nitrate.

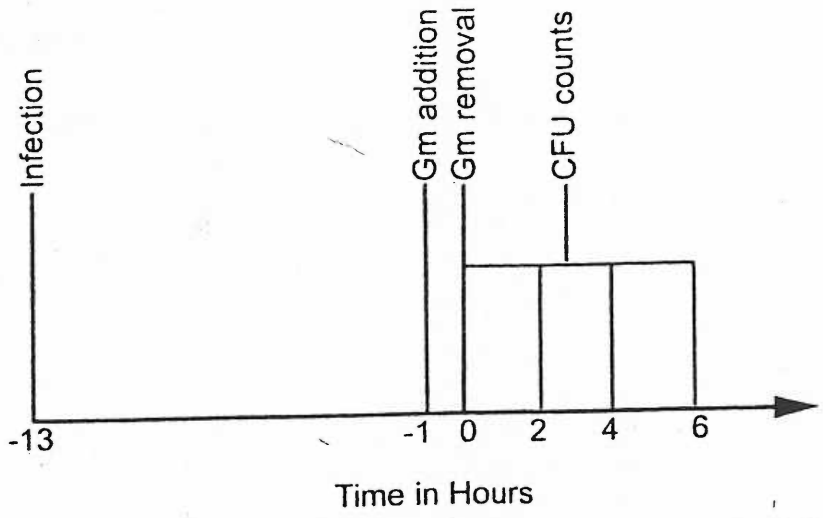

FIG. 1. Gentamicin protection assays were initiated by infecting A431 cells with bacteria as noted ($T = -13$). After 12 h of infection cultures were incubated with gentamicin for 1 h. They were then rinsed thoroughly and incubated further without gentamicin ($T = 0$). At various times, cells were disrupted, and intracellular bacteria were quantified as described in Materials and Methods. Desferal or ferric nitrate was added to some cultures after gentamicin removal ($T = 0$). Some mutants required ferric nitrate to promote growth during the invasion phase of the assay ($T = -13$ to -1).

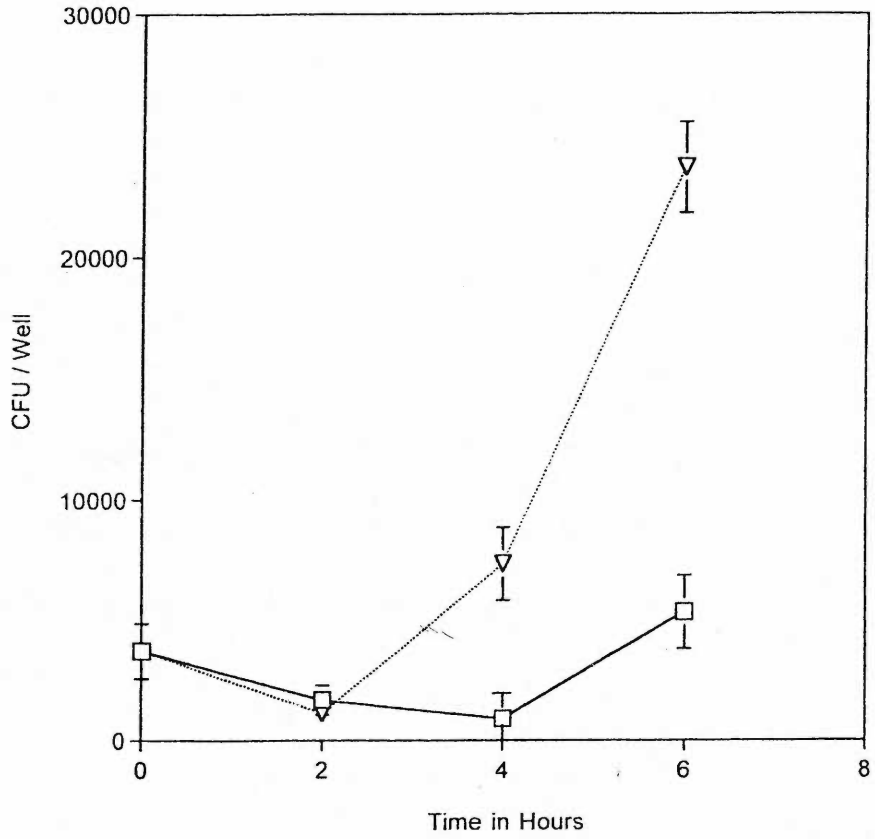

FIG. 2. Intraepithelial replication of MC 8013.6 was monitored by using a gentamicin protection assay (described in Fig. 1). The addition of 100 μM Desferal to the infected cultures after gentamicin removal ($T = 0$ in Fig. 1) resulted in an inhibition of intracellular replication (□) compared to the no-Desferal control (▽). The datum points represent the averages of three or more cultures, with error bars representing the standard deviation.

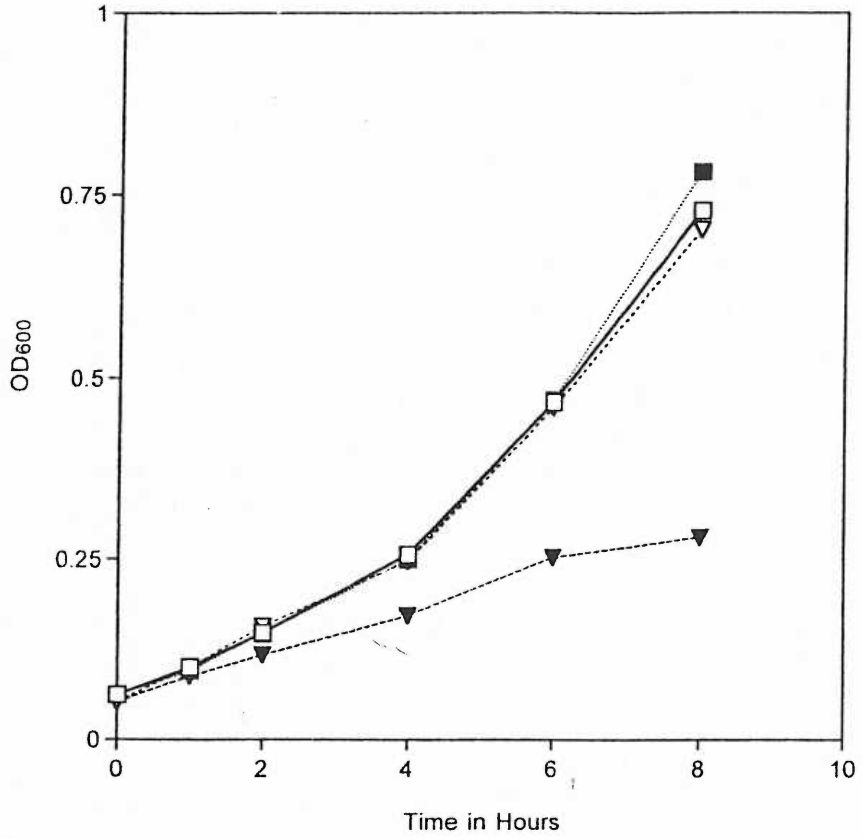

FIG. 3. Replication rates of MC 8013.6 (■), 8013.6 *hmbR*::Kan (▼) in DMEM–10% FCS (without phenol red) were monitored by measuring the OD_{600} for 8 h. Iron supplementation of the media with ferric nitrate rescued the replication defect of the *hmbR* mutant (▽). Growth of the wt strain was not enhanced by ferric nitrate supplementation (□). The viability of the bacteria was determined by plating cultures for the enumeration of CFU. An increase in the OD_{600} corresponded with a comparable increase in the numbers of CFU (data not shown).

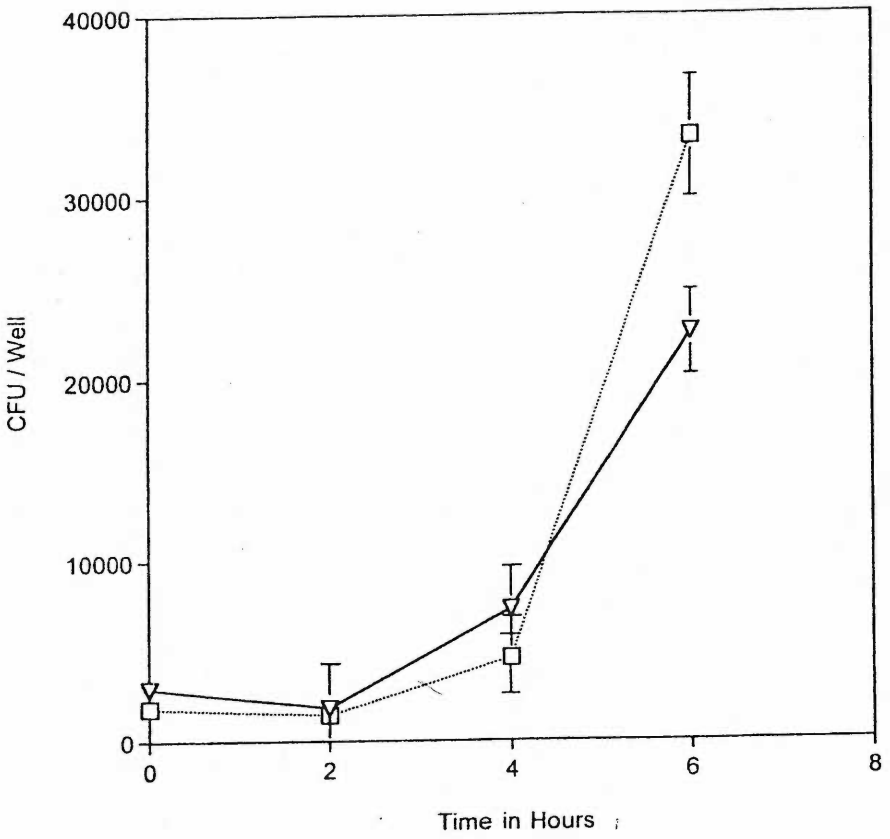

FIG. 4. Intracellular replication rates of MC 8013.6 (▽) and MC 8013.6 *hmbR*::Kan (□) as determined by gentamicin protection assay. Ferric nitrate was included in the invasion media ($T = -13$ to -1 in Fig. 1) to allow efficient growth and entry of the mutant. MC 8013.6 *hmbR*::Kan replicated faster than wt MC 8013.6 within cells. The datum points represent the averages of three or more cultures, with error bars representing the standard deviation.

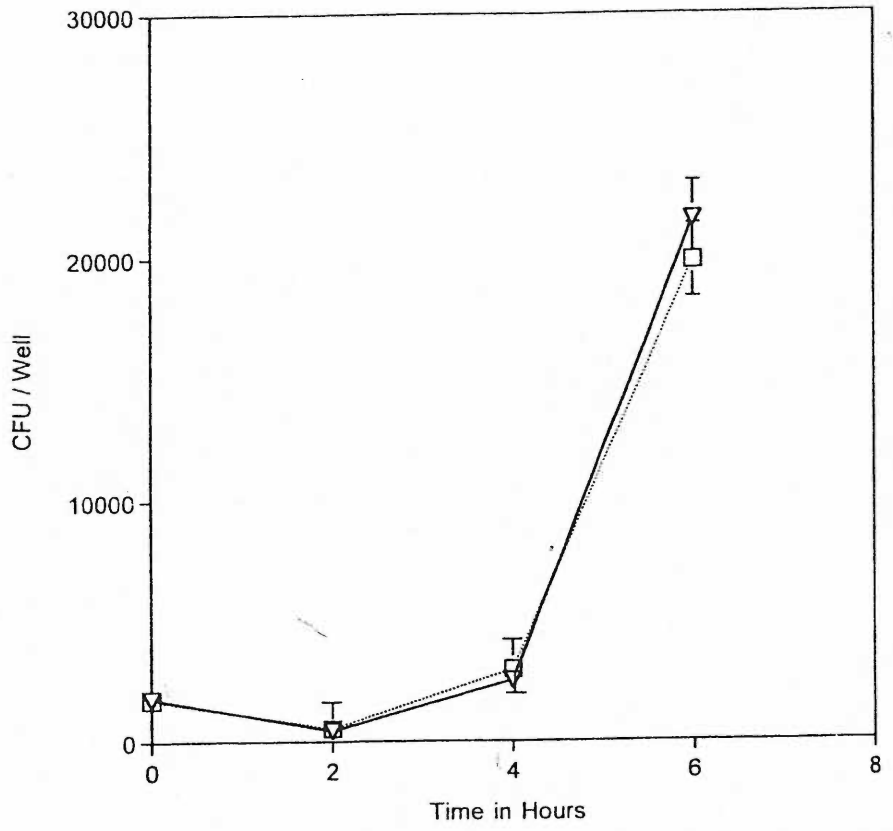

FIG. 5. Intracellular replication rates of MC 8013.6 (▽) and MC 8013.6 *fbpA*::Ω (□) as determined by gentamicin protection assay. MC 8013.6 *fbpA*::Ω replicated similarly to wt MC 8013.6 inside of cells. The datum points represent the averages of three or more cultures, with error bars representing the standard deviation.

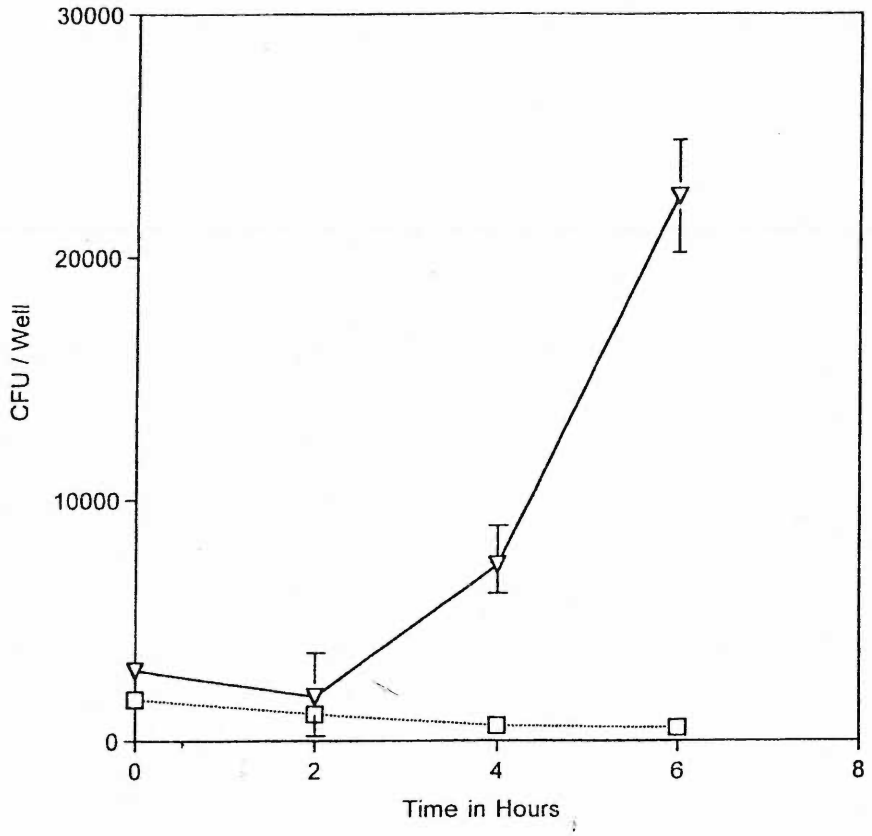

FIG. 6. Intracellular replication rates of MC 8013.6 (▽) and MC 8013.6 *tonB*::Kan (□) determined by the gentamicin protection assay. Ferric nitrate was included in the invasion media ($T = -13$ to -1 in Fig. 1) to allow efficient growth and entry of the mutant. MC 8013.6 *tonB*::Kan failed to replicate within A431 cells. The datum points represent the averages of three or more cultures, with error bars representing the standard deviation.

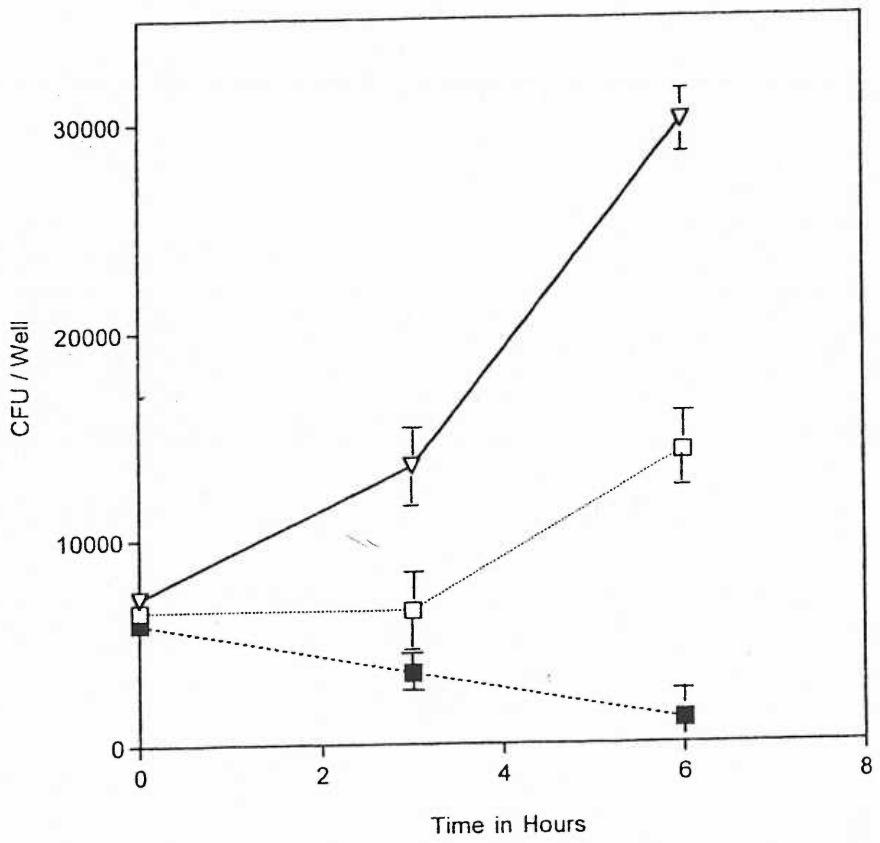

FIG. 7. Intracellular replication of MC 8013.6 *tonB*::Kan was rescued by ferric nitrate supplementation after gentamicin killing ($T = 0$ to 6 in Fig. 1). Supplementation of the media with ferric nitrate after gentamicin killing ($T = 0$ in Fig. 1) rescued intracellular replication (□) compared to non-iron-supplemented cultures (■). Replication was not rescued to wt (▽) level. The datum points represent the average of three or more cultures, with error bars representing the

Chapter 3: Manuscript 2

Neisseria meningitidis accelerates ferritin degradation in host epithelial cells to yield an essential iron source

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Summary

In order to colonize humans and cause disease, pathogenic bacteria must assimilate iron from their host. The vast majority of non-heme iron in humans is localized intracellularly, within the storage molecule ferritin. Despite the vast reserves of iron within ferritin, no pathogen has previously been demonstrated to exploit this molecule as an iron source. Here we show that the gram-negative diplococcus *Neisseria meningitidis* can trigger rapid redistribution and degradation of cytosolic ferritin within infected epithelial cells. Indirect immunofluorescence microscopy revealed that cytosolic ferritin is aggregated and recruited to intracellular meningococci (MC). The half-life of ferritin within cultured epithelial cells was found to decrease from 20.1 to 5.3 hours upon infection with MC. Supplementation of infected epithelial cells with ascorbic acid abolished ferritin redistribution and degradation and prevented intracellular MC from replicating. The lysosomal protease inhibitor leupeptin slowed ferritin turnover and also retarded MC replication. Our laboratory has recently shown that MC reduce transferrin uptake in infected cells (Bonnah *et al.*, 2000), and that the infected cells have a transcriptional profile that indicates iron starvation (Bonnah *et. al.* submitted). In light of these findings, we conclude that accelerated ferritin degradation occurs as a response to an iron starvation state induced by MC infection and that ferritin degradation provides intracellular MC with a critical source of iron.

Introduction

The gram-negative diploccocus *Neisseria meningitidis* remains a major cause of meningitis and septicemia throughout the world. One key determinant in the pathogenesis of this organism is its ability to acquire iron from the human host. In nearly all organisms, iron serves as an essential cofactor for a wide range of functions such as nucleotide biosynthesis and electron transport. In higher organisms, the low solubility of ferric iron and its potential toxicity necessitate a complex network of molecules to coordinate its uptake, transport, and storage (Ponka, 1999a).

Free iron is present in extremely low concentrations within the human body. This presents a nutritional barrier to invading microbes. In order to colonize humans, bacteria must acquire iron from host proteins (Weinberg, 1985). Many bacteria acquire iron by secreting small iron-binding molecules called siderophores that compete with host carrier-molecules for iron and deliver it to bacterial surface receptors (Crosa, 1989),

MC lives exclusively within the human host. Perhaps due to its highly specific niche MC does not secrete siderophores. Instead MC has evolved outer membrane receptors that confiscate iron directly from host proteins (Schryvers and Stojiljkovic, 1999). The importance of iron acquisition in the development of

meningococcal disease has been established in animal models that simulate the late stages of MC infection in humans (Calver *et al.*, 1976; Klee *et al.*, 2000; Schryvers and Gonzalez, 1989; Stojiljkovic *et al.*, 1995). In all of these models, the inability of MC to acquire iron corresponded with a strong attenuation of virulence.

MC deploys several outer membrane receptors that allow it to access iron at specific locations within the host. These receptors share a requirement for the TonB complex, a multimeric energy transduction system located in the neisserial inner membrane and periplasm. This complex transduces the proton motive force across the inner membrane to facilitate the uptake of molecules, such as iron, by outer membrane receptors (Braun and Braun, 2002a).

The first significant interaction between MC and its host occurs on the nasopharyngeal mucosa. On the human mucosal surface, iron is bound by the glycoprotein lactoferrin. The neisserial LbpA/B two-component receptor is believed to allow MC to obtain iron from lactoferrin while colonizing the nasopharynx (Schryvers and Stojiljkovic, 1999).

In order to produce disease, MC must disseminate to the bloodstream, where it can cause septicemia, and sometimes cross the blood-brain barrier to the cerebrospinal fluid where it can trigger meningitis (Nassif and So, 1995). In the

bloodstream most iron is contained within red blood cells as a component of hemoglobin. Spontaneous lysis of red blood cells results in a low basal level of hemoglobin within serum that is sequestered by the abundant human glycoprotein haptoglobin (Schryvers and Stojiljkovic, 1999). The neisserial HpuA/B receptor complex allows MC to liberate heme from free hemoglobin or hemoglobin-haptoglobin and thereby utilize heme as an iron source (Lewis *et al.*, 1997). A second receptor, HmbR, allows removal of heme from free hemoglobin, but not from hemoglobin-haptoglobin (Stojiljkovic *et al.*, 1996). HmbR is likely to be important during septicemia, when increased red blood cell lysis can elevate free hemoglobin levels in the serum (Rohde and Dyer, 2003).

Serum also contains high levels of transferrin, a glycoprotein that transports iron throughout the body. MC removes transferrin iron via the TbpA/B receptor complex (Schryvers and Stojiljkovic, 1999). Transferrin is also present in the cerebrospinal fluid (Bradbury, 1997), so it is likely to serve as an iron source during the progression of meningitis.

Dissemination of MC to the bloodstream is believed to occur via transcytosis of the nasopharyngeal epithelial barrier (Merz and So, 2000). MC has the ability to invade cultured epithelial cells and replicate within them (Lin *et al.*, 1997). Intracellular replication increases the transcytosis frequency of *Neisseria* through polarized epithelial monolayers (Hopper *et al.*, 2000) and may, therefore,

promote dissemination from the epithelial barrier to deeper tissues and the bloodstream.

The intracellular behavior of MC is currently receiving intense focus. In addition to being the launching point for dissemination, intra-epithelial MC is protected from antibody-mediated immune surveillance. The intracellular environment may also provide nutrients to the bacteria that are not available on the mucosal surface. The ability of MC to invade and replicate within host epithelial cells may, therefore, be a critical determinant in both the establishment of the carrier state and the development of disease.

We recently demonstrated that intracellular MC must acquire iron from the epithelial cell in order to replicate. This process was shown to be TonB-dependent, yet none of the previously characterized outer membrane receptors were involved in intracellular iron uptake (Larson *et al.*, 2002). Thus, MC appears to utilize a novel iron source for intracellular replication through an unidentified TonB-dependent receptor.

We have further examined the parameters of intracellular iron acquisition by MC. This examination focused on ferritin, which stores the vast majority of non-heme iron in the human body (Stojiljkovic and Perkins-Balding, 2002). Ferritins are ubiquitous molecules found in both prokaryotic and eukaryotic cells that store

iron in the ferric form to prevent iron-catalyzed free-radical generation. Human ferritin is a large molecule (MW 480,000) composed of 24 subunits assembled into a spherical cage. Ferritin subunits of two types, heavy and light chain, assemble in various ratios to allow the storage of up to 4,000 iron atoms within the core as an iron-oxide biomineral (Theil, 1990, 2003).

Human cells rely upon ferritin to supply iron for their metabolic needs. Under iron limiting conditions, cytosolic ferritin is autophagocytized and subsequently degraded within lysosomes (Bridges, 1987; Radisky and Kaplan, 1998; Roberts and Bomford, 1988). Iron released from degraded ferritin is redistributed to the cytoplasm through an undefined pathway (Radisky and Kaplan, 1998). Ascorbic acid (Vitamin C) inhibits autophagy of ferritin and its subsequent degradation (Bridges, 1987). Ascorbate can also release iron from the ferritin core via reduction. It is probable that iron can be recycled from ferritin directly within the cytoplasm when a sufficient amount of ascorbate is present, thus bypassing the need for the degradative pathway.

In this report we describe additional parameters of intracellular iron uptake by MC. We demonstrate that MC triggers rapid degradation of ferritin within infected epithelial cells. We show that ferritin degradation and intracellular replication of MC are inhibited by ascorbic acid, and by leupeptin, which inhibits lysosomal proteases. We also provide further evidence that iron derived from ferritin

supports intracellular replication of MC. To the best of our knowledge, this is the first evidence of ferritin iron utilization by a human pathogen.

Results

Heme is not an intracellular iron source for MC

Iron acquisition by MC within cultured epithelial cells is TonB-dependent. Neisserial TonB-dependent receptors can be separated into two general classes: those that remove and transport free iron and those that remove and transport iron incorporated into heme. We previously reported that the iron chelator Desferal inhibits intracellular replication of MC (Larson *et al.*, 2002). Desferal cannot chelate iron incorporated into heme, making heme an unlikely candidate for an intracellular iron source. In another recent study, it was shown that a neisserial mutant defective in porphyrin synthesis could not replicate within A431 cells (Turner *et al.*, 1998). This mutant requires an exogenous porphyrin source such as heme in order to replicate. This strain, therefore, does not appear to acquire heme from host cells.

In order to directly rule out heme as an intracellular iron source, we examined the replication efficiency of a *hemO* mutant using a Gentamicin protection assay (Larson *et al.*, 2002). This mutant strain lacks a functional heme oxygenase enzyme that breaks down heme within the bacterial cytosol and allows iron

removal (Zhu *et al.*, 2000). This strain cannot grow with heme as a sole iron source. The *hemO* mutant replicated with the same efficiency as wildtype MC within A431 cells (Fig. 1), providing further evidence that heme is not utilized by intracellular MC.

Transferrin inhibits intracellular replication of MC

In a previous report we demonstrated that an *fbpA* mutant that cannot utilize transferrin as a sole iron source replicates normally within A431 cells (Larson *et al.*, 2002). This provided evidence that MC cannot utilize transferrin-bound iron within cells. Epithelial cells take up transferrin via receptor-mediated endocytosis. The low pH of transferrin-containing endosomes facilitates the release of iron from transferrin (Ponka, 1999a). Iron is then transported out of the endosome to the cytosol via the membrane protein Nramp2/Dmt1 (Tabuchi *et al.*, 2000). It is therefore possible that transferrin-derived iron is indirectly utilized by intracellular MC after its removal from transferrin.

In order to investigate this possibility, we examined the effect of iron-loaded (holo) transferrin on the replication of intracellular MC. Unexpectedly, we found that holotransferrin inhibited intracellular replication of MC in a dose-dependent manner (Fig. 1). Cells supplemented with 40μg/ml human holotransferrin were

unable to support MC replication. This result led us to hypothesize that MC replicate more efficiently in an iron-starved host cell.

Iron starvation of host cells increases the replication rate of intracellular MC

To test our hypothesis that MC replicates faster in iron starved host cells, we excluded serum from the tissue culture medium during a portion of the assay. Non-supplemented DMEM contains only nanomolar levels of iron. We compared the replication rate in cells grown in serum-free medium to that in medium with 10% fetal calf serum (FCS). The replication of MC within serum-starved cells was dramatically increased (Fig. 1). To verify that this effect was iron-specific, holotransferrin was added to the serum-free medium. The replication of MC was reduced to below the control level after supplementation with either bovine or human holotransferrin at 20μM (Fig. 1). Iron-starved host cells, therefore, appear to be a more conducive environment for MC replication.

Serum starvation has been demonstrated to increase the Desferal-chelatable iron pool in cultured hepatocytes (Ollinger and Roberg, 1997). We have previously shown that Desferal prevents replication of MC within A431 cells (Larson *et al.*, 2002). This effect was also observed in serum-starved cells (data not shown). The Desferal-chelatable iron pool, therefore, should contain the meningococcal iron source. The increased Desferal-chelatable iron pool in serum-starved hepatocytes is derived from degraded ferritin (Ollinger and

Roberg, 1997). In many cell lines, degradation of cytosolic ferritin during iron starvation occurs via autophagy followed by lysosomal degradation (Ollinger and Roberg, 1997; Radisky and Kaplan, 1998; Tabuchi *et al.*, 2000). We next explored the possibility that ferritin was being degraded within MC-infected cells.

MC infection triggers rapid degradation of host epithelial cell ferritin

Using an Enzyme Linked Immunosorbent Assay (ELISA), we monitored ferritin within infected A431 cells. Ferritin levels rapidly declined in infected cells, with less than 10% remaining after 12 hrs (Fig. 2). Ferritin levels did not change in uninfected cells during the same period. The decrease in ferritin levels upon infection was not due to a reduction in ferritin mRNA levels. Using quantitative real time RT-PCR, we found that the levels of ferritin heavy and light chain transcripts decreased only slightly throughout the infection (Fig. 2).

Ferritin levels in human cells are also regulated post-transcriptionally by IRP's (Iron Regulatory Proteins) that bind to ferritin mRNA and repress ferritin translation (Ponka, 1999a). Our lab has found that MC infection reduces IRP binding activity (Bonnah *et.al.* manuscript submitted), a result that shows post-transcriptional regulation in the opposite direction of ferritin reduction. Since the diminished levels of ferritin shown by the ELISA were not due to the repression of mRNA or translation, we next determined whether ferritin turnover was accelerated in infected cells.

In order to determine the half-life of ferritin, pulse-chase experiments were performed using [^{35}S] cysteine and methionine, followed by immunoprecipitation with a polyclonal ferritin antibody (Fig. 3). A431 cells express predominately ferritin heavy chain. We found that infected cells displayed a rapid turnover of ferritin ($t_{1/2}$ = 5.3 hrs compared to $t_{1/2}$ = 20.1 hrs in uninfected cells). The diminished ferritin levels observed with the ELISA are, therefore, a result of degradation triggered by MC infection. A 37-kDa protein doublet was also precipitated by the ferritin antibody. Unlike ferritin heavy chain, the faster migrating of these two proteins remained constant throughout the infection, providing an internal control for variability in gel loading.

The observed effect of MC infection on ferritin occurs in media supplemented with FCS, which contains bovine transferrin. MC cannot utilize bovine holotransferrin an iron source (Schryvers and Gonzalez, 1989). The depletion of ferritin was, therefore, not due to iron starvation brought about by direct competition for iron between the cells and bacteria.

Our laboratory reported previously that MC disrupt transferrin uptake by epithelial cells (Bonnah *et al.*, 2000). MC infection reduces transferrin receptor mRNA and slows receptor cycling. Based on these observations, we hypothesized that disruption of epithelial cell transferrin uptake by MC induces an iron starvation

response in host cells that results in the lysosomal degradation of cytosolic ferritin.

Cytosolic ferritin is aggregated and recruited to intracellular MC upon infection

Iron starvation has been shown to induce autophagy and subsequent lysosomal degradation of cytosolic ferritin in several cultured cell lines (Konijn *et al.*, 1999; Ollinger and Roberg, 1997; Roberts and Bomford, 1988). In order to determine if MC infection was stimulating a similar process in A431 cells we performed indirect immunofluorescence microscopy to determine if cytosolic ferritin is transported to lysosomes during infection. Using a polyclonal antibody against ferritin and a monoclonal antibody against the lysosomal membrane protein Lamp1, we examined the distribution of cellular ferritin and lysosomes. Cells treated with 100μM Desferal served as a control to study the iron-starvation response in A431 cells.

In uninfected, iron-supplemented cells, ferritin and Lamp1 signals were small and punctate and were distributed throughout the cytosol (Fig. 4A). Ferritin and Lamp1 signals rarely colocalized. In Desferal-treated cells, ferritin and Lamp1 signals colocalized in large aggregates (Fig. 4B). Our results demonstrate that A431 cells respond to iron starvation in a similar way to other cell culture systems. In cells infected with MC for 8 hrs, ferritin signals in the cytosol were greatly diminished. Instead, ferritin signals were clustered at the site of

intracellular bacteria (white arrow, Fig. 4C,D). The ferritin signals did not colocalize with Lamp1 signals. We have previously shown that the neisserial IgA protease can cleave Lamp1 and reduce the levels of a number of lysosomal markers in infected cells (Ayala *et al.*, 1998; Lin *et al.*, 1997). It is, therefore, possible that the punctate ferritin staining in infected cells represents a compartment that is degradative, but does not have typical lysosomal markers.

The lysosomal protease inhibitor leupeptin reduces MC-induced ferritin degradation

The degradation of ferritin that occurs in iron-starved K562 cells was shown to be reduced by the lysosomal protease inhibitor leupeptin (Roberts and Bomford, 1988). We found that treatment of infected A431 cells with 10mM leupeptin partially prevented ferritin degradation (Fig. 5). This observation suggests that lysosomal proteases are at least partially responsible for the degradation of ferritin in MC-infected epithelial cells.

Ferritin iron is redistributed to a low molecular weight pool upon MC infection

Having demonstrated that ferritin is degraded in infected cells, we next investigated the possibility that ferritin-derived iron could support replication of intracellular MC. Ferritin is a large molecule (outside diameter 12-13 nm). It is

unlikely that removal of its protein shell by degradation would yield a molecule small enough to be taken up by a TonB-dependent receptor. Our next experiment was designed to determine the fate of ferritin-encaged iron in infected cells. If ferritin serves as an iron source for MC, the iron within it should be freed from its massive core during infection.

Using ^{55}Fe-labeling and gel filtration we monitored the relative molecular weight of ^{55}Fe-containing molecules in A431 cells. Lysates from 8-hr infected and uninfected cells were fractionated with a Sephacryl S400 column. Ferritin is very large (MW 480kDa) compared to other iron-containing molecules and eluted early from the column. Early fractions collected from uninfected cells displayed a large peak of ^{55}Fe radioactivity (Fig. 6). These fractions were found by ELISA to contain the majority of cellular ferritin. Infected cell extracts lacked both ELISA-measurable ferritin and ^{55}Fe in these early fractions. In addition, infected cell extracts displayed an increase in ^{55}Fe in the low molecular weight fractions. This result demonstrates that iron previously contained within ferritin is released to a low molecular weight pool during infection. MC could potentially take up a low molecular weight ferritin degradation product via a TonB-dependent receptor. Our next goal was to inhibit ferritin degradation in infected cells and quantify its influence on replication of intracellular MC.

Inhibition of ferritin degradation with ascorbic acid, holotransferrin or leupeptin prevents intracellular replication of MC

Ascorbic acid (Vitamin C) has been shown to prevent autophagy of ferritin and its subsequent degradation in several cell types (Bridges, 1987; Hoffman *et al.*, 1991; Ollinger and Roberg, 1997). We studied the effect of ascorbic acid on ferritin degradation in MC-infected A431 cells using an ELISA. In contrast to our results with non-supplemented cells, we found that ferritin levels in ascorbate-treated cells slowly increased throughout 12 hrs of MC infection (Fig. 7).

Ascorbic acid also prevented the replication of intracellular MC (Fig. 1). The replication rate could be greatly improved by supplementing the ascorbate-treated infected cells with ferric nitrate. These results provide evidence that MC-induced ferritin degradation occurs via autophagy, and suggest that the iron released from degraded ferritin serves as an intracellular iron source for MC.

Earlier, we described the ability of the protease inhibitor leupeptin to reduce ferritin degradation in infected cells. We found that this treatment also retarded replication of intracellular MC (Fig. 1). The replication efficiency could be rescued by supplementing the cells with ferric nitrate, providing additional evidence that ferritin degradation yields an intracellular iron source for MC.

We had also previously found that holotransferrin inhibited intracellular replication of MC. We next measured the ferritin levels in infected cells treated with 40mM human holotransferrin. We found that ferritin levels remained stable in the iron-loaded cells (Fig. 7). In summary, three separate treatments that prevent ferritin turnover in infected cells show a corresponding ability to prevent intracellular replication of MC. Taken together, these results strongly suggest that iron derived from degraded ferritin stimulates intracellular replication of MC.

Both transferrin and ascorbic acid prevent ferritin redistribution in MC-infected cells

Having shown that holotransferrin and ascorbate can prevent ferritin turnover in infected cells, we next used indirect immunofluorescence microscopy to examine the distribution of ferritin in infected cells treated with these reagents. In infected cells, ferritin signals are found in large aggregates around intracellular MC (Fig. 8B). In infected cells supplemented with ascorbate, ferritin signals do not cluster around the bacteria. Rather, they are diffusely distributed throughout the cytosol, much like the signals observed in uninfected cells (compare Fig. 8C and A). A similar distribution of ferritin signals was also observed in infected cells supplemented with holotransferrin (Fig. 8D). Thus, ascorbate and holotransferrin appear to prevent the cellular redistribution of ferritin in infected cells. In addition, in agreement with intracellular replication results (Fig. 1), these reagents resulted in lower numbers of visible intracellular bacteria (data not shown).

The ability of ascorbate to prevent both ferritin degradation and redistribution suggests that these two phenomena are linked. Ascorbate has been shown to prevent ferritin autophagy and subsequent degradation in iron-starved cells (Bridges, 1987; Ollinger and Roberg, 1997). The effect of ascorbate in our experiments, therefore, suggests that ferritin autophagy occurs in MC-infected cells.

Discussion

The obligate human pathogen *Neisseria meningitidis* possesses a remarkably diverse iron acquisition strategy. MC has well defined uptake systems that allow removal of iron from several proteins found in the extracellular milieu (Schryvers and Stojiljkovic, 1999). This report predicts an additional mechanism that this bacterium utilizes to exploit the vast reserves of iron that are stored within human cells. While MC is not generally considered an intracellular bacterium, recent studies have shown that invasion of epithelial cells and replication within them is likely to play an important role in the pathogenesis of this organism.

Our previous studies demonstrated that MC does not replicate within cultured epithelial cells unless iron is acquired from the host cell (Larson *et al.*, 2002). The present study was intended to identify the intracellular iron source that stimulates MC replication. The results of our study strongly suggest that iron mobilized from degraded ferritin supports intracellular replication by MC.

In this report we have ruled out heme as an intracellular iron source. We also found that the rate of intracellular MC replication is inversely proportional to the amount of transferrin iron available to the host cells. This information, in addition to previous studies from our lab demonstrating impaired transferrin uptake by MC infected cells (Bonnah *et al.*, 2000), led us to make the following hypothesis: We believe that MC infection of epithelial cells triggers an iron-starvation response. As a consequence, the host cell degrades cytosolic ferritin, releasing iron in order to meet its own metabolic needs. Further, we believe that intracellular MC must access ferritin-derived iron in order to replicate. The iron starvation response was recently demonstrated by a microarray analysis of iron-related gene expression in MC-infected A431 cells (Bonnah *et.al.* submitted).

Additional components of our hypothesis are supported by this study. We have verified that ferritin is degraded in infected cells. ELISAs supported by pulse-chase experiments showed that the majority of cellular ferritin is degraded within

12 hrs of infection. Gel filtration of lysates from ^{55}Fe-labeled cells demonstrated that iron is mobilized from the core of degraded ferritin during infection.

Indirect immunofluorescence microscopy of infected cells revealed that ferritin is redistributed from the cytosol to aggregates that closely associate with intracellular MC. We believe this punctate staining represents compartments where ferritin is degraded. We were unable to demonstrate that these compartments are lysosomes because none of the lysosomal markers examined (Lamp1, Lamp2, CD63) colocalized with ferritin. The fact that MC can alter multiple lysosomal constituents (Ayala *et al.*, 2001) may have interfered with our efforts. The process of ferritin degradation described in this report closely resembles the iron starvation-induced pathway characterized in many eukaryotic cell systems. In all cases, ferritin degradation was inhibited by ascorbic acid, iron loaded transferrin, or leupeptin, and involved a change in distribution of ferritin from the cytosol to cellular compartments.

We can alter the iron metabolism of infected epithelial cells by adding holotransferrin, ascorbate or leupeptin. In all cases, blocking degradation of ferritin correlates with disruption of intracellular replication by MC. These experiments support our hypothesis that the degradation of ferritin yields an iron source for MC.

The ability of holotransferrin to prevent ferritin degradation suggests that the MC-induced starvation response can be overcome. One interpretation of our results is that high levels of transferrin can saturate the few remaining transferrin receptors on MC-infected cells and deliver enough iron to the cells to prevent starvation.

It should be noted that we are not the first to investigate ferritin as a potential iron source for MC. Over two decades ago, several researchers reported that ferritin could not serve as a sole iron source for MC and that in some conditions ferritin was inhibitory to MC growth (Archibald and DeVoe, 1980; Calver *et al.*, 1979). Our data strongly suggest that the host cell must first degrade ferritin before MC can utilize the iron contained within the core. Our results, therefore, are not in conflict with earlier reports.

In future studies we will further test and extend our hypothesis. The most direct interpretation of our results is that a ferritin degradation product is serving as an intracellular iron source for MC. However, the formal possibility remains that ferritin degradation leads to the production of another iron-binding protein that is used by the bacteria. For instance, the protein may be a transport molecule that redistributes iron from degraded ferritin to the cellular cytosol. The identification of such an iron-containing species would be an important advance in the study of cellular iron metabolism. In addition to identifying the exact iron source, we hope

to clearly define the nature of the compartment that ferritin is sequestered within during infection. This may be a novel degradative compartment that is unique to MC-infected cells. We are also attempting to identify the TonB-dependent receptor that MC uses to acquire its iron source.

Although this is the first evidence that the ferritin iron pathway is exploited by a human pathogen, there is reason to believe that MC is not the only bacterium with this ability. The intracellular pathogens *Mycobacterium tuberculosis* (Olakanmi *et al.*, 2002), and *Legionella pneumophila* (Byrd and Horwitz, 1991) can access intracellular iron and ferritin was not ruled out as a possible iron source for either of these organisms. The reduction in epithelial cell transferrin uptake triggered by MC may be a general aspect of cellular immunity such as that seen in IFNg and LPS-activated macrophages (Ludwiczek *et al.*, 2003). This macrophage response has been shown to limit transferrin iron availability to intracellular pathogens and may result in mobilization of ferritin iron to meet cellular metabolic needs. Pathogens with diverse iron-scavenging tactics such as *Neisseria meningitidis* are likely to exploit this process by tapping into ferritin iron reserves and bypassing the need for transferrin iron within cells.

Experimental Procedures

Cell Culture

The A431 human endocervical epithelial cell line, obtained from S. Schmid, was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco BRL). Cells were used for bacterial infection experiments between the 8^{th} and 20^{th} passages. Cells were incubated in a 37° C, 5% CO_2 environment.

Bacterial strains

All MC strains were maintained on GCB agar plates with Kellog's Supplement I and II. Strains were passed no more than 3 times before assays and bacterial inocula were harvested from plates 12-16 hours after passage. MC strain 8013.6 is a serogroup C isolate from a patient at the Pasteur Hospital (Nassif *et al.*, 1993). 8013.6 is a capsulated, piliated strain producing high-adhesive pilin. MC 8013 *hemO*::kan was obtained by transformation of 8013.6 with chromosomal DNA from a published mutant (Zhu *et al.*, 2000). Inactivation of *hemO* was verified by PCR. This strain was also piliated and Opa negative as judged by immunoblotting. The iron-uptake phenotype of 8013 *hemO*::kan was verified by growth on GCB plates containing 100μM Desferal and spotted with 25mg of heme and ferric nitrate.

Intracellular replication-rate assay

A431 cells were seeded into 12-well plates and grown to 80% confluency before infection. WT 8013.6 or its *hemO* derivative were swabbed from GCB plates, directly suspended in DMEM+10% FCS and diluted in the same medium to allow for infection at a multiplicity of infection (MOI) of 2 (2 bacteria per cell). Infected cultures were incubated for 12 hrs then rinsed 3X in Phosphate Buffered Saline (PBS, Gibco BRL) to remove non-adherent bacteria. Cultures were then incubated for 1 hr in fresh pre-warmed media containing 50 μg/ml Gentamicin (Gibco BRL) to kill extracellular bacteria. Gentamicin was then removed and the cells gently rinsed 3X in PBS. Cells were either harvested immediately after Gentamicin treatment (T=0), or after a 5 hr intracellular replication phase (T=5). During the 5 hr phase, fresh media was added. In some cases serum was excluded, and/or supplements added as noted (Fig 1). Sodium ascorbate, iron-loaded human and bovine transferrin and leupeptin were purchased from Sigma. Epithelial cells were lifted and disrupted by incubating them for 3 min in PBS containing 2mM EDTA and 0.05% Saponin (Sigma), followed by transfer to Eppendorff tubes and vortexing at high speed for 30 sec. The lysed host cells were plated at appropriate dilutions on GCB agar to determine MC colony forming units (CFUs). Values for each time point were derived from 3-5 infected cultures. Replication efficiency was calculated as CFUs at T=5/ CFUs at T=0. The efficiencies were normalized for each assay by dividing the replication efficiency by that for wildtype 8013.6 under control conditions (DMEM+10%

FCS). Error bars represent standard deviation of the mean. All assays were repeated at least 3X with similar results.

Indirect immunofluorescence microscopy

A431 epithelial cells seeded onto glass coverslips were grown to 60% confluence and infected with 8013.6 at an MOI of 10 for 8 hrs. They were then rinsed 3X with PBS to remove adherent bacteria and fixed in PBS with 4% paraformaldehyde (Electron Microscopy Sciences) for 8 hrs at 4°C. After rinsing 3X with PBS, cells were blocked and permeablized in PBS containing 3% (vol/vol) normal goat serum (Gibco BRL) and 0.03% saponin for 2 hrs. Cells were incubated with primary antibodies [polyclonal rabbit ferritin antiserum (Sigma) diluted 1:1000 and monoclonal mouse anti LAMP-1 antibodies (H4A3 Developmental Hybridoma Bank) diluted 1:100] in blocking solution overnight at 4°C. After rinsing 3X with PBS, cells were incubated with secondary antibodies [Red and Green Alexa conjugates (Molecular Probes) diluted 1:5,000 in blocking solution] for 45 min. DAPI diluted 1:5,000 was included to label bacteria and nuclei. Cells were rinsed in PBS 3X and prepared for microscopy by mounting in Fluoromount G (Fisher Scientific). Optical sections in the z-axis plane were obtained with a Nikon 60x oil immersion objective and the images were processed using a Deltavision Restoration Microscope (Applied Precision Instruments, Inc.) and Silicon Graphics workstation with accompanying API

software. The images were subsequently exported to Adobe Photoshop for manuscript preparation.

Ferritin ELISA

A431 cells were seeded into 6 well plates and grown to 70% confluence. After infecting the cells for various times with 8013.6 at an MOI of 10, the cells were lysed in 125 ml of NET (150mM NaCl, 10mM EDTA, 10mM Tris pH 7.4) with 1% Triton X-100 (Sigma) and protease inhibitors (Roche complete mini tablets). The lysates were transferred to Eppendorff tubes and microcentrifuged for 2 min on high speed to remove cell debris. 100μl of the supernatant was transferred to a human ferritin ELISA kit (Biotech Diagnostic LLC) and analyzed per the manufacturer's instructions. Samples were analyzed in triplicate. All experiments were repeated at least three times with similar results.

Real-Time PCR analysis of ferritin levels

A431 cells grown to 75% confluence in 6 well plates were infected with 8013.6 at an MOI of 10 for various times. Following infection, cells were lysed in buffer RLT (Qiagen), and then homogenized by centrifugation in QiaShredder columns (Qiagen). Total RNA was isolated using the RNeasy total RNA kit (Qiagen), followed by DNase digestion with DNAfree (Ambion) as recommended by the manufacturer. Spectrophotometric analysis was used to assess RNA quality and quantity. 1μg of total RNA was subsequently reverse-transcribed to generate

cDNA using the iScript cDNA synthesis kit (BioRad). Quantitative real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The expression of H-ferritin heavy chain (HC) and light chain (LC) was monitored with oligonucleotide primers 179F (ctcatgaggagagggaacatgc)/289R (cgctctcccagtcatcacagt) and 147F (ccagcaccgtttttgtggtta)/227R (cacgtcggtggaataattctga) respectively. The expression of GAPDH was monitored with primers 868F (acccactcctccacctttga) /968R (ctgttgctgtagccaaattcgt) and used as a control to normalize all samples. All primers were designed, verified and generously donated by Paige Davies. Amplification was carried out using SYBR-green master mix (ABI), according to the manufacturers instructions. Reactions were performed in triplicate in a 20μl volume containing the indicated primers at a final concentration of 100nM, with the following cycle parameters: 95°C/15 seconds, 55°C/30 seconds, 72°C/30 seconds for 40 cycles. A dissociation curve verified the presence of a single product at the correct Tm. Data analysis was performed using the comparative Ct method (Applied Biosystems) to determine relative expression levels. Reported values are the mean of two experiments, each done in triplicate +/- the standard deviation.

Gel filtration

A Sephacryl S400 (Pharmacia) column with dimensions of 25 cm height and 1 cm diameter was poured and equilibrated in gel filtration buffer (GFB 50mM Tris

pH 7.4 150mM NaCl 0.1% Triton X-100). 2 mg each of standards; horse spleen ferritin, hemoglobin, and heme (all from Sigma) were diluted in 1ml of GFB and applied to the column. 1ml fractions were collected while adding GFB on top of the standards. The standards were tracked by visual inspection and their peak of elution is noted in figure 6. A431 cells grown to 50% confluence in 10 cm dishes were labeled by incubating with 10mCi of ^{55}Fe in serum free DMEM for 12 hrs. The cells were then incubated for a 12 hr recovery period in DMEM+10% FCS to stimulate ferritin production. The cells were next rinsed 3X with PBS and incubated with DMEM+10% FCS with or without infection by 8013.6 at an MOI of 10. After 8 hrs the cells were lysed in 3 ml GFB containing 1% Triton X-100 and protease inhibitors. 1ml of each lysate was applied to the column followed by GFB. 26x1ml fractions were collected after which 100μl of the even numbered fractions were analyzed by ELISA and scintillation counting with a Beckman LS 5000TD. The column was rinsed with 200ml of GFB between each use.

Immunoprecipitations

All steps were carried out at 4° C. A431 cells grown in 6 well plates were lysed in 1 ml of NET containing 1% Triton X-100 and protease inhibitors. Lysates were transferred to 1.5 ml Eppendorff tubes and microcentrifuged at full speed for 5 min to remove cell debris. Supernatants were transferred to fresh tubes to which 2μl of polyclonal ferritin rabbit antiserum (Sigma) was added. After rotating the tubes overnight, 20μl of Protein G+ agarose (Oncogene) was added to each

tube. The tubes were rotated for 2 hrs and microcentrifuged at full speed for 5 min to precipitate immune complexes. The pellets were rinsed twice in 1ml of NET+1% Triton X-100 and twice in 1ml of NET. The pellets were then resuspended in 50μl of Laemmli buffer and boiled for 10 min prior to loading on 12% SDS-PAGE gels.

Ferritin half-life assay

A431 cells were grown to 70% confluence in 6-well plates and pulse labeled with 100μCi of ^{35}S-labeled cysteine/methionine (Tran^{35}S-label ICN) for two hours in Cys/met free DMEM (ICN) containing 50μg/ml human transferrin. The cells were rinsed 3X with PBS and the medium replaced with DMEM+10% FCS containing excess cold cysteine and methionine. After a 2 hr chase the medium was replaced with fresh DMEM+10% FCS and the cells infected with 8013.6 at an MOI of 2. Infected and uninfected cells were lysed in triplicate at 0, 6, 12, and 18 hrs post-chase. In some cases 10μM leupeptin (Sigma) was added to the infected cells every 6 hrs during an 18 hr infection. Immunoprecipitations were then performed as described above. 12% SDS-PAGE gels were soaked in Amplify (NAMP-100 Amersham) and dried under vacuum and heat. Dried gels were used to expose Kodak Biomax film in autoradiography cassettes for 3 days at –80°C. Immunoprecipitated ferritin from A431 cells displayed approximately a 10:1 ratio of heavy to light ferritin chain. Heavy chain was quantified by densitometry of scanned images using NIH Image 1.63 f. In order to calculate

ferritin half-lives the data from densitometry was plotted (Fig.3) and lines were drawn by interpolation of the data. The slope and y-intercept of the lines calculated using Cricketgraph were used to determine the half-life.

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References

Abboud, S., and Haile, D.J. (2000) A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J Biol Chem* **275**: 19906-19912.

Aisen, P., Enns, C., and Wessling-Resnick, M. (2001) Chemistry and biology of eukaryotic iron metabolism. *Int J Biochem Cell Biol* **33**: 940-959.

Al-Younes, H.M., Rudel, T., Brinkmann, V., Szczepek, A.J., and Meyer, T.F. (2001) Low iron availability modulates the course of Chlamydia pneumoniae infection. *Cell Microbiol* **3**: 427-437.

Anderson, G.J. (2001) Ironing out disease: inherited disorders of iron homeostasis. *IUBMB Life* **51**: 11-17.

Archibald, F.S., and DeVoe, I.W. (1980) Iron acquisition by Neisseria meningitidis in vitro. *Infect Immun* **27**: 322-334.

Archibald, F.S., and Duong, M.N. (1986) Superoxide dismutase and oxygen toxicity defenses in the genus Neisseria. *Infect Immun* **51**: 631-641.

Ayala, B.P., Vasquez, B., Clary, S., Tainer, J.A., Rodland, K., and So, M. (2001) The pilus-induced Ca2+ flux triggers lysosome exocytosis and increases the amount of Lamp1 accessible to Neisseria IgA1 protease. *Cell Microbiol* **3**: 265-275.

Ayala, P., Lin, L., Hopper, S., Fukuda, M., and So, M. (1998) Infection of epithelial cells by pathogenic neisseriae reduces the levels of multiple lysosomal constituents. *Infect Immun* **66**: 5001-5007.

Barnewall, R.E., and Rikihisa, Y. (1994) Abrogation of gamma interferon-induced inhibition of Ehrlichia chaffeensis infection in human monocytes with iron-transferrin. *Infect Immun* **62**: 4804-4810.

Belland, R.J., Morrison, S.G., Carlson, J.H., and Hogan, D.M. (1997) Promoter strength influences phase variation of neisserial opa genes. *Mol Microbiol* **23**: 123-135.

Bisaillon, J.G., Dubois, G., Beaudet, R., Sylvestre, M., Charbonneau, R., and Gagnon, M. (1985) Quantitative determination of catalase activity produced by Neisseria gonorrhoeae, Staphylococcus epidermidis, Neisseria meningitidis and other bacterial strains using the Catalasemeter. *Exp Biol* **43**: 225-230.

Bonnah, R.A., Lee, S., Vasquez, B.L., Enns, C., and So, M. (2000) Alteration of epithelial cell transferrin-iron homeostasis by the pathogenic Neisseriae. *Cell Microbiol* **In press.**

Boyer, E., Bergevin, I., Malo, D., Gros, P., and Cellier, M.F. (2002) Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. *Infect Immun* **70**: 6032-6042.

Bradbury, M.W. (1997) Transport of iron in the blood-brain-cerebrospinal fluid system. *J Neurochem* **69**: 443-454.

Braun, V., and Braun, M. (2002a) Active transport of iron and siderophore antibiotics. *Curr Opin Microbiol* **5**: 194-201.

Braun, V., and Braun, M. (2002b) Iron transport and signaling in Escherichia coli. *FEBS Lett* **529**: 78-85.

Breuer, W., Epsztejn, S., and Cabantchik, Z.I. (1995) Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II). *J Biol Chem* **270**: 24209-24215.

Bridges, K.R., and Hoffman, K.E. (1986) The effects of ascorbic acid on the intracellular metabolism of iron and ferritin. *J Biol Chem* **261**: 14273-14277.

Bridges, K.R. (1987) Ascorbic acid inhibits lysosomal autophagy of ferritin. *J Biol Chem* **262**: 14773-14778.

Brock, J.H., Williams, P.H., Liceaga, J., and Wooldridge, K.G. (1991) Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of Escherichia coli and to other microorganisms. *Infect Immun* **59**: 3185-3190.

Bucci, C., Lavitola, A., Salvatore, P., Del Giudice, L., Massardo, D.R., Bruni, C.B., and Alifano, P. (1999) Hypermutation in pathogenic bacteria: frequent phase variation in meningococci is a phenotypic trait of a specialized mutator biotype. *Mol Cell* **3**: 435-445.

Byrd, T.F., and Horwitz, M.A. (1989) Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of Legionella pneumophila by limiting the availability of iron. *J Clin Invest* **83**: 1457-1465.

Byrd, T.F., and Horwitz, M.A. (1991) Chloroquine inhibits the intracellular multiplication of Legionella pneumophila by limiting the availability of iron. A potential new mechanism for the therapeutic effect of chloroquine against intracellular pathogens. *J Clin Invest* **88**: 351-357.

Cable, H., and Lloyd, J.B. (1999) Cellular uptake and release of two contrasting iron chelators. *J Pharm Pharmacol* **51**: 131-134.

Calver, G.A., Kenny, C.P., and Lavergne, G. (1976) Iron as a replacement for mucin in the establishment of meningococcal infection in mice. *Can J Microbiol* **22**: 832-838.

Calver, G.A., Kenny, C.P., and Kushner, D.J. (1979) Inhibition of the growth of Neisseria meningitidis by reduced ferritin and other iron-binding agents. *Infect Immun* **25**: 880-890.

Cane, D.E., and Walsh, C.T. (1999) The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases. *Chem Biol* **6**: R319-325.

Carson, S.D., Klebba, P.E., Newton, S.M., and Sparling, P.F. (1999) Ferric enterobactin binding and utilization by Neisseria gonorrhoeae. *J Bacteriol* **181**: 2895-2901.

Cascales, E., Lloubes, R., and Sturgis, J.N. (2001) The TolQ-TolR proteins energize TolA and share homologies with the flagellar motor proteins MotA-MotB. *Mol Microbiol* **42**: 795-807.

Chen, C.Y., and Morse, S.A. (1999) Neisseria gonorrhoeae bacterioferritin: structural heterogeneity, involvement in iron storage and protection against oxidative stress. *Microbiology* **145 (Pt 10)**: 2967-2975.

Clemens, D.L., and Horwitz, M.A. (1996) The Mycobacterium tuberculosis phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. *J Exp Med* **184**: 1349-1355.

Conrad, M.E., and Umbreit, J.N. (2000) Iron absorption and transport-an update. *Am J Hematol* **64**: 287-298.

Conrad, M.E., and Umbreit, J.N. (2002) Pathways of iron absorption. *Blood Cells Mol Dis* **29**: 336-355.

Cooper, C.E., Lynagh, G.R., Hoyes, K.P., Hider, R.C., Cammack, R., and Porter, J.B. (1996) The relationship of intracellular iron chelation to the inhibition and regeneration of human ribonucleotide reductase. *J Biol Chem* **271**: 20291-20299.

Crichton, R.R., and Ward, R.J. (1992) Iron metabolism--new perspectives in view. *Biochemistry* **31**: 11255-11264.

Crosa, J.H. (1989) Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiol Rev* **53**: 517-530.

Dabernat, H., Delmas, C., and Lareng, M.B. (1984) [Antibiotic sensitivity of meningococci isolated from patients and carriers]. *Pathol Biol (Paris)* **32**: 532-535.

Dautry-Varsat, A., Ciechanover, A., and Lodish, H.F. (1983) pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc Natl Acad Sci U S A* **80**: 2258-2262.

Dehio, C., Gray-Owen, S.D., and Meyer, T.F. (1998) The role of neisserial Opa proteins in interactions with host cells. *Trends Microbiol.* **6**: 489-495.

Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S.J., Moynihan, J., Paw, B.H., Drejer, A., Barut, B., Zapata, A., Law, T.C., Brugnara, C., Lux, S.E., Pinkus, G.S., Pinkus, J.L., Kingsley, P.D., Palis, J., Fleming, M.D., Andrews, N.C., and Zon, L.I. (2000) Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* **403**: 776-781.

Eklund, H., Uhlin, U., Farnegardh, M., Logan, D.T., and Nordlund, P. (2001) Structure and function of the radical enzyme ribonucleotide reductase. *Prog Biophys Mol Biol* **77**: 177-268.

Ellison, R.T., 3rd (1994) The effects of lactoferrin on gram-negative bacteria. *Adv Exp Med Biol* **357**: 71-90.

Feder, J.N., Gnirke, A., Thomas, W., Tsuchihashi, Z., Ruddy, D.A., Basava, A., Dormishian, F., Domingo, R., Jr., Ellis, M.C., Fullan, A., Hinton, L.M., Jones, N.L., Kimmel, B.E., Kronmal, G.S., Lauer, P., Lee, V.K., Loeb, D.B., Mapa, F.A., McClelland, E., Meyer, N.C., Mintier, G.A., Moeller, N.,

Moore, T., Morikang, E., Wolff, R.K., and et al. (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* **13**: 399-408.

Ganz, T. (2003) Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* **102**: 783-788.

Goetz, D.H., Holmes, M.A., Borregaard, N., Bluhm, M.E., Raymond, K.N., and Strong, R.K. (2002) The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell* **10**: 1033-1043.

Goodman, S.D., and Scocca, J.J. (1988) Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae. Proc. Natl. Acad. Sci. USA* **85**: 6982-6986.

Gray-Owen, S.D., and Schryvers, A.B. (1996) Bacterial transferrin and lactoferrin receptors. *Trends Microbiol* **4**: 185-191.

Harrison, P.M., and Arosio, P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* **1275**: 161-203.

Hart, C.A., and Cuevas, L.E. (1997) Meningococcal disease in Africa. *Ann Trop Med Parasitol* **91**: 777-785.

Headley, V., Hong, M., Galko, M., and Payne, S.M. (1997) Expression of aerobactin genes by Shigella flexneri during extracellular and intracellular growth. *Infect Immun* **65**: 818-821.

Hermes-Lima, M., Storey, J.M., and Storey, K.B. (1998) Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. *Comp Biochem Physiol B Biochem Mol Biol* **120**: 437-448.

Hoffman, K.E., Yanelli, K., and Bridges, K.R. (1991) Ascorbic acid and iron metabolism: alterations in lysosomal function. *Am J Clin Nutr* **54**: 1188S-1192S.

Holbein, B.E., Jericho, K.W., and Likes, G.C. (1979) Neisseria meningitidis infection in mice: influence of iron, variations in virulence among strains, and pathology. *Infect Immun* **24**: 545-551.

Holbein, B.E. (1980) Iron-controlled infection with Neisseria meningitidis in mice. *Infect Immun* **29**: 886-891.

Holbein, B.E. (1981) Enhancement of Neisseria meningitidis infection in mice by addition of iron bound to transferrin. *Infect Immun* **34**: 120-125.

Hopper, S., Wilbur, J.S., Vasquez, B.L., Larson, J., Clary, S., Mehr, I.J., Seifert, H.S., and So, M. (2000) Isolation of Neisseria gonorrhoeae mutants that show enhanced trafficking across polarized T84 epithelial monolayers. *Infect Immun* **68**: 896-905.

Isberg, R.R., and Falkow, S. (1985) A single genetic locus encoded by Yersinia pseudotuberculosis permits invasion of cultured cells by Escherichia coli K-12. *Nature* **317**: 262-264.

Jack, D.L., Dodds, A.W., Anwar, N., Ison, C.A., Law, A., Frosch, M., Turner, M.W., and Klein, N.J. (1998) Activation of complement by mannose-binding lectin on isogenic mutants of Neisseria meningitidis serogroup B. *J Immunol* **160**: 1346-1353.

Jarvis, G.A. (1995) Recognition and control of neisserial infection by antibody and complement. *Trends Microbiol* **3**: 198-201.

Kakhlon, O., Gruenbaum, Y., and Cabantchik, Z.I. (2001) Repression of ferritin expression increases the labile iron pool, oxidative stress, and short-term growth of human erythroleukemia cells. *Blood* **97**: 2863-2871.

Kallstrom, H., Liszewski, M.K., Atkinson, J.P., and Jonsson, A.B. (1997) Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria. *Mol Microbiol* **25**: 639-647.

Kammler, M., Schon, C., and Hantke, K. (1993) Characterization of the ferrous iron uptake system of Escherichia coli. *J Bacteriol* **175**: 6212-6219.

Kaplan, J. (2002) Mechanisms of cellular iron acquisition: another iron in the fire. *Cell* **111**: 603-606.

Khun, H.H., Kirby, S.D., and Lee, B.C. (1998) A Neisseria meningitidis fbpABC mutant is incapable of using nonheme iron for growth. *Infect Immun* **66**: 2330-2336.

Klee, S.R., Nassif, X., Kusecek, B., Merker, P., Beretti, J.L., Achtman, M., and Tinsley, C.R. (2000) Molecular and biological analysis of eight genetic islands that distinguish Neisseria meningitidis from the closely related pathogen Neisseria gonorrhoeae. *Infect Immun* **68**: 2082-2095.

Klein, N.J., Ison, C.A., Peakman, M., Levin, M., Hammerschmidt, S., Frosch, M., and Heyderman, R.S. (1996) The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by Neisseria meningitidis. *J Infect Dis* **173**: 172-179.

Kline, K.A., Sechman, E.V., Skaar, E.P., and Seifert, H.S. (2004) Recombination, repair and replication in the pathogenic Neisseriae: the 3 R's of molecular genetics of two human-specific bacterial pathogens. *Mol Microbiol* **51**: 297.

Kolb-Maurer, A., Kurzai, O., Goebel, W., and Frosch, M. (2003) The role of human dendritic cells in meningococcal and listerial meningitis. *Int J Med Microbiol* **293**: 241-249.

Konijn, A.M., Glickstein, H., Vaisman, B., Meyron-Holtz, E.G., Slotki, I.N., and Cabantchik, Z.I. (1999) The cellular labile iron pool and intracellular ferritin in K562 cells. *Blood* **94**: 2128-2134.

Koppenol, W.H. (2001) The Haber-Weiss cycle--70 years later. *Redox Rep* **6**: 229-234.

Larsen, R.A., Letain, T.E., and Postle, K. (2003) In vivo evidence of TonB shuttling between the cytoplasmic and outer membrane in Escherichia coli. *Mol Microbiol* **49**: 211-218.

Larson, J.A., Higashi, D.L., Stojiljkovic, I., and So, M. (2002) Replication of Neisseria meningitidis within epithelial cells requires TonB-dependent acquisition of host cell iron. *Infect Immun* **70**: 1461-1467.

Lee, S.W., Bonnah, R.A., Higashi, D.L., Atkinson, J.P., Milgram, S.L., and So, M. (2002) CD46 is phosphorylated at tyrosine 354 upon infection of epithelial cells by Neisseria gonorrhoeae. *J Cell Biol* **156**: 951-957.

Letain, T.E., and Postle, K. (1997) TonB protein appears to transduce energy by shuttling between the cytoplasmic membrane and the outer membrane in Escherichia coli. *Mol Microbiol* **24**: 271-283.

Lewis, L.A., Gray, E., Wang, Y.P., Roe, B.A., and Dyer, D.W. (1997) Molecular characterization of hpuAB, the haemoglobin-haptoglobin- utilization operon of Neisseria meningitidis. *Mol Microbiol* **23**: 737-749.

Lewis, L.A., Sung, M.H., Gipson, M., Hartman, K., and Dyer, D.W. (1998) Transport of intact porphyrin by HpuAB, the hemoglobin-haptoglobin utilization system of Neisseria meningitidis. *J Bacteriol* **180**: 6043-6047.

Lieu, P.T., Heiskala, M., Peterson, P.A., and Yang, Y. (2001) The roles of iron in health and disease. *Mol Aspects Med* **22**: 1-87.

Lin, L., Ayala, P., Larson, J., Mulks, M., Fukuda, M., Carlsson, S.R., Enns, C., and So, M. (1997) The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. *Mol Microbiol* **24**: 1083-1094.

Ludwiczek, S., Aigner, E., Theurl, I., and Weiss, G. (2003) Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood* **101**: 4148-4154.

McKie, A.T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T.J., Farzaneh, F., Hediger, M.A., Hentze, M.W., and Simpson, R.J. (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell* **5**: 299-309.

Mehr, I.J., and Seifert, H.S. (1997) Random shuttle mutagenesis: gonococcal mutants deficient in pilin antigenic variation. *Mol Microbiol* **23**: 1121-1131.

Merz, A.J., Enns, C.A., and So, M. (1999) Type IV pili of pathogenic neisseriae elicit cortical plaque formation in epithelial cells. *Mol Microbiol* **32**: 1316-1332.

Merz, A.J., and So, M. (2000) Interactions of pathogenic neisseriae with epithelial cell membranes. *Annual reviews in Cell and Developmental Biology* **16**: 423-457.

Meyer, T.F. (1991) Evasion mechanisms of pathogenic Neisseriae. *Behring Inst Mitt*: 194-199.

Miyazaki, E., Kato, J., Kobune, M., Okumura, K., Sasaki, K., Shintani, N., Arosio, P., and Niitsu, Y. (2002) Denatured H-ferritin subunit is a major constituent of haemosiderin in the liver of patients with iron overload. *Gut* **50**: 413-419.

Moeck, G.S., and Coulton, J.W. (1998) TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. *Mol Microbiol* **28**: 675-681.

Moran, A.P., Prendergast, M.M., and Appelmelk, B.J. (1996) Molecular mimicry of host structures by bacterial lipopolysaccharides and its contribution to disease. *FEMS Immunol Med Microbiol* **16**: 105-115.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., and So, M. (1993) Antigenic variation of pilin regulates adhesion of Neisseria meningitidis to human epithelial cells. *Mol Microbiol* **8**: 719-725.

Nassif, X., and So, M. (1995) Interaction of pathogenic neisseriae with nonphagocytic cells. *Clin Microbiol Rev* **8**: 376-388.

Olakanmi, O., Britigan, B.E., and Schlesinger, L.S. (2000) Gallium disrupts iron metabolism of mycobacteria residing within human macrophages. *Infect Immun* **68**: 5619-5627.

Olakanmi, O., Schlesinger, L.S., Ahmed, A., and Britigan, B.E. (2002) Intraphagosomal Mycobacterium tuberculosis acquires iron from both extracellular transferrin and intracellular iron pools. Impact of interferon-gamma and hemochromatosis. *J Biol Chem* **277**: 49727-49734.

Ollinger, K., and Roberg, K. (1997) Nutrient deprivation of cultured rat hepatocytes increases the desferrioxamine-available iron pool and augments the sensitivity to hydrogen peroxide. *J Biol Chem* **272**: 23707-23711.

Pathan, N., Faust, S.N., and Levin, M. (2003) Pathophysiology of meningococcal meningitis and septicaemia. *Arch Dis Child* **88**: 601-607.

Ponka, P. (1999a) Cellular iron metabolism. *Kidney Int Suppl* **69**: S2-11.

Ponka, P. (1999b) Cell biology of heme. *Am J Med Sci* **318**: 241-256.

Ponka, P., and Lok, C.N. (1999) The transferrin receptor: role in health and disease. *Int J Biochem Cell Biol* **31**: 1111-1137.

Ponka, P., Sheftel, A.D., and Zhang, A.S. (2002) Iron targeting to mitochondria in erythroid cells. *Biochem Soc Trans* **30**: 735-738.

Pope, C.D., O'Connell, W., and Cianciotto, N.P. (1996) Legionella pneumophila mutants that are defective for iron acquisition and assimilation and intracellular infection. *Infect Immun* **64**: 629-636.

Postle, K., and Kadner, R.J. (2003) Touch and go: tying TonB to transport. *Mol Microbiol* **49**: 869-882.

Radisky, D.C., and Kaplan, J. (1998) Iron in cytosolic ferritin can be recycled through lysosomal degradation in human fibroblasts. *Biochem J* **336**: 201-205.

Ratledge, C., and Dover, L.G. (2000) Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol* **54**: 881-941.

Raymond, K.N., Dertz, E.A., and Kim, S.S. (2003) Enterobactin: an archetype for microbial iron transport. *Proc Natl Acad Sci U S A* **100**: 3584-3588.

Reeves, S.A., Torres, A.G., and Payne, S.M. (2000) TonB is required for intracellular growth and virulence of Shigella dysenteriae. *Infect Immun* **68**: 6329-6336.

Reshilov, L.N., Borovkova, V.M., Salov, V.F., Basnak'ian, I.A., and Lopyrev, I.V. (1986) [Formation of microvesicles in meningococcal cells during batch cultivation]. *Zh Mikrobiol Epidemiol Immunobiol*: 12-16.

Richardson, A.R., and Stojiljkovic, I. (2001) Mismatch repair and the regulation of phase variation in Neisseria meningitidis. *Mol Microbiol* **40**: 645-655.

Roberts, S., and Bomford, A. (1988) Ferritin iron kinetics and protein turnover in K562 cells. *J Biol Chem* **263**: 19181-19187.

Robey, M., and Cianciotto, N.P. (2002) Legionella pneumophila feoAB promotes ferrous iron uptake and intracellular infection. *Infect Immun* **70**: 5659-5669.

Robinson, K., Neal, K.R., Howard, C., Stockton, J., Atkinson, K., Scarth, E., Moran, J., Robins, A., Todd, I., Kaczmarski, E., Gray, S., Muscat, I., Slack, R., and Ala'Aldeen, D.A. (2002) Characterization of humoral and cellular immune responses elicited by meningococcal carriage. *Infect Immun* **70**: 1301-1309.

Rohde, K.H., and Dyer, D.W. (2003) Mechanisms of iron acquisition by the human pathogens Neisseria meningitidis and Neisseria gonorrhoeae. *Front Biosci* **8**: d1186-1218.

Rouault, T., Rao, K., Harford, J., Mattia, E., and Klausner, R.D. (1985) Hemin, chelatable iron, and the regulation of transferrin receptor biosynthesis. *J Biol Chem* **260**: 14862-14866.

Roy, C.N., and Enns, C.A. (2000) Iron homeostasis: new tales from the crypt. *Blood* **96**: 4020-4027.

Runyen-Janecky, L.J., Reeves, S.A., Gonzales, E.G., and Payne, S.M. (2003) Contribution of the Shigella flexneri Sit, Iuc, and Feo iron acquisition systems to iron acquisition in vitro and in cultured cells. *Infect Immun* **71**: 1919-1928.

Schryvers, A.B., and Gonzalez, G.C. (1989) Comparison of the abilities of different protein sources of iron to enhance Neisseria meningitidis infection in mice. *Infect Immun* **57**: 2425-2429.

Schryvers, A.B., and Gonzalez, G.C. (1990) Receptors for transferrin in pathogenic bacteria are specific for the host's protein. *Can J Microbiol* **36**: 145-147.

Schryvers, A.B., and Stojiljkovic, I. (1999) Iron acquisition systems in the pathogenic Neisseria. *Mol Microbiol* **32**: 1117-1123.

Smith, H.O., Gwinn, M.L., and Salzberg, S.L. (1999) DNA uptake signal sequences in naturally transformable bacteria. *Res Microbiol* **150**: 603-616.

Stephens, D.S., Hoffman, L.H., and McGee, Z.A. (1983) Interaction of *Neisseria meningitidis* with human nasopharyngeal mucosa: attachment and entry into columnar epithelial cells **148**: 369-376.

Stephens, D.S., Whitney, A.M., Melly, M.A., Hoffman, L.H., Farley, M.M., and Frasch, C.E. (1986) Analysis of damage to human ciliated nasopharyngeal epithelium by Neisseria meningitidis. *Infect Immun* **51**: 579-585.

Stojiljkovic, I., Hwa, V., de Saint Martin, L., O'Gaora, P., Nassif, X., Heffron, F., and So, M. (1995) The Neisseria meningitidis haemoglobin receptor: its role in iron utilization and virulence. *Mol Microbiol* **15**: 531-541.

Stojiljkovic, I., Larson, J., Hwa, V., Anic, S., and So, M. (1996) HmbR outer membrane receptors of pathogenic Neisseria spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. *J Bacteriol* **178**: 4670-4678.

Stojiljkovic, I., and Srinivasan, N. (1997) Neisseria meningitidis tonB, exbB, and exbD genes: Ton-dependent utilization of protein-bound iron in Neisseriae. *J Bacteriol* **179**: 805-812.

Stojiljkovic, I., and Perkins-Balding, D. (2002) Processing of heme and heme-containing proteins by bacteria. *DNA Cell Biol* **21**: 281-295.

Tabuchi, M., Yoshimori, T., Yamaguchi, K., Yoshida, T., and Kishi, F. (2000) Human NRAMP2/DMT1, which mediates iron transport across endosomal membranes, is localized to late endosomes and lysosomes in HEp-2 cells. *J Biol Chem* **275**: 22220-22228.

Theil, E.C. (1990) The ferritin family of iron storage proteins. *Adv Enzymol Relat Areas Mol Biol* **63**: 421-449.

Theil, E.C., and Eisenstein, R.S. (2000) Combinatorial mRNA regulation: iron regulatory proteins and iso-iron-responsive elements (Iso-IREs). *J Biol Chem* **275**: 40659-40662.

Theil, E.C. (2003) Ferritin: at the crossroads of iron and oxygen metabolism. *J Nutr* **133**: 1549S-1553S.

Thomas-Keprta, K.L., Clemett, S.J., Bazylinski, D.A., Kirschvink, J.L., McKay, D.S., Wentworth, S.J., Vali, H., Gibson Jr, E.K., Jr., and Romanek, C.S. (2002) Magnetofossils from ancient Mars: a robust biosignature in the martian meteorite ALH84001. *Appl Environ Microbiol* **68**: 3663-3672.

Tobiason, D.M., and Seifert, H.S. (2001) Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. *Microbiology* **147**: 2333-2340.

Torti, F.M., and Torti, S.V. (2002) Regulation of ferritin genes and protein. *Blood* **99**: 3505-3516.

Turner, P.C., Thomas, C.E., Elkins, C., Clary, S., and Sparling, P.F. (1998) Neisseria gonorrhoeae heme biosynthetic mutants utilize heme and hemoglobin as a heme source but fail to grow within epithelial cells. *Infect Immun* **66**: 5215-5223.

Velayudhan, J., Hughes, N.J., McColm, A.A., Bagshaw, J., Clayton, C.L., Andrews, S.C., and Kelly, D.J. (2000) Iron acquisition and virulence in Helicobacter pylori: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol Microbiol* **37**: 274-286.

Vogel, U., and Frosch, M. (1999) Mechanisms of neisserial serum resistance. *Mol Microbiol* **32**: 1133-1139.

Vulpe, C.D., Kuo, Y.M., Murphy, T.L., Cowley, L., Askwith, C., Libina, N., Gitschier, J., and Anderson, G.J. (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nat Genet* **21**: 195-199.

Vyoral, D., and Petrak, J. (1998) Iron transport in K562 cells: a kinetic study using native gel electrophoresis and 59Fe autoradiography. *Biochim Biophys Acta* **1403**: 179-188.

Weinberg, E.D. (1985) Roles of iron in infection and neoplasia. *J Pharmacol* **16**: 358-364.

Welch, K.D., Van Eden, M.E., and Aust, S.D. (2001) Modification of ferritin during iron loading. *Free Radic Biol Med* **31**: 999-1006.

Wenger, J.D., Hightower, A.W., Facklam, R.R., Gaventa, S., and Broome, C.V. (1990) Bacterial meningitis in the United States, 1986: report of a multistate surveillance study. The Bacterial Meningitis Study Group. *J Infect Dis* **162**: 1316-1323.

Wildes, S.S., and Tunkel, A.R. (2002) Meningococcal vaccines: a progress report. *BioDrugs* **16**: 321-329.

Yancey, R.J., and Finkelstein, R.A. (1981) Assmilation of iron by pathogenic Neisseria spp. *Infect Immun* **32**: 592-599.

Yang, J., Goetz, D., Li, J.Y., Wang, W., Mori, K., Setlik, D., Du, T., Erdjument-Bromage, H., Tempst, P., Strong, R., and Barasch, J. (2002) An iron delivery pathway mediated by a lipocalin. *Mol Cell* **10**: 1045-1056.

Zhu, W., Wilks, A., and Stojiljkovic, I. (2000) Degradation of heme in gram-negative bacteria: the product of the hemO gene of Neisseriae is a heme oxygenase. *J Bacteriol* **182**: 6783-6790.

Figure Legends

Figure 1

Intracellular replication assays

Replication of MC within A431 epithelial cells was quantified under various conditions. A431 cells were infected with MC for the times indicated and intracellular colony forming units were determined using the Gentamicin protection assay as described in Experimental Procedures. The Replication Efficiency of MC under each experimental condition was calculated as a ratio of the number of intracellular bacteria at 5 hrs post Gentamicin treatment to that immediately after Gentamicin treatment. The efficiency is normalized to that of the control, which is wildtype 8013.6 infecting A431 cells grown in DMEM+10% FCS. Each data bar is the average of 3-5 cultures with error bars representing standard deviation. Mutant bacteria or supplements were used as noted in the figure. hTf = human holotransferrin and bTf = bovine holotransferrin. Both transferrins were used at a concentration of 20μg/ml unless noted otherwise in the figure. Leupeptin was used at a concentration of 10μM. Ascorbate was used

at a concentration of 200mM. Fe = ferric nitrate and was used at a concentration of 25μM.

Figure 2

Quantification of ferritin protein and mRNA in A431 cells infected with MC

ELISA quantification of ferritin within A431 cells infected with 8013.6 (triangles). Lysates of infected cells were quantified throughout 12 hrs of infection. Data points represent the average of 3 cultures with error bars representing standard deviation. Data is expressed as the amount of ferritin normalized to uninfected cells. This assay was repeated 3 times with similar results. Real time RT-PCR quantification of relative ferritin mRNA in infected cells. Ferritin heavy chain (filled squares) and light chain (outlined squares) are expressed as the amount of mRNA relative to uninfected cells at each time-point. Data points represent the average of 2 experiments with error bars representing standard deviation.

Figure 3

Determination of the half-life ($t_{1/2}$) of ferritin in uninfected and MC-infected cells

The half-life of ferritin was measured by immunoprecipitation of ferritin from ^{35}S-pulse-labeled A431 cells. A) Autoradiograms of immunoprecipitated material from uninfected cells harvested at 0,6,12, and 18 hrs post-chase. Each time

point was performed in triplicate as shown. The (H) marks ferritin heavy chain. B) Autoradiography of immunoprecipitates from 8013.6-infected cells harvested at 0,6,12, and 18 hrs post-chase. Each time point was performed in triplicate as shown. The (H) marks ferritin heavy chain. C) Densitometric quantification of ferritin heavy chain from autoradiograms was used to calculate the average quantity of ferritin heavy chain in uninfected (squares) and infected cells (circles). Lines representing interpolation of the data points were used to calculate the half-life of ferritin. The $t_{1/2}$ of ferritin in uninfected cells is 20.1 hrs and the $t_{1/2}$ of ferritin in infected cells is 5.3 hrs. This assay was repeated 3 times with similar results.

Figure 4

Indirect immunofluorescence microscopy of ferritin and LAMP-1

Indirect immunofluorescence microscopy of A431 cells using a polyclonal ferritin antibody (red) and a monoclonal LAMP-1 antibody (green). Nuclei and bacteria are visualized with DAPI (blue). A) Uninfected cells B) Uninfected cells treated with 100μM Desferal for 8 hrs C) Cells infected with 8013.6 at an MOI of 10 for 8 hrs. Punctate ferritin staining was frequently visualized in close proximity to intracellular MC (white arrow). D) A DAPI only image of the same field of cells shown in (C). A cluster of intracellular MC is located in the bottom right quadrant (white arrow).

Figure 5

Leupeptin inhibits ferritin degradation in MC-infected cells

Autoradiograms of material immunoprecipitated from ^{35}S-labeled A431 cells with a polyclonal ferritin antibody. The (H) marks the location of ferritin heavy chain. A) Uninfected cells lysed immediately after a 2-hr chase. B) Infected cells lysed 18 hrs post-chase. C) Leupeptin-treated infected cells lysed 18 hrs post-chase.

Figure 6

Analysis of iron-containing molecules in A431 lysates by gel filtration

Gel filtration of lysates from uninfected A431 cells (squares) infected for 8 hrs with 8013.6 (triangles). Cells were labeled for 12 hrs with ^{55}Fe prior to the assay. Iron-containing molecules were separated by size using a Sephacryl S400 column. Even numbered fractions were monitored for radioactivity and ferritin levels by ELISA. Data points represent ^{55}Fe counts per minute in each fraction. Gray bars indicate ferritin levels in uninfected fractions. No ferritin was detected in fractions from infected cell lysates. The peak elution of the following standards: Horse spleen ferritin (FT) MW 450kDa, hemoglobin (Hb) MW 64.5kDa and heme (H) MW 652Da, are marked on top of the figure.

Figure 7

Ascorbic acid and human holotransferrin inhibit ferritin degradation in MC-infected cells

ELISA quantification of ferritin within A431 epithelial cells treated with 200mM Na Ascorbate (squares) or 40μM human holotransferrin (diamonds) prior to infection with 8013.6. Lysates of infected cells were quantified throughout 12 hrs of infection. Data points represent the average of 3 cultures with error bars representing standard deviation. Data is expressed as relative ferritin levels normalized to those of uninfected cells. Ferritin levels from infected untreated cells are shown for comparison (triangles). This assay was repeated 3 times with similar results.

Figure 8

Localization of ferritin in MC-infected cells

Indirect immunofluorescence microscopy of A431 cells using a polyclonal antibody to visualize ferritin (red) and DAPI (blue) to visualize nuclei and intracellular MC. A) Uninfected A431 cells. B) A431 cells infected with 8013.6 for 8 hrs at an MOI of 10 display ferritin aggregation around 3 clusters of intracellular MC. C) A431 cells infected with 8013.6 for 8 hrs in the presence of 200mM Na ascorbate. D) A431 cells infected with 8013.6 for 8 hrs in the presence of 40μM human holotransferrin.

Figure 1

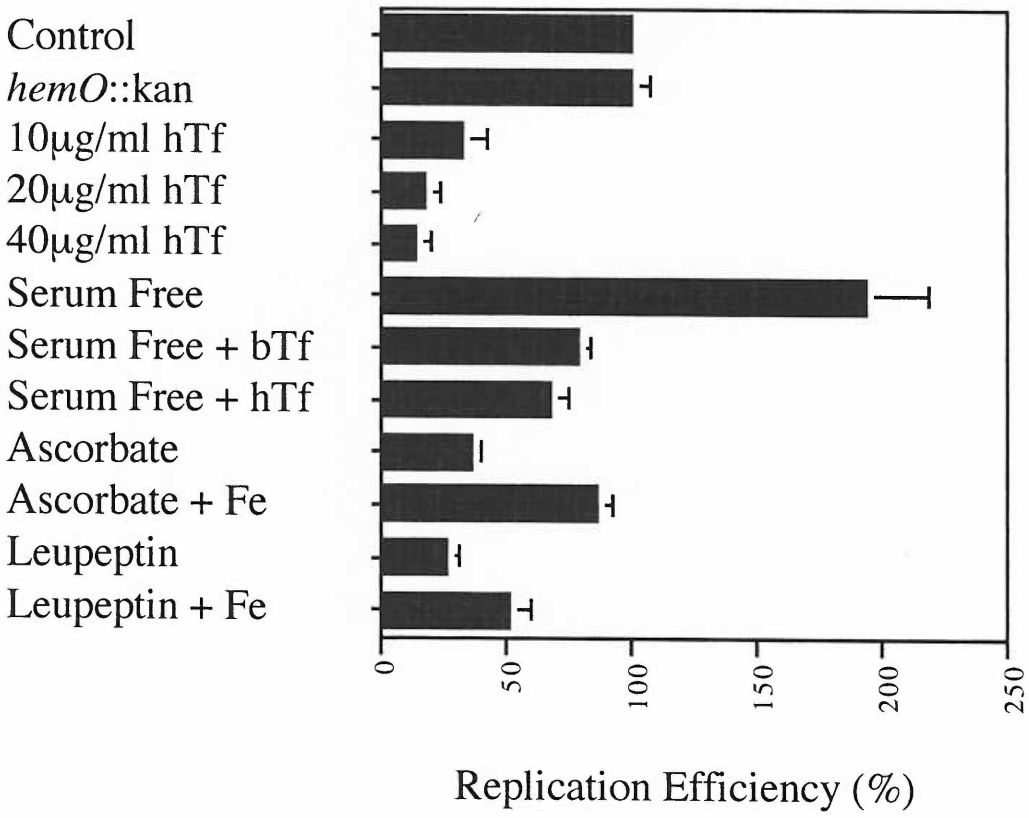

Control
hemO::kan
10μg/ml hTf
20μg/ml hTf
40μg/ml hTf
Serum Free
Serum Free + bTf
Serum Free + hTf
Ascorbate
Ascorbate + Fe
Leupeptin
Leupeptin + Fe
0
50
100
150
200
250
Replication Efficiency (%)

Figure 2

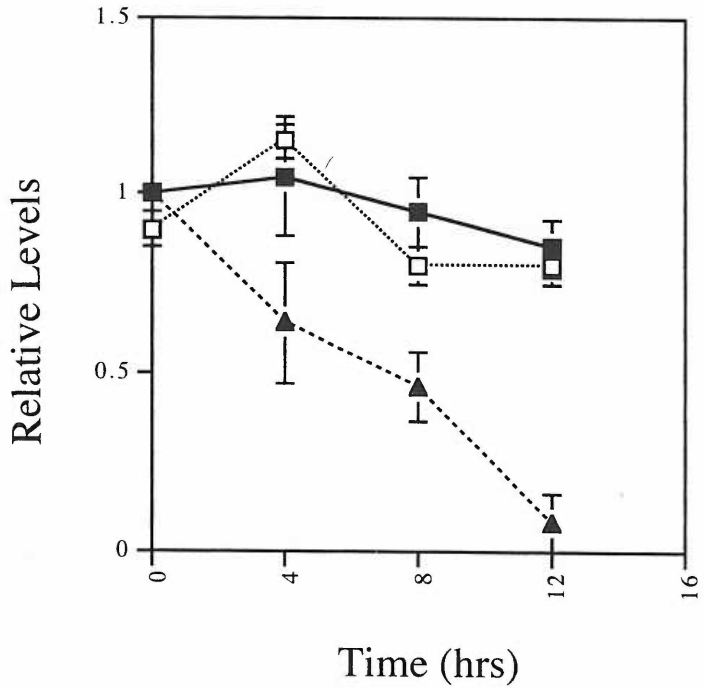


Figure 3

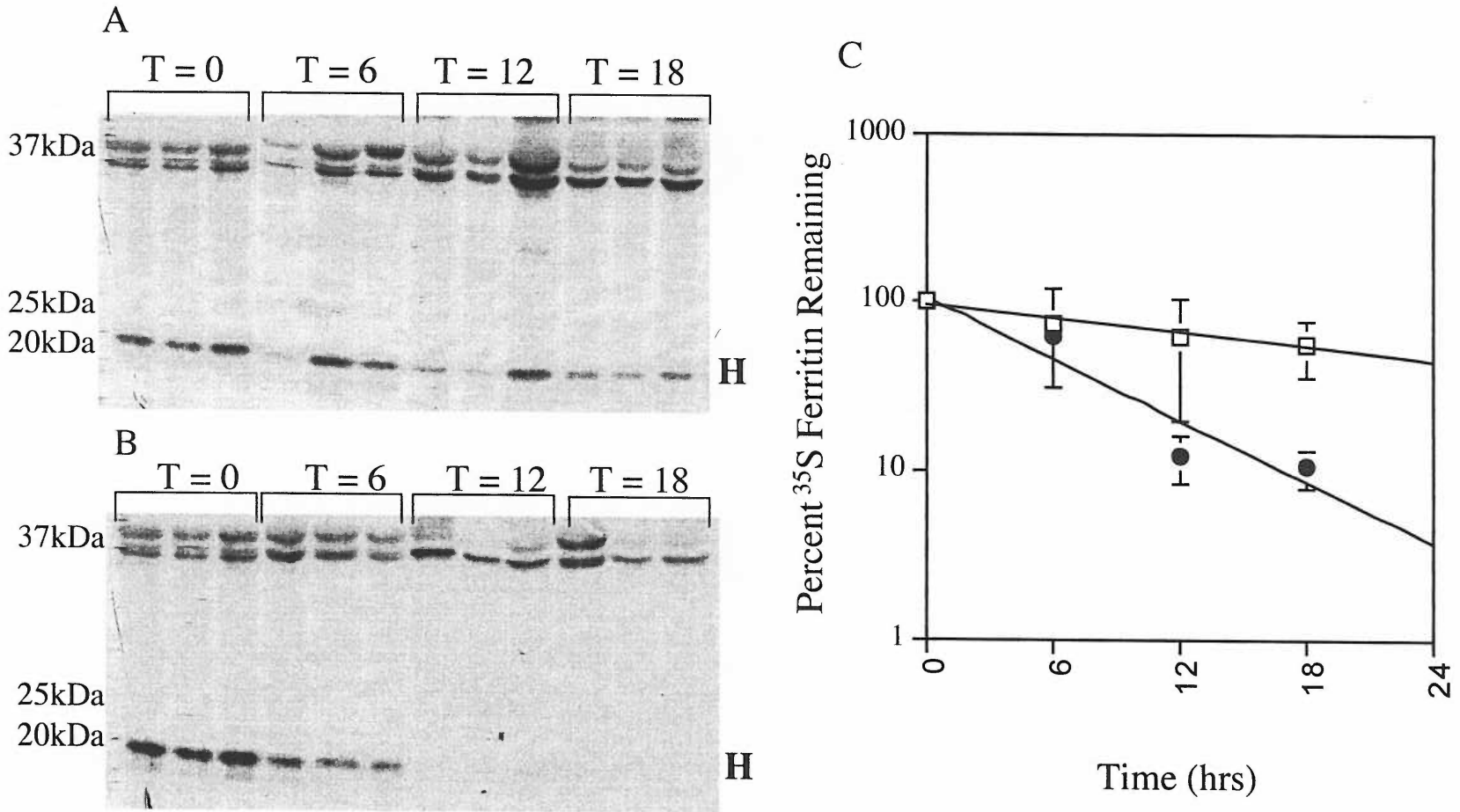

A
T = 0
T = 6
T = 12
T = 18
37kDa
25kDa
20kDa
H
B
T = 0
T = 6
T = 12
T = 18
37kDa
25kDa
20kDa
H
C
1000
100
10
1
Percent 35S Ferritin Remaining
0
6
12
18
24
Time (hrs)

Figure 4

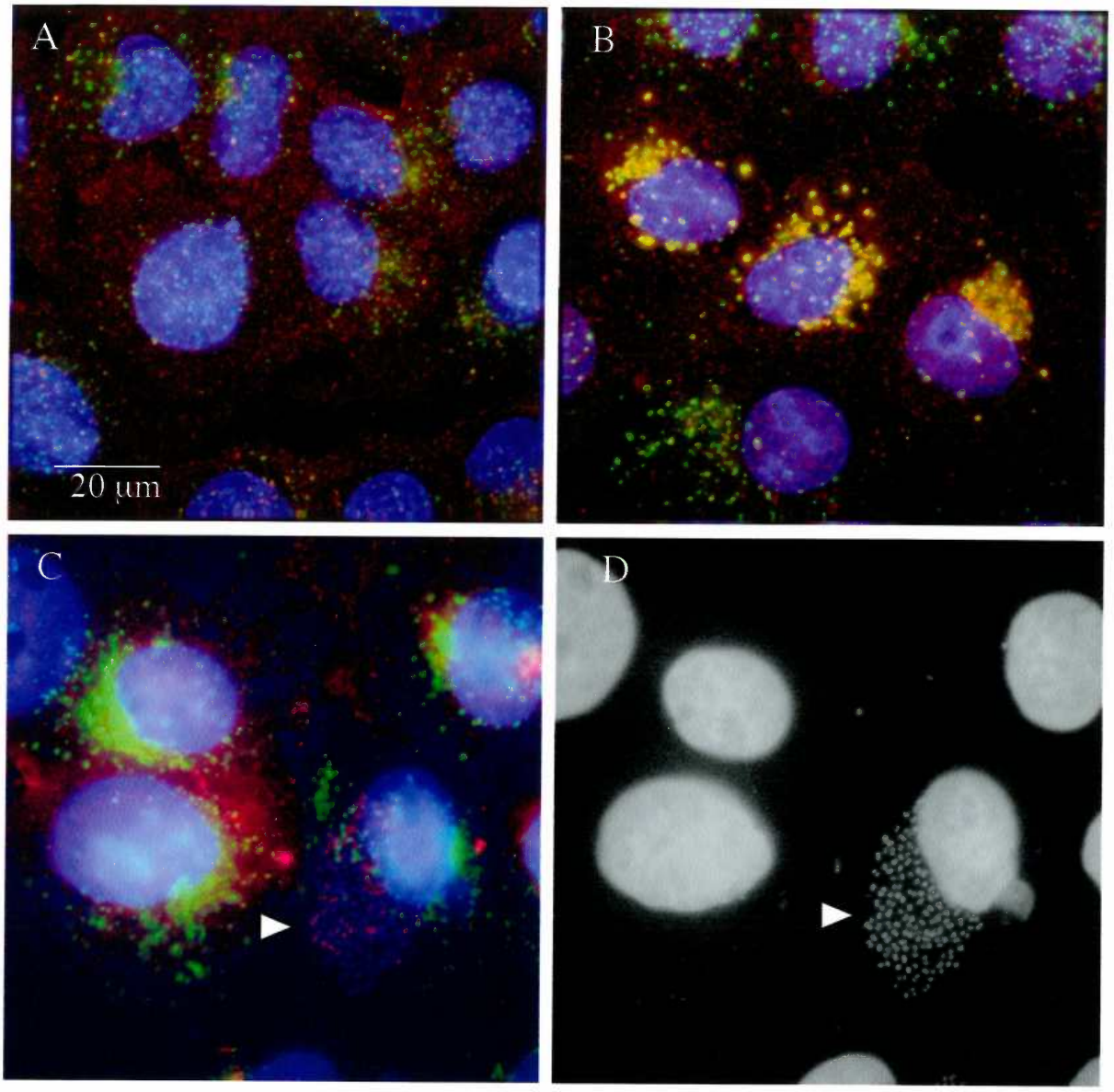


Figure 5

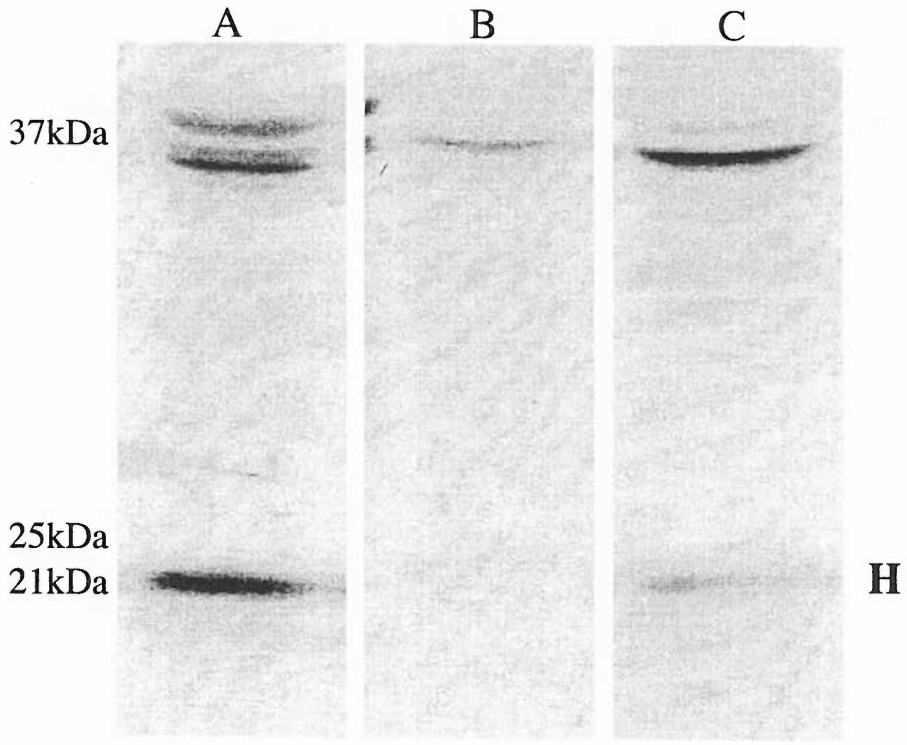


Figure 6

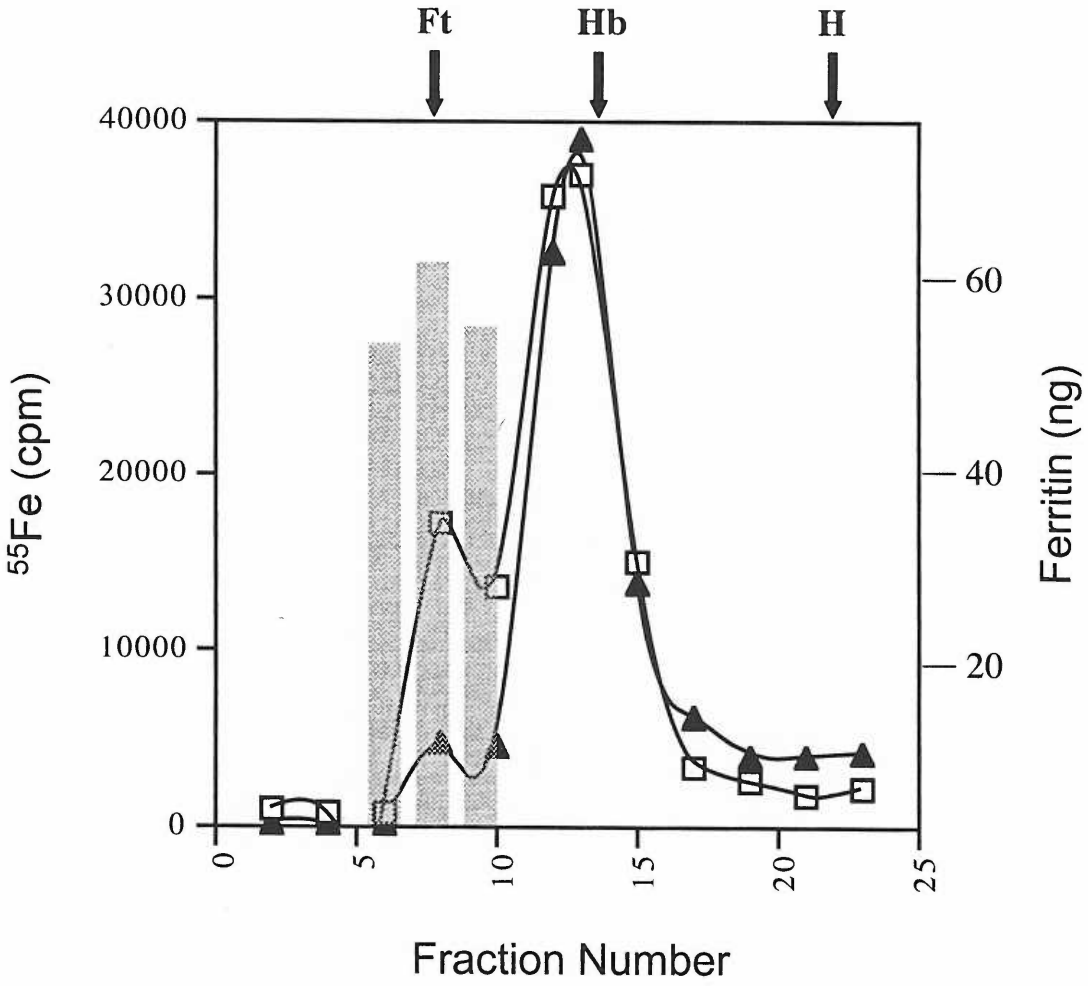

Ft
Hb
H
40000
30000
20000
10000
0
^{55}Fe (cpm)
60
40
20
Ferritin (ng)
0
5
10
15
20
25
Fraction Number

Figure 7

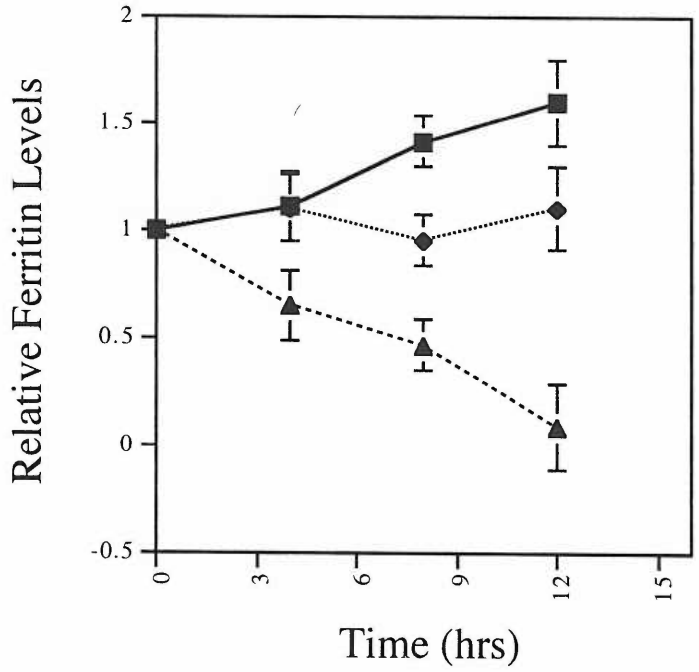
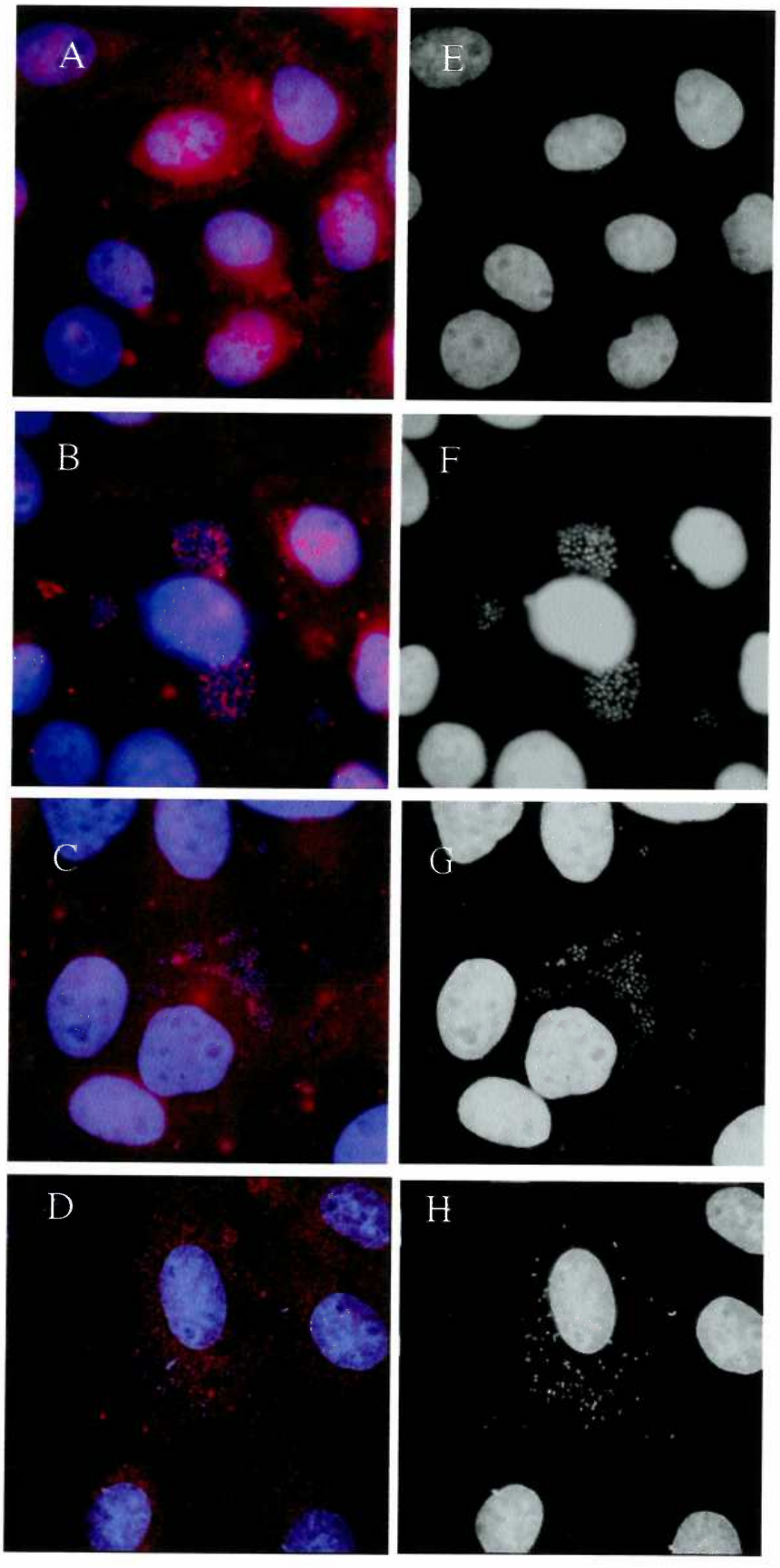


Figure 8



Chapter 4: Conclusions and Prospects

Neisseria meningitis is perhaps the most versatile iron-scavenger that infects humans. What it lacks in its inability to produce siderophores, it more than makes up for via its repertoire of outer membrane receptors that remove iron directly from host proteins. While the reasons for the tactics that MC uses to gain access to iron can only be speculated, it seems logical that this is a product of their single environment, the human host.

Siderophore-mediated iron acquisition is inefficient at best, because it supposes that secreted siderophores will return to the species that secreted them. The fact that many bacteria can utilize xenosiderophores coupled to the recent identification of a human siderophore scavenging protein, suggests that most siderophores fail to return to their species of origin. However, siderophores are able to scavenge iron from a wide range of environments, which could explain their prevalence in bacteria such as E. coli that have diverse habitats.

MC has well-documented mechanisms to obtain iron from transferrin, lactoferrin, heme, hemoglobin, and the xenosiderophore enterobactin (Rohde and Dyer, 2003; Schryvers and Stojiljkovic, 1999). This thesis presents data that suggests the existence of an additional iron-scavenging mechanism, one that can access the vast reserves of iron within cells. Ninety-nine percent of total body iron is

located intracellularly (Stojiljkovic and Perkins-Balding, 2002). It would be almost naive to assume that a pathogen that is so successful at exploiting the human environment cannot obtain iron from within cells. Our recent studies suggest that MC cannot only access this iron but does so through manipulating the host cell and taking advantage of previously existing host cell mechanisms.

We demonstrated that the iron chelator Desferal inhibits intracellular replication of MC. This effect was iron-dependent, as ferric nitrate supplementation rescued replication of MC. Desferal, a water-soluble chelator, is known to enter cells via fluid-phase endocytosis. Desferal does not enter the cytoplasm during short incubations but can affect the labile iron pool, most likely by interfering with transferrin iron uptake or blocking the flux of iron from lysosomes (Cable and Lloyd, 1999). Our result suggests that the meningococcal iron source is within the endosomal-lysosomal system. We also showed that intracellular MC must acquire iron from the host cell in order to replicate and that this process involves an unidentified TonB-dependent receptor and an unknown iron source.

We showed that MC-infected cells rapidly degrade ferritin, that degraded ferritin is recruited to intracellular MC and that this degradation promotes intracellular MC replication. The ferritin degradation and MC replication was diminished in transferrin-loaded cells and enhanced in iron-starved cells. Our laboratory previously showed that MC-infected cells express low levels of transferrin

receptors and that their transferrin cycling is slowed (Bonnah *et al.*, 2000). Taken together these results suggest that MC induces an iron-starvation response in host cells that forces ferritin degradation. This degradation results in the mobilization of iron from the ferritin core, and may be intended to supply iron for host cell metabolic needs. This process, however, appears to produce a meningococcal iron source that is required by intracellular MC in order to replicate.

We also demonstrated that ascorbic acid inhibits the process of ferritin degradation and reduces the ability of intracellular MC to replicate. This result stimulates speculation that Vitamin C may be beneficial in combating MC infection. To the best of our knowledge, nobody has investigated the role of Vitamin C in preventing meningococcal carriage or alleviating the effects of MC-related disorders. It is not clear how well our tissue culture model can predict interactions within the host, but it may be worthy of investigation.

There are several aspects of intracellular iron acquisition by MC that have yet to be addressed. Foremost is the identity of the iron-containing species that is utilized. There may, of course, be more than one. The simplest possibility is that direct products of ferritin degradation are utilized. The products of ferritin degradation have never been characterized *in vivo* or *in vitro*. The only product of the lysosomal degradation of ferritin that has been reported is hemosiderin.

Hemosiderin is a degradation product of ferritin that accumulates within cells during pathological iron overload. Its structure is poorly defined, and it has been described as an amorphous globule of ferric oxide with both protein and lipid constituents. Some of the protein components react with ferritin H-chain antibodies, but no other proteins have been pinpointed as a constituent (Miyazaki *et al.*, 2002). Hemosiderin is mostly associated with the liver, spleen, bone marrow, heart, and brain, suggesting cells that frequently have major iron deposits may have specific mechanisms for hemosiderin formation. Hemosiderin is insoluble, and in some cases the iron is precipitated in the form of geothite, which does not freely release iron (Harrison and Arosio, 1996). Hemosiderin may, therefore, be at a metabolic endpoint and not used as an iron source.

MC infection of A431 cells triggered mobilization of iron from ferritin to a low molecular weight pool, a process that is not consistent with hemosiderin formation. Further research on this phenomenon should lead to the identification of the iron-containing species within this low molecular weight pool. In the simplest model for ferritin degradation, ferric oxide polymers would be broken down into smaller ferric oxide molecules that may have phosphate associated with them. MC may have one or more TonB-dependent receptors for ferritin degradation products.

If ferritin degradation products can support growth of MC, this raises another possibility, that of iron cannibalization. *Neisseria*, like most bacteria, stores iron within bacterioferritin, a functional homologue of human ferritin (Chen and Morse, 1999). Although bacterioferritins show little homology to ferritin on the protein level, they are structurally similar and can store iron in the same form as mammalian ferritins (Harrison and Arosio, 1996). MC frequently undergoes autolysis in stationary phase (Reshilov *et al.*, 1986). It is, therefore, possible that bacterioferritin released from autolysed MC could support growth of living MC. In support of this theory, iron-depleted cultures of MC will reach a high optical density after several days of incubation. Subsequent plating of these cultures reveals that most of the MC are dead. The presence of Desferal in these cultures will prevent them from reaching saturation (personal unpublished data).

Another possible means of ferritin iron acquisition by MC is via host iron-binding molecules that may be intended to redistribute ferritin-derived iron to the cellular cytosol. One potential mechanism for iron redistribution from degraded ferritin that would be an extremely significant finding is one mediated by a human siderophore. There is some new evidence that makes this idea seem plausible. As described earlier, the human lipocalin, NGAL, can bind catecholate siderophores (Goetz *et al.*, 2002). Its ability to alter epithelial tissue development suggests that there is a chatecholate siderophore produced by human epithelial cells (Yang *et al.*, 2002). A very exciting simplified model for intracellular iron

acquisition by MC is the following: 1) MC induces iron starvation in host cells by blocking transferrin-mediated uptake; 2) Iron-starved cells degrade ferritin within MC-altered lysosomes in order to meet their own metabolic needs; 3) Host cell siderophores bind iron released from degraded ferritin in order to redistribute it to the cytosol; 4) MC intercepts iron-loaded siderophores and takes them up via one of the TonB-dependent receptors.

We have currently initiated a collaborative investigation with Dr. Roland Strong in order to address the possibility that a siderophore is delivering iron to intracellular MC. Purified NGAL from Dr. Strong's lab will be added to infected cultures during the intracellular replication phase. NGAL may bind a mammalian siderophore and diminish MCs access to the iron complex, thus limiting growth. NGAL that is preloaded with the siderophore enterobactin will serve as a control to verify that NGAL's activity is based only on its iron-scavenging abilities. Successful identification of a human siderophore would be a huge contribution to the field of mammalian iron physiology.

A critical component of any model for intracellular iron acquisition by MC is the identity of the TonB-dependent receptor(s) involved in intracellular iron acquisition. Although not yet included in a manuscript, some progress has been made in this area. As mentioned in the introduction, six different putative TonB-dependent receptors have been annotated on the meningococcal genome

projects. Because it was the first available, the genome from the serogroup A isolate Z2491 was used to identify potential receptors (http://www.sanger.ac.uk/Projects/N_meningitidis/).

All of these candidate receptors share homology to known TonB-dependent receptors including putative B-barrel pores, N-terminal plugs, and "TonB boxes". Primers were generated to all six putative receptors and cloning of each was attempted via PCR. Full-length PCR products were not obtained from two of these putative receptors, NMA0575 and NMA1662, indicating that they have been partially deleted from our serogroup C isolate MC8013.6.

The remaining four putative receptors were cloned and mutated via transposon mutagenesis with the mini transposon mTnEGNS (Mehr and Seifert, 1997), which contains an erythromycin resistance marker. The mutated plasmids were sequenced to verify transposon insertion and transformed into MC8013.6.

Multiple isolates of each mutant were assayed for their ability to replicate within A431 cells using the protocol described in Manuscript 2. Two of these putative receptor mutants, NMA2193 and NMA1558, replicated with the same efficiency as wildtype MC (data not shown). The two remaining putative receptor mutants, NMA1161 and NMA1700, had pronounced intracellular replication defects (appendix 1). However, compared to the TonB mutant examined in Manuscript

1, the replication efficiency of these mutants did not rescue as well upon supplementation with ferric nitrate. While the reasons for this are unknown, it does cast some doubt on whether or not these receptors are involved in intracellular iron uptake.

In order to verify the nature of the defects displayed by NMA1161 and NMA1700 it will be necessary to isolate the iron containing species that is produced upon ferritin degradation and perform an *in vitro* assay for its utilization with each of the mutants, along with the TonB mutant and wildtype MC8013.6 as controls. Ideally, the NGAL experiments will suggest the presence of a siderophore. This would allow the isolation of the siderophore using an NGAL affinity purification method.

The observations made in this thesis have other implications that transcend intracellular iron acquisition by MC. One question raised by the MC-induced redistribution of cytosolic ferritin to compartments and its degradation is whether or not this process generates toxic free radicals that may affect the host cell. MC expresses catalase and superoxide dismutase that may protect it from oxidative insult in such an environment (Archibald and Duong, 1986; Bisaillon *et al.*, 1985), however, the host cell may not have this level of protection.

There may be some evidence for oxidative damage to host cells. MC infection results in a massive reduction of lysosomal levels that is due in part to the ability of IgA protease to cleave the lysosomal membrane protein Lamp1 (Lin *et al.*, 1997). Other lysosomal constituents such as Lamp2 and CD63 are also reduced during infection and they are not believed to be substrates for IgA protease (Ayala *et al.*, 1998). One possibility is that lysosomal destruction is also facilitated by the iron-catalyzed production of free radicals.

Serum starvation of hepatocytes was recently shown to increase ferritin turnover within lysosomes and increase the cellular sensitivity to oxidative damage (Ollinger and Roberg, 1997). Iron-catalyzed Fenton chemistry leading to lysosomal rupture was believed to be the culprit in hepatocyte death (Ollinger and Roberg, 1997). In MC-infected cells IgA protease and iron may act synergistically to destroy lysosomal constituents. This view is supported by the observation that ascorbate-treated cells infected with MC have much higher levels of Lamp1-positive compartments (personal unpublished observation).

The iron starvation-induced movement of iron may play another important role during meningococcal infection. One hallmark of MC-induced septic shock and meningitis is massive endothelial damage within capillaries. This effect was shown to be dependent on the presence of PMNs, which secrete hydrogen peroxide when activated (Klein *et al.*, 1996). If endothelial cells respond similarly

to epithelial cells upon MC infection, the hydrogen peroxide could be taken up into iron-containing compartments within the endothelial cells, contributing to the observed damage. In agreement with this theory, I found that A431 cells infected for 12 hours with MC were eight-fold more sensitive to hydrogen peroxide killing. The effect was not seen in ascorbate-treated cells (personal unpublished observations).

One last implication of the present study is that iron may be released from infected cells. MC infection triggers exocytosis of lysosomes from epithelial cells (Ayala *et al.*, 2001), and may also lead to exocytosis of iron-containing vesicles. This process could increase the availability of this vital nutrient to MC on the mucosal surface.

Hopefully, this thesis will contribute to the understanding of MC interactions with the human host. Minimally, it will bring further appreciation to the diverse means that *Neisseria meningitidis* will go to in order to get iron from its host. To extend upon the quote in the acknowledgements by Igor Stojiljkovic – " These damn bugs always want to eat iron. Sometimes they use a fork, sometimes a spoon." "Sometimes they wait to be served and other times they break into your house, smash your refrigerator, steal your food, and don't bother to say thank you."

Chapter 4 References

Archibald, F.S., and Duong, M.N. (1986) Superoxide dismutase and oxygen toxicity defenses in the genus Neisseria. *Infect Immun* **51**: 631-641.

Ayala, B.P., Vasquez, B., Clary, S., Tainer, J.A., Rodland, K., and So, M. (2001) The pilus-induced Ca2+ flux triggers lysosome exocytosis and increases the amount of Lamp1 accessible to Neisseria IgA1 protease. *Cell Microbiol* **3**: 265-275.

Ayala, P., Lin, L., Hopper, S., Fukuda, M., and So, M. (1998) Infection of epithelial cells by pathogenic neisseriae reduces the levels of multiple lysosomal constituents. *Infect Immun* **66**: 5001-5007.

Bisaillon, J.G., Dubois, G., Beaudet, R., Sylvestre, M., Charbonneau, R., and Gagnon, M. (1985) Quantitative determination of catalase activity produced by Neisseria gonorrhoeae, Staphylococcus epidermidis, Neisseria meningitidis and other bacterial strains using the Catalasemeter. *Exp Biol* **43**: 225-230.

Bonnah, R.A., Lee, S., Vasquez, B.L., Enns, C., and So, M. (2000) Alteration of epithelial cell transferrin-iron homeostasis by the pathogenic Neisseriae. *Cell Microbiol* **In press.**

Cable, H., and Lloyd, J.B. (1999) Cellular uptake and release of two contrasting iron chelators. *J Pharm Pharmacol* **51**: 131-134.

Chen, C.Y., and Morse, S.A. (1999) Neisseria gonorrhoeae bacterioferritin: structural heterogeneity, involvement in iron storage and protection against oxidative stress. *Microbiology* **145 (Pt 10)**: 2967-2975.

Goetz, D.H., Holmes, M.A., Borregaard, N., Bluhm, M.E., Raymond, K.N., and Strong, R.K. (2002) The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell* **10**: 1033-1043.

Harrison, P.M., and Arosio, P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* **1275**: 161-203.

Klein, N.J., Ison, C.A., Peakman, M., Levin, M., Hammerschmidt, S., Frosch, M., and Heyderman, R.S. (1996) The influence of capsulation and lipooligosaccharide structure on neutrophil adhesion molecule expression and endothelial injury by Neisseria meningitidis. *J Infect Dis* **173**: 172-179.

Lin, L., Ayala, P., Larson, J., Mulks, M., Fukuda, M., Carlsson, S.R., Enns, C., and So, M. (1997) The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells. *Mol Microbiol* **24**: 1083-1094.

Mehr, I.J., and Seifert, H.S. (1997) Random shuttle mutagenesis: gonococcal mutants deficient in pilin antigenic variation. *Mol Microbiol* **23**: 1121-1131.

Miyazaki, E., Kato, J., Kobune, M., Okumura, K., Sasaki, K., Shintani, N., Arosio, P., and Niitsu, Y. (2002) Denatured H-ferritin subunit is a major constituent of haemosiderin in the liver of patients with iron overload. *Gut* **50**: 413-419.

Ollinger, K., and Roberg, K. (1997) Nutrient deprivation of cultured rat hepatocytes increases the desferrioxamine-available iron pool and augments the sensitivity to hydrogen peroxide. *J Biol Chem* **272**: 23707-23711.

Reshilov, L.N., Borovkova, V.M., Salov, V.F., Basnak'ian, I.A., and Lopyrev, I.V. (1986) [Formation of microvesicles in meningococcal cells during batch cultivation]. *Zh Mikrobiol Epidemiol Immunobiol*: 12-16.

Rohde, K.H., and Dyer, D.W. (2003) Mechanisms of iron acquisition by the human pathogens Neisseria meningitidis and Neisseria gonorrhoeae. *Front Biosci* **8**: d1186-1218.

Schryvers, A.B., and Stojiljkovic, I. (1999) Iron acquisition systems in the pathogenic Neisseria. *Mol Microbiol* **32**: 1117-1123.

Stojiljkovic, I., and Perkins-Balding, D. (2002) Processing of heme and heme-containing proteins by bacteria. *DNA Cell Biol* **21**: 281-295.

Yang, J., Goetz, D., Li, J.Y., Wang, W., Mori, K., Setlik, D., Du, T., Erdjument-Bromage, H., Tempst, P., Strong, R., and Barasch, J. (2002) An iron delivery pathway mediated by a lipocalin. *Mol Cell* **10**: 1045-1056.

Appendix 1

Intracellular Replication of MC8013 and Putative TonB-dependent Receptor Mutants