Microbial Induction of Programmed Cell Death in the Gastrointestinal Tract

by

Nicola L. Jones

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Molecular and Medical Genetics, University of Toronto.

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Dissemination of Work Arising from this Thesis

Chapter 3 has been accepted for publication pending revision in American Journal of Physiology:


Chapter 4 was published as detailed below:


Chapter 5 was published as outlined:


Studies from our laboratory discussed in the Introduction of the thesis were taken from the following publications:


List of Abbreviations

Apaf-1 apoptotic protease releasing factor-1  
BabA blood group antigen binding adhesin  
BH Bcl-2 homology  
CAD caspase-activated DNase  
CagA cytotoxin associated gene product  
CARD caspase recruitment domains  
eaeA *Escherichia coli* attaching and effacing gene A  
ELISA enzyme-linked immunosorbent assay  
EPEC enteropathogenic *Escherichia coli*  
EspA *Escherichia coli* secreted protein A  
EspB *Escherichia coli* secreted protein B  
EspD *Escherichia coli* secreted protein D  
FADD Fas-associating protein with death domains  
Gb-3 globotriaosylceramide  
gld generalized lymphoproliferation disease  
GVHD graft-versus-host disease  
HUS Hemolytic uremic syndrome  
ICAD inhibitor of caspase-activated DNase  
ICE interleukin-1b converting enzyme  
IFN-γ gamma-interferon  
IL-1 interleukin 1  
IL-2 interleukin 2  
IL-8 interleukin 8  
IP3 inositol trisphosphate  
ipaB invasin plasmid antigen B gene
kDa  kilodalton
LeB  Lewis blood group B
LEE  locus of enterocyte effacement
LeX  Lewis blood group x
LeY  Lewis blood group y
lpr  lymphoproliferation
LPS  lipopolysaccharide
MALT mucosa-associated lymphoid tissue
MHC major histocompatibility complex
NF-κB nuclear factor kappa B
PARP poly(ADP-ribose) polymerase
RDEC-1 rabbit diarrheagenic Escherichia coli
STEC Shigatoxin-producing Escherichia coli
Stx1  Shigatoxin-1
Stx2  Shigatoxin-2
TGF-β transforming growth factor beta
Tir  translocated intimin receptor
TNF-α tumor necrosis factor alpha
TTP Thrombotic thrombocytopenic purpura
TUNEL terminal transferase-mediated biotinylated dUTP nick end-labeling
VacA vacuolating cytotoxin
VTEC Verotoxin-producing Escherichia coli
WHO World Health Organization

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Abstract

Infection with *Helicobacter pylori* and Shigatoxin-producing *Escherichia coli* is associated with a number of diseases in humans. The mechanisms underlying disease pathophysiology remain unclear. Recent evidence suggests that induction of apoptosis could be a pathogenic trait of specific bacterial pathogens. Therefore, the objectives of this thesis were 1) to determine if gastrointestinal pathogens or their products induce an apoptotic response in epithelial cells following infection and 2) to delineate the mechanisms by which infection triggers the cell's apoptotic machinery.

I determined if infection with the gastric pathogen, *H. pylori*, induced apoptosis of gastric epithelial cells. *In vivo* studies documented that infection was associated with an increase in both proliferation and apoptosis of gastric epithelial cells in association with enhanced expression of the tumor suppressor p53. These findings indicate that induction of apoptosis occurs during infection and could influence disease outcome.

I began to explore the mechanisms responsible for the increase in apoptosis observed during *H. pylori* infection *in vivo*. I determined that the bacteria could directly trigger apoptosis of epithelial cells *in vitro*. In addition, infection with *H. pylori* increased expression of the Fas death receptor on gastric epithelial cells in association with an increased sensitivity to Fas-triggered cell death. Taken
together, these findings indicate that *H. pylori*-mediated apoptosis occurs by more than one pathway. The bacterium directly stimulates apoptosis and infiltrating immune cells can induce apoptosis through the Fas signaling pathway.

Finally, I demonstrated that epithelial cells expressing globotriaosylceramide undergo apoptosis following incubation with Shigatoxins produced by *E. coli*. The induction of apoptosis is associated with an alteration in Bcl-2 family member expression. Furthermore, the toxin-mediated apoptosis can be inhibited by overexpression of the anti-apoptotic homologue Bcl-2 or by inhibition of downstream caspase activation. Inhibition of the apoptotic cascades defined in these studies could ameliorate the disease complications associated with infection.

These findings indicate that bacterial pathogens and their products are capable of inducing apoptosis in epithelial cells. Therefore, the induction of apoptosis could be a common mechanism by which bacteria interact with host cells.
Chapter 1:

Introduction
Apoptosis

Definition and Features

Cells die by one of two morphologically distinct processes, necrosis or apoptosis (Figure 1-1). In general, necrosis is considered to be a pathologic process usually following a severe insult to the cell (reviewed in Kerr et al., 1994). During necrosis the plasma membrane loses selective permeability and the cell begins to swell. The organellar membranes also lose integrity and are unable to maintain normal function. Condensation of chromatin may occur but it tends to be irregular. Leakage of cytoplasmic contents evokes an inflammatory response in the surrounding tissue. Overall, the configuration of the necrotic cell is maintained until its removal by professional phagocytes.

The process of apoptosis was first discovered by Kerr (1971; 1972) during the investigation of ischaemic injury to hepatocytes. Kerr (1971) initially described the form of cell death observed following mild ischaemic injury as "shrinkage necrosis". Upon further characterization, recognizing that this type of cell death was different from necrosis the term "apoptosis" was coined (Kerr et al., 1972). Apoptosis is derived from an ancient Greek word for "falling off of leaves from trees" and is identified most readily by specific morphologic features. In the past two decades, there has been an explosion of scientific literature investigating this form of cell death.
Figure 1-1: Transmission electron micrographs of (A) an apoptotic HEP-2 cell and (B) a HEP-2 cell undergoing necrosis. (A) The apoptotic cell shows the characteristic features of apoptosis including condensed and margined nuclear chromatin (arrows) and cell membrane blebbing. In comparison, (B) the necrotic cell is swollen with loss of membrane integrity.
Morphology

During apoptosis the cell receives a stimulus which triggers the death-signaling cascade (Kerr et al., 1994). The cell begins to shrink in association with cytoplasmic condensation and vacuolation. The surface of the cell is altered with loss of microvilli and formation of blebs. In addition to these morphologic events, the outer aspects of the plasma membrane constituents change such that phosphotidylserine is exposed to the outer leaflet of the lipid bilayer (Fadok et al., 1992). During this time an increase in tissue transglutaminase allows for cross linking of cytoplasmic and membrane proteins to maintain cell membrane integrity (Fesus et al., 1987; Melino et al., 1994). Nuclear changes, including condensation and margination of chromatin around the nuclear envelope are observed (reviewed in Kerr et al., 1995). Ultimately, the cell separates into discrete, membrane bound apoptotic bodies (reviewed in Kerr et al., 1995). These alterations in surface constituents allow for the recognition and phagocytosis of apoptotic bodies by either neighboring cells or professional scavengers (Fadok et al., 1992). It is generally considered that due to maintenance of cell membrane integrity, the absence of leakage of cytoplasmic constituents into the extracellular space, and the rapid removal of apoptotic bodies, programmed cell death is not associated with a marked degree of inflammation (Cohen, 1994).

A characteristic, although not universal, biochemical feature of apoptosis is the fragmentation of the nuclear DNA (Montague and Cidlowski, 1996). The DNA initially undergoes fragmentation into larger 50 kilobase pair fragments and then 180-200 base pair internucleosomal fragments (Peitsch et al., 1993). The
presence of fragmented DNA is often exploited to detect apoptotic cells both in situ and in vitro.

**Apoptosis signaling cascades**

Investigation of the molecular regulation of apoptosis in *Caenorhabditis elegans*, drosophila and mammals demonstrates a large degree of conservation across species (Thornberry, 1998). A number of different stimuli and signaling pathways are able to initiate the final common process of programmed cell death (Thompson, 1995; Green 1998). Many of these pathways interact with each other to modulate the cell death signal and demonstrate both the complexity and redundancy of the apoptotic process. It is likely that this redundancy allows for a finer regulation of apoptosis by providing cell type-specific and stimulus-specific apoptotic pathways. Reviewed below are the key signaling pathways investigated in the studies outlined in this thesis.

**Caspases**

Caspases are a family of cysteine proteases that play a key role in mediating apoptosis (Thornberry and Lazebnik 1998; Green et al., 1998). The importance of caspase activation was initially identified in the nematode *C. elegans* (Xue and Horvitz, 1995; Yuan et al., 1993). The caspase, CED-3, is essential for the physiologic cell death that occurs in 131 of the 1090 somatic cells during development of the nematode (Xue and Horvitz, 1995). Mutation of CED-3 or expression of p35, a caspase inhibitor, abrogates apoptosis of the cells that were predestined to die. Following this pivotal discovery, the identification of ten
human homologues to date (Table 1-1) have confirmed the importance of the
caspase cascade in the apoptotic pathway in mammalian cells.

Caspases are synthesized as inactive proenzymes which are cleaved to form a
large subunit (17-21 kDa) and a small subunit (10-13 kDa) which interact to
form heterotetramers that function as the active enzyme (Cohen, 1997) (Figure
1-2). Caspases are unique in that they require an aspartic acid at the cleavage
site (Fraser and Evan, 1996). Cleavage of the large polypeptide to the active
form of the enzyme occurs at aspartic acid residues suggesting that caspases
can be autocatalytically activated. The aggregation of procaspases mediated by
adapter proteins is thought to aid in autocatalytic cleavage (Muzio et al.,
1998). For example, following ligation of a cell death receptor such as Fas,
adapter proteins, including FADD in this case, recruit procaspases into a
complex termed the "apoptosome" (Green et al., 1998). This results in
activation of the caspase cascade.

The caspase family can be divided into three groups based on their
requirement of four specific amino acids at the cleavage site (reviewed in
Thornberry, 1998). Group I caspases require the sequence Trp-Glu-His-Asp
and include caspase-1,-4, and -5. Group I enzymes play a role in mediating
inflammation by activating pro-inflammatory cytokines. For example, caspase 1
cleaves pro-interleukin 1β to form the biologically active interleukin 1β (Black et
al. 1989). Group II caspases recognize the sequence Asp-Glu-X-Asp and
generally function as effectors of programmed cell death by cleaving crucial
structural or regulatory proteins such as inhibitor of caspase-activated DNase
(ICAD)(Green and Kroemer, 1998). Group III family members prefer the
Table 1-1. Mammalian caspases. (Adapted from Thornberry, 1998)

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<th>Caspase group</th>
<th>Putative substrates of group</th>
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<td>Caspase 1</td>
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<td>(eg. retinoblastoma protein)</td>
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<td>Caspase 2</td>
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<td><strong>Group III: activators of apoptosis</strong></td>
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<td>Caspase 6</td>
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<tr>
<td>Caspase 8</td>
<td>and nuclear lamins</td>
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<td>Caspase 10</td>
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Figure 1-2: Enzymatic cleavage of caspase proenzyme to form active caspase. Cleavage of the large polypeptide occurs at aspartic acid residues. The large and small subunits then interact to form a heterotetramer which functions as the active enzyme. (Adapted from Thornberry and Lazebnik, 1998)
sequence Val/ Leu- Glu- X- Asp and serve as upstream activators of group II enzymes. Thus, in addition to autocatalytic cleavage, caspases can activate other members of the family in a cascade which induces programmed cell death.

The crucial role for caspases in programmed cell death is illustrated by the phenotypes of various caspase knockout mice. Mutants of caspases 1, 2, 3, 11 and, most recently, 9 have been generated (Hakem et al., 1998; Kuida et al., 1998). Although some phenotypic similarities exist, each of the mutant mice display cell-type and stimulus- specific alterations in apoptosis. This finding indicates that under certain circumstances caspases can function independently. For example, the majority of both caspase 9 and caspase 3 deficient mice die perinatally and display abnormal cortical development in association with decreased programmed cell death of neurons in the cerebral cortex (Hakem et al., 1998; Kuida et al., 1998). However, T cells obtained from caspase 3 deficient mice are resistant to Fas-mediated cell death (Hakem et al., 1998; Kuida et al., 1998). In contrast, caspase 9 deficient T cells remain sensitive to Fas ligation. Similarly, caspase 3-negative thymocytes undergo apoptosis upon exposure to dexamethasone whereas caspase 9-negative thymocytes are resistant to this apoptotic stimulus (Hakem et al., 1998; Kuida et al., 1998). These data suggest that caspase activation to induce cell death may not always occur in a sequential fashion.

Recent evidence indicates that additional factors regulate caspase-mediated apoptosis (Figure 1-3). Release of mitochondrial cytochrome c is a potent activator of caspases (Zhivotisky et al., 1998). Cytochrome c associates with apoptotic protease releasing factor 1 (Apaf-1, the human homologue of CED-4)
**Figure 1-3:** Schematic representation of the apoptotic signal cascade triggered by two pathways, ligation of death receptor or alternate death signal. Ligation of the Fas death receptor by the Fas ligand causes trimerization and mediates binding of the adaptor protein FADD through death domains. The death effector domain of FADD then causes oligomerization of procaspase 8 autocatalytically activating caspase 8 and triggering the caspase cascade. Cleavage of key substrates by the caspases results in apoptosis. Alternatively, the cell receives a death signal which results in the release of mitochondrial cytochrome c. Cytochrome c associates with Apaf-1, likely resulting in a conformational change causing exposure of the CARD domain allowing binding to procaspase 9. Activation of the caspase cascade then mediates programmed cell death. The two death signaling pathways are linked at an as yet unidentified step. (Adapted from Green, 1998)
DEATH RECEPTOR

DEATH SIGNAL

FADD

Isl

CYTOCHROME C

APAF-1

MITOCHONDRIA

PROCASPASE 8

PROCASPASE 9

CASPASE CASCADE

APOPTOSIS

FAS
and pro-caspase 9 to form a multiprotein complex also known as the mammalian apoptosome (Li et al., 1997). The physical interaction of Apaf-1 and caspase 9 is mediated through their terminal caspase recruitment domains (CARD) and ultimately causes proteolytic activation of caspase 3. The crucial role for Apaf-1 in the apoptotic process is demonstrated by two recent studies of knockout mice with targeted disruption of Apaf-1 (Cecconi et al., 1998; Yoshida et al., 1998). Phenotypically these mice display severe craniofacial abnormalities with cleft palate and brain hypertrophy, persistence of interdigital webs and alterations in the eye including the lens and retina. Consistent with divergent signaling pathways, signaling through the death receptor Fas remained intact in the Apaf-1/-/- mice.

**Cytochrome c**

Normally localized to the inner mitochondrial membrane, cytochrome c appears to be released to the cytoplasmic compartment by the disruption of the mitochondrial transmembrane potential during the apoptotic process (Zhivotsky et al., 1998). Since disruption of mitochondrial membrane integrity is observed prior to caspase activation in some forms of apoptosis it has been suggested that cytochrome c acts upstream of caspase 9 (Bossy-Wetzel et al., 1998). However, caspases are also capable of disrupting mitochondrial function indicating that cytochrome c and caspase activation may act as self-amplifiers of the initial apoptotic signal (Marzo et al., 1998). The Bcl-2 family of pro and anti-apoptotic regulatory proteins also plays a role in regulation of this pathway since Bcl-2 and Bcl-xL inhibit the release of cytochrome c (Kluck et al., 1997; Yang et al., 1997). Thus, it is clear that the complex process of caspase
activation plays a crucial role in regulating cell death which is stimulus- and cell type- dependent.

**Fas ligand/Fas receptor**

The Fas ligand is a type II membrane protein belonging to the tumor necrosis factor family which, when bound to the Fas receptor, triggers an apoptotic signal within the cell (Suda et al., 1993) (Figure 1-3). Fas ligand undergoes metalloproteinase-mediated proteolytic cleavage which generates a soluble, less active form (Tanaka et al., 1996). Thus, under physiologic conditions the membrane-bound form of Fas ligand is believed to induce apoptosis via cell-to-cell interactions, while the soluble form attenuates cell death.

The Fas receptor (also known as CD95 or APO-1) is a type I membrane protein which belongs to the tumor necrosis factor receptor family (Nagata and Golstein, 1995). Similar to other receptor-mediated effects, the cell death signal via Fas receptor is transduced by a series of protein-protein interactions. Activation of the Fas receptor is caused by binding of the natural Fas ligand or by agonistic antibodies which result in receptor clustering through interactions of the Fas intracellular death domain (Itoh and Nagata, 1993). This activation initiates clustering with Fas-associating protein with death domain (FADD, also known as MORT1) (Chinnaiyan et al., 1995). The C-terminal region of FADD contains a homologous death domain which interacts with active Fas receptor while the N-terminus region contains a death effector domain which is necessary for transducing the death signal. Overexpression of FADD results in apoptosis, while expression of FADD mutants lacking the N-terminal region blocks Fas-mediated apoptosis (Chinnaiyan et al., 1995). The death effector
domain of FADD binds to the cysteine protease caspase 8 (also known as FLICE/ MACH) (Muzio et al., 1996; Boldin et al., 1996). The N-terminal region of caspase 8 contains two death effector domains which mediate binding to FADD and allows oligomerization of the protein resulting in self-activation of its protease domain. The resulting pattern of caspase activation in response to Fas-mediated signaling is not entirely clear. However, inhibitors of capase 3 and caspase 1 block Fas-mediated cell death (Enari et al., 1996). In addition, sequential activation of caspase 1 and caspase 3 is observed during Fas-stimulated death (Enari et al., 1996). Apoptosis is then executed by caspase-mediated proteolysis of important functional and structural proteins such as poly(ADP-ribose) polymerase (PARP), actin, and caspase-activated DNase (CAD) (Enari et al., 1998).

A physiologic role implicated for Fas-induced cell death is downregulation of the immune response by eliminating activated T cells (Lenardo, 1996). In addition, Fas also may be involved in the deletion of activated B cells (Rothstein et al., 1995). Virus-infected cells and malignant cells are also eliminated through Fas-mediated apoptosis (Nagata and Golstein, 1995).

Recent evidence indicates that Fas signaling also is involved in the pathogenesis of certain gastrointestinal diseases. The Fas receptor is normally expressed on epithelial cells within the gastrointestinal tract (Leithauser et al., 1993). The Fas ligand is only expressed on Paneth cells and occasionally on cells within the lamina propria (Moller et al., 1996). In contrast, an increase in infiltrating T lymphocytes expressing the Fas ligand is observed in mucosal biopsy specimens of affected areas of the colon obtained from individuals with ulcerative colitis (Ueyama et al., 1998). In a murine model of autoimmune
gastritis, enhanced epithelial cell Fas receptor expression is identified in association with increased apoptosis (Nishio et al., 1996).

The importance of Fas signaling in humans is made evident by the development of disease in individuals who carry mutations within the Fas gene (Rieux-Laucat et al., 1995; Drappa et al., 1996; Fisher et al., 1995; Le Deist et al., 1996). The Canale-Smith syndrome is a rare disease in children which causes lymphoproliferation with lymphadenopathy and hepatosplenomegaly often in association with autoimmune diseases such as hemolytic anemia. The accumulated lymphocytes are CD4-CD8+, T cell receptor positive cells expressing high levels of IL-2 receptor α, suggesting that they are chronically activated T cells. Genetic analysis has identified several different Fas mutations which accompany this syndrome and result in genes encoding Fas proteins which are unable to activate the normal signal transduction cascade.

The phenotype of these patients mimics those found in two inbred strains of mice, lpr (for lymphoproliferation) and gld (for generalized lymphoproliferative disease) mice (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). lpr mice have an autosomal recessive mutation in the Fas receptor gene resulting in impaired transcription of the gene. The mutation is leaky as a low level of full length Fas receptor mRNA can be detected in the thymus and liver of these mice (Watanabe-Fukunaga et al., 1992). gld mice have a point mutation in the Fas ligand which disrupts binding to the Fas receptor (Takahashi et al., 1994). Similar to the human counterpart, lpr and gld mice display lymphadenopathy and hepatosplenomegaly. The mice produce high levels of antibodies and usually succumb to either nephritis or arthritis before six months of age.
Bcl-2 family

Proteins in the Bcl-2 family are key regulators of the programmed cell death cascade and consist of both pro- and anti-apoptotic homologues (Table 1-2) (Chao and Korsmeyer, 1998). Bcl-2 was originally identified during investigation of the chromosomal translocation t(14; 18), a translocation characteristic of B cell lymphomas (Tsujimoto et al., 1984). The juxtaposition of Bcl-2 to the immunoglobulin heavy chain locus results in deregulated expression of Bcl-2. Subsequent in vitro (Vaux et al., 1988; Hockenberry et al., 1990) and in vivo analyses (McDonnell et al., 1989; McDonnell et al., 1991) documented that Bcl-2 functions to prevent cell death.

The important role of several of the Bcl-2 family members has been addressed by knock-out studies in mice. Bcl-2 homozygous knockout mice die at a few weeks of age due to renal failure (Veis et al., 1993). Morphologically the kidneys display features of polycystic kidney disease indicating that Bcl-2 plays a key role in renal development. In addition, Bcl-2/-/- mice become hypopigmented due to decreased survival of melanocytes. In contrast, Bax knockout mice appear phenotypically normal (Knudson et al., 1995). However, Bax/-/- mice are infertile due to alterations in the cell death pathways that occur during the formation of sperm cells and ovaries.

The mechanisms by which the Bcl-2 family regulates cell death are beginning to be characterized. Following the discovery of Bcl-2, additional family members were discovered by identifying proteins which interact with Bcl-2 (Oltvai et al.,
Table 1-2: The mammalian Bcl-2 family (Adapted from Adams and Cory, 1998).

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<tr>
<th>Anti-apoptotic</th>
<th>Pro-apoptotic</th>
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<tr>
<td>Bcl-2</td>
<td>Bax</td>
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<td>Bcl-xL</td>
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</table>
Thus the ability of the proteins to form hetero and homodimers suggests that, through competitive dimerization, pro- and anti-apoptotic members regulate cell death. Indeed, the ratio of Bcl-2 to the apoptotic agonist Bax determines the sensitivity of some cells to specific apoptotic stimuli such as cytokine withdrawal (Oltvai et al., 1993). The Bcl-2 family members contain various conserved regions designated Bcl-2 homology regions BH1, BH2, BH3 and BH4 which are required for protein-protein interactions (Chao and Korsmeyer, 1998). Mutational analysis demonstrated that these domains are necessary both for hetero and homodimerization and, in some instances, for regulation of apoptosis (Yin et al., 1994; Chittenden et al., 1995).

Although these mutational studies support the role for competitive dimerization of positive and negative homologues in regulating cell death, more recent studies indicate that additional, more complex factors also are involved (Hentgartner et al., 1998). Both Bcl-2 and Bax are co-localized to mitochondria and disruption of the mitochondrial transmembrane potential is a common feature observed during apoptosis. Therefore, a role for Bcl-2 and Bax in disrupting mitochondrial function has also been considered.

Bcl-2 and Bax can insert into artificial lipid bilayer membranes and form channels with selective ion conductivity (Adams and Cory, 1998; Chao and Korsmeyer, 1998). It remains unclear precisely how these pores may function to mediate the cell death signal. Bcl-2 channels could alter the permeability transition pore of mitochondria thereby regulating the release of cytochrome c, an activator of the caspase signaling cascade (Zhivotsky et al., 1998). The ability of Bax to mediate release of cytochrome c provides support for this
hypothesis (Rosse et al., 1998). Bcl-2 is capable of blocking cell death and cytochrome c under certain conditions (Kluck et al., 1997; Yang et al., 1997). However, in rat embryo fibroblasts or human melanoma cells which overexpress Bax, Bcl-2 inhibits DNA degradation characteristic of apoptosis but does not block cytochrome c release (Rosse et al., 1998). Thus regulation of cell death by these positive and negative homologues involves complex as yet undetermined pathways.

p53

The importance of the p53 gene is evident when one considers that approximately half of all human cancers contain mutations of p53 (Levine et al., 1997). In addition, humans with the Li-Fraumeni syndrome who are heterozygous for p53 develop a higher rate of malignancies at an earlier age (Malkin et al., 1992). Furthermore, p53 null mice are susceptible to the development of a wide spectrum of tumors (Donehower et al., 1992). The p53 protein is often referred to as the "gatekeeper of the genome" since its biologic roles include cell cycle regulation and the induction of apoptosis in response to DNA damage (Levine, 1997).

The exact mechanisms by which p53 signals a cell to undergo programmed cell death following DNA damage are not known. In certain cell types, p53 transcriptionally activates the pro-apoptotic protein Bax after DNA damage (Miyashita and Reed, 1995). In addition, Bax expression correlates with apoptosis suggesting that Bax mediates p53-dependent apoptosis. However, in the absence of p53, overexpression of Bax does not restore DNA-damage
mediated apoptosis (Brady et al., 1996). These findings suggest that Bax is not the only factor involved in mediating p53 dependent cell death.

An additional contributor to p53-triggered apoptosis has recently been described. KILLER/DR5 (Wu et al., 1997) is a receptor for TRAIL, a member of the tumor necrosis family which can stimulate programmed cell death in vitro (Pan et al., 1997). Overexpression of KILLER/DR5 also can stimulate apoptosis in the absence of TRAIL (Wu et al., 1997). Transfection of wildtype p53 and DNA damage induces transcription of KILLER/DR5 (Wu et al., 1997). Furthermore, mutation of p53 blocks DNA damage KILLER/DR5 induction (Wu et al., 1997).

**Physiologic Apoptosis in the Gastrointestinal Tract**

The epithelium of the gastrointestinal tract has a highly stereotyped organization and is under a constant state of renewal (Potten, 1995). In humans the villus-crypt epithelial unit is renewed approximately every 5 to 6 days, whereas in mice the epithelium is renewed every 3 days. Stem cells, which are relatively undifferentiated cells capable of cell cycle progression, while at the same time maintaining their numbers, give rise to more differentiated progeny and allow for regeneration following tissue injury. The topographic organization of the proliferative compartment within different regions of the gut is clearly defined in humans.

For the maintenance of tissue homeostasis, the proliferative rate must be balanced by cell loss through the morphologically distinct process of apoptosis (Hall et al., 1994). Apoptosis also occurs in a spatially oriented manner in the...
gastrointestinal epithelium. In the small and large intestine, the rate of apoptosis increases as the cells move up the crypt villus axis away from the proliferative compartment (Jones and Gore, 1997; Hall et al., 1994; Shibahara et al., 1995). Apoptosis is also observed in the region of the stem cells presumably thereby removing cells with DNA damage (Hall et al., 1994).

In the stomach, a bidirectional flux of proliferating cells is observed. Cells destined to become parietal cells slowly migrate downwards to the base of the gastric gland, while the majority of cells derived from the stem cell migrate upwards (Hall et al., 1994). Thus, apoptotic cells are observed in the stomach both at the epithelial surface and, to a lesser degree, in the base of the gland.

Current evidence indicates that several mediators including apoptotic regulatory proteins, dietary factors, components of the extracellular matrix as well as adhesion molecules are involved in the physiologic regulation of apoptosis in the intestine. Alterations in the same pathways also could be involved in disease processes, including carcinogenesis (Schulte-Hermann et al., 1995).

The topographic expression of Bcl-2 family members correlates with the observed proliferative and apoptotic compartments in the intestine. Expression of the anti-apoptotic member Bcl-2 is highest in the proliferative zone of the large intestine (Merritt et al., 1995). In contrast the proapoptotic homologue Bax is expressed at the luminal surface (Krajewski et al., 1994). Of interest, in the small intestine Bcl-2 is not expressed in the crypt region (Merritt et al., 1995). It has been suggested that the lack of Bcl-2 could decrease the apoptotic
threshold in the small intestine and thereby account for the lower incidence of small bowel malignancies (Merritt et al., 1995).

The localized expression of p53 also implicates a role for this apoptotic regulatory molecule in the control of cell turnover rates in the intestine (Merritt et al., 1994). However, p53 deficient mice develop normally suggesting that p53 either does not play a specific role in physiologic cell death or there is a redundancy in the system controlling apoptosis (Donehower et al., 1992). p53 likely plays a role in DNA damage-induced cell death since Merritt et al. (1994) showed that intestinal cells in p53 null mice lack the normal apoptotic response to radiation exposure.

Indirect evidence implicates the Fas ligand/ Fas receptor system in the normal physiologic removal of intestinal epithelial cells. Fas receptor is expressed in human intestinal epithelial cells and a small proportion of T lymphocytes in the lamina propria exhibit Fas ligand expression (Ueyama et al., 1998; De Maria et al., 1996). An increase in Fas ligand expressing cells are detected in the colonic mucosa obtained from patients with ulcerative colitis (Ueyama et al., 1998).

In other anchorage-dependent cell types, alterations in the extracellular matrix regulate apoptosis (Boudreau et al., 1995). Components of the extracellular matrix likely also play a role in regulation of programmed cell death in the intestine. Upon loss of anchorage, isolated human intestinal epithelial cells undergo apoptosis in association with activation of the caspase cascade (Grossmann et al., 1998). This detachment-induced cell death mimics the normal physiologic process of cell loss at the luminal surface (Hall et al., 1994).
Several mediators are thought to contribute to physiologic detachment induced cell death in the intestine. The extracellular matrix and its components differ along the crypt villus axis (Beaulieu, 1992; Probstmeier et al., 1990). Transforming growth factor- beta (TGF-β) expression is increased at the luminal surface (Avery et al., 1993) and TGF-β is capable of inducing apoptosis in intestinal cell lines (Wang et al., 1995). Cell to cell contact also is crucial since expression of a dominant-negative E-cadherin mutant along the murine intestinal crypt-villus axis disrupts intercellular and cell-matrix contact and results in increased cell migration, loss of differentiation and enhanced apoptosis (Hermiston and Gordon, 1995).

Deregulation of Apoptosis

Cancer

For many years it was considered that deregulation of proliferation played a key role in carcinogenesis. Recent evidence suggests that the multistage process of tumorigenesis involves deregulation of both proliferation and apoptosis (Evan and Littlewood, 1998). Investigations using animal models as well as studies in humans provide evidence to support the role of altered cell death in the initiation and progression of neoplasms. Furthermore, therapeutic agents directed against cancers work most effectively through the induction of apoptosis (Kerr et al., 1994).

The p53 gene is frequently altered in human cancers and is now known to regulate apoptosis. Individuals with the Li-Fraumeni syndrome who are
heterozygous for the p53 gene develop malignancies at an increased frequency (Malkin et al., 1990). The loss of p53-dependent cell death could allow propagation of abnormal cells. In support of this hypothesis, in a murine model of choroid plexus oncogenesis, p53-/ mice develop more rapid tumor growth due to a reduction in apoptosis (Symonds et al., 1994). Furthermore, the responsiveness of tumor cells to chemotherapeutic agents or radiation often depends on the presence of functional p53 (Lowe et al., 1993).

Alteration in the expression of the anti-apoptotic protein Bcl-2 is frequently observed during the adenoma to carcinoma sequence of colonic carcinogenesis (Bronner et al., 1995). Increased levels of Bcl-2 are detected in dysplastic, adenomatous areas adjacent to tumors suggesting that Bcl-2 plays a role early in the neoplastic process (Bronner et al., 1995). In a p53-mediated model of choroid plexus tumors in mice, deficiency of the apoptotic homologue Bax results in accelerated tumor growth, further supporting a role for induction of apoptosis in modulating the neoplastic process (Yin et al., 1997).

The recognition and removal of tumor cells by Fas signaled cell death may also modulate the malignant process (Nagata, 1996). However, some tumor cells have developed mechanisms to evade this immune surveillance. For example, colonic tumor cells display resistance to Fas-triggered cell death by a reduction in Fas receptor expression (O'Connell et al., 1997). The expression of functional Fas ligand by tumor cells triggers apoptosis of T lymphocytes and could allow escape from recognition by infiltrating immune cells (Strand et al., 1996; O'Connell et al., 1999). Additional, as yet undefined, disruptions in Fas signaled cell death of tumor cells also have been described (von Reyher et al., 1998).
In summary, these studies indicate that modulation of programmed cell death plays a role in tumor development. However, the exact mechanisms by which deregulated apoptosis mediates the development of human malignancies is yet to be determined.

**Bacterial Infection**

Infectious agents— including viruses and bacteria— have developed mechanisms to exploit the apoptotic machinery of the eukaryotic cell which can result in the induction of programmed cell death (Zychlinsky and Sansonetti, 1997). Virus encoded proteins can interact with several apoptotic pathways to either inhibit or induce apoptosis to maximize viral replication. For example, Kaposi’s-sarcoma-associated human herpesvirus 8 FLICE inhibitory proteins (v-FLIPS) interact with FADD to block cell death mediated by the Fas pathway (Thome et al., 1997). Homologues of the Bcl-2 family, such as the Epstein Barr virus BHRF-1, inhibit apoptosis induced by the BCL-2 pro-apoptotic family member Bik (Henderson et al., 1993).

A growing list of bacterial pathogens have also been recognized as mediators of the cell death cascade (Table 1-3). Perhaps the best characterized bacterial modulator of cell death is *Shigella flexneri*, the agent responsible for bacillary dysentery. *S. flexneri* is an invasive pathogen which translocates epithelial cells and M cells, multiplies and then spreads to adjacent cells within lymphoid follicles in the lamina propria. *S. flexneri* infects macrophages with resulting tissue destruction, infiltration of phagocytic cells, and the formation of abscesses (Anand et al., 1986).
Table 1-3: Summary of bacterial pathogens shown to mediate programmed cell death in eukaryotic cells.

- *Staphylococcus aureus*
- Enterogaegregative *Escherichia coli*
- *Bordetella pertussis*
- *Shigella flexneri*
- *Pseudomonas aeruginosa*
- *Salmonella enteritidis*
- *Mycobacterium tuberculosis*
- *Yersinia enterocolitica*
- *Actinobacillus actinomycetemcomitans*
- *Listeria monocytogenes*
Shigella infection induces apoptosis of infected macrophages both in vitro (Zychlinsky et al., 1992) and in vivo (Zychlinsky et al., 1994). Characterization of the bacterial factors which mediate cell death identified the bacterial invasin gene *ipaB* (invasin plasmid antigen B) as essential since isogenic mutants of *ipaB* are unable to stimulate programmed cell death (Zychlinsky et al., 1994). Furthermore, microinjection of an IpaB-GST fusion product induces apoptosis in macrophages (Chen et al., 1996). By utilizing affinity purification and immunoprecipitation, the IpaB fusion product bound to macrophage proteins (1996). *S. flexneri*-mediated cell death is inhibited by ICE-specific inhibitors (Chen et al., 1996). Taken together, these findings indicate that there is an interaction of IpaB with host cell ICE which mediates apoptosis. However, it remains unclear if IpaB is capable of directly activating the ICE cell death cascade. It is of interest that *S. flexneri* does not stimulate apoptosis during infection of an epithelial cell line (HeLa) in vitro (Mantis et al., 1996). The factors which account for this cell type specificity for apoptosis have not been elucidated.

Recent evidence also indicates that induction of apoptosis during infection of immune cells, including macrophages, may be a common mechanism employed by bacterial pathogens. For example, in vitro studies indicate that several different pathogens including *Salmonella* (Monack et al., 1996), *Pseudomonas* (Baran et al., 1996), and *Yersinia* (Ruckdeschel et al., 1997) stimulate cell death of immunocytes. In contrast to the investigation of infected immune cells, few studies have addressed the apoptotic response of epithelial cells, the first line of defense encountered during infection.
For many of these pathogens, the mechanism(s) involved in transducing the cell death signal are not known. Zychlinsky (1994) proposed several mechanisms by which bacteria, or their products, might activate the cell death cascade. Binding of the bacteria, or its product, to a host cell receptor could activate the apoptotic program. Entry of an invasive organism or a secreted molecule could mimic second messengers in the cell death cascade or be directly toxic to the cell. Alternatively, the microorganism or a component of the bacterium could disrupt a constitutively expressed anti-apoptotic signal within the host cell.

In summary, the recent discovery of the induction of apoptosis in response to bacterial infections has resulted in a intense interest in the field. Knowledge gained from these studies should elucidate the role of microbial-mediated cell death in disease pathogenesis and allow for the development of novel therapies.
Helicobacter pylori

*Helicobacter pylori* is a gram negative microaerophilic bacterium which infects more than half of the world population (Cover, 1997). Infection with this microbe is the major causal factor resulting in the development of chronic-active (type B) gastritis and peptic ulcer disease, including both duodenal ulcers and those gastric ulcers unrelated to the ingestion of aspirin and other non-steroidal anti-inflammatory agents (Bourke et al., 1996).

Chronic infection is also considered a risk factor for gastric adenocarcinoma and lymphoma. Prospective case control studies identify a higher rate of serologic evidence of *H. pylori* infection amongst individuals with cancers in the body and antrum of the stomach compared with age-matched controls (Forman et al., 1991; Parsonnet et al., 1991; Nomura et al., 1991). Epidemiologic studies also have documented an association between *H. pylori* infection and the development of gastric lymphomas including MALT lymphoma (Parsonnet et al., 1994). Eradication studies document the regression of low grade MALT lymphoma following anti-helicobacter eradication therapy (Bayerdoffer et al., 1995; Rogerro et al., 1995; Neubauer et al., 1997). A working group of the World Health Organization concluded there is sufficient evidence from studies in humans to declare *H. pylori* a type 1 carcinogen (IARC, 1994). Recently, an animal model which develops gastric cancer during chronic infection with *H. pylori* substantiates the role of the bacterium in the initiation of gastric malignancies (Watanabe et al., 1998).

Since its initial discovery by Drs. Marshall and Warren in the early 1980's (Warren and Marshall, 1983), an explosion of scientific information including the
recent sequencing of the entire genome of two *H. pylori* strains (Tomb et al.,
1997; Alm et al., 1999) has focused on enhancing our understanding of the
bacterium. Although the factors involved in disease pathogenesis remain
unknown, it is likely that both host and bacterial factors play a role (Mobley,
1997).

**Pathogenesis**

**Adhesion**

*Helicobacter pylori* binds specifically to gastric epithelial cells lining the
stomach or in areas of gastric metaplasia (Clyne and Drumm, 1997). The
mechanisms responsible for this tissue tropism remain unclear but likely are
related to specific bacterial adhesins. Although the precise factors mediating *H.
pylori* adherence are unclear, it is generally considered that multiple adhesins
are involved (Sherman, 1994). The recent sequencing of the entire genome of
one *H. pylori* strain identified the presence of a large number of genes
potentially encoding outer membrane proteins and lipoproteins which may
serve as putative adhesins (Tomb et al., 1998).

A number of other putative attachment factors also have been investigated.
These include urease, heat shock proteins, blood group antigens and major
histocompatibility complex molecules. Initial reports suggested that urease
mediates bacterial attachment to the gastric epithelium (Tsuda et al., 1994).
However, current evidence indicates that this surface-exposed enzyme does
not play a role in the attachment of *H. pylori* to gastric epithelia. No detectable
difference in bacterial binding to either primary gastric epithelial cells or a
gastric epithelial cell line in vitro was observed when adhesion was compared between an isogenic urease-negative mutant and the parent strain (Clynne and Drumm, 1996).

Heat shock proteins also have been implicated in mediating H. pylori adhesion. A brief period of acid shock altered H. pylori binding specificity with an increase in bacterial adherence to sulfogalactosyl ceramide as assessed by using a thin-layer chromatography overlay binding assay (Huesca et al., 1996). This effect was abrogated by incubation with either inhibitors of protein synthesis or anti-heat shock protein antibodies, suggesting a role for heat shock proteins in mediating adhesion.

In situ studies suggest that the fucosylated blood group antigen Lewis b mediates binding of H. pylori to gastric epithelial cells (Boren et al., 1993). Utilizing a receptor activity-directed affinity tagging method, Ilver et al. (1998) purified and cloned BabA (blood group antigen-binding adhesin), the putative H. pylori adhesin which binds to Lewis b. Yet, the importance of Le\textsuperscript{b} as a receptor for H. pylori has been questioned since in vitro studies demonstrate that adhesion of H. pylori to isolated human gastric epithelial cells is independent of the expression of Lewis antigens (Clynne and Drumm, 1996).

More recently, the class II major histocompatibility complex (MHC) has been suggested to mediate attachment of H. pylori to gastric cells in vitro (Fan et al., 1998). The expression of MHC class II by gastric epithelial cell lines correlates with bacterial binding and is inhibited by co-incubation of bacteria with monoclonal antibodies that recognize the class II MHC domain. Furthermore,
transfection of MHC class II deficient cells with the gene encoding HLA-DR4 enhanced \textit{H. pylori} binding (Fan et al., 1998).

The significance of bacterial adhesion in mediating disease was recently demonstrated in a murine model (Guruge et al., 1998). \textit{H. pylori}-infected transgenic mice expressing the putative receptor Le\textsuperscript{b} in the gastric pit and mucous cells show an altered pattern of bacterial adhesion and disease manifestations compared with normal littermates. Although colonization is equivalent in both groups of mice, bacteria bind only to the mucous layer in normal mice whereas bacteria are adherent to both the mucous layer and the epithelial cells of Le\textsuperscript{b}-expressing mice. Transgenic mice have more severe gastritis with greater development of mucosal-associated lymphoid tissue (MALT). Furthermore, the presence of parietal cell autoantibodies, in association with more pronounced reactive atypia and parietal cell loss, was detected more frequently in transgenic mice (Guruge et al., 1998).

\textbf{\textit{H. pylori}-mediated Signal Transduction}

In addition to promoting binding to specific receptors on host gastric epithelial cells, adhesins can mediate a cascade of signals that are transmitted to the cytosol and nucleus of the infected eukaryotic cell. For example, following intimate adherence of \textit{H. pylori} to epithelial cells \textit{in vitro} there is an elevation in the second messenger inositol trisphosphate (IP\textsubscript{3}) (Dytoc et al., 1993; Pucciarelli et al., 1995).

The activation of IP\textsubscript{3} signaling is similar to the cascade of events observed during infection with the diarrheal pathogen enteropathogenic \textit{Escherichia coli}

-34-
EPEC (Dytoc et al., 1993). EPEC infection of eukaryotic cells induces elevation of inositol phosphate, calcium flux, and tyrosine phosphorylation of a 90 kDa protein in association with rearrangements of the cytoskeleton underneath the adherent bacteria (known as the attaching/effacing lesion) (reviewed in McDaniel and Kaper, 1997). However, it remains controversial whether or not tyrosine phosphorylation of host proteins and alteration of the cytoskeleton to form the attaching and effacing lesion occurs during H. pylori infection. Two groups (Smoot et al. 1993; Segal et al., 1997) detected F-actin accumulation and pedestal formation in gastric epithelial cells infected with H. pylori in association with tyrosine phosphorylation of two host proteins distinct from the bacterial translocated protein Tir. Isogenic mutants generated in the cag pathogenicity island abrogated the observed protein phosphorylation (Segal et al., 1997). In contrast, Dytoc et al. (1993) and Pucciarelli et al. (1995) identified microvilli effacement in the absence of redistribution of host cell cytoskeletal elements in infected epithelial cells.

H. pylori infection also causes activation and nuclear translocation of the transcription factor NF-κB in gastric epithelial cell lines. Activation of NF-κB results in increased transcription of chemokines such as the potent neutrophil chemoattractant interleukin-8 (IL-8) (Keates et al., 1997). Enhanced IL-8 production is detected in H. pylori-infected human gastric biopsy specimens (Crabtree et al., 1994) and tissue culture cells (Crowe et al., 1995). Activation of NF-κB also is detected in gastric tissue obtained from H. pylori-infected humans (Keates et al., 1997). Utilizing transfected reporter gene constructs, Aihara et al. (1997) demonstrated that NF-κB and AP-1 bind to the IL-8 promotor region to upregulate IL-8 production. Findings obtained from mutagenesis studies infer
a role for picB (for promote the induction of cytokines; and also known as cagE) in addition to other genes encoded on the cag pathogenicity island in mediating IL-8 secretion (Censini et al., 1996; Tummuru et al., 1995). It is not known if PicB can directly upregulate IL-8 or whether it is involved in the export or secretion of another activator.

Vacuolating cytotoxin

Approximately fifty percent of H. pylori strains produce a cytotoxin (VacA) which induces the vacuolation of epithelial cells in vitro (Cover, 1996). Although the gene encoding the vacuolating cytotoxin (vacA) is present in all H. pylori strains, allelic variation exists within the gene such that different alleles encoding the signal sequence (eg. s1a, s1b and s2 alleles) and the midregion (m1, and m2 alleles) are identified (Atherton et al., 1995). In vitro studies indicate that the expression of the VacA protein correlates with the presence of specific vacA alleles (Atherton, 1997).

Although initial studies suggested a link between infection with VacA expressing strains and more severe disease, subsequent studies were unable to support this association. More recently, the role of vacA genotypes in disease pathogenesis has been explored. Atherton et al. (1995) identified a higher percentage of patients with a past or present history of peptic ulceration were infected with strains possessing the s1a signal sequence allele (89%) than either the s1b (50%) or the s2 (26%) allele. A subsequent study identified more severe gastric inflammation in gastric biopsy specimens obtained from H. pylori-infected subjects harboring an s1A strain than those with s1b or s2 strains
(Atherton et al., 1997). However, these associations have not been consistently identified in other populations (Go et al., 1997).

Elucidation of the cellular mechanisms involved in cytotoxin-mediated vacuolation indicates that the toxin induces disruption in endocytic trafficking at a late stage. Markers of the late endosomal compartments, including the vacuolar-ATPase and the GTPase rab7, as well as lysosomal markers are identified on vacuole membranes (Papini et al., 1997; Molinari et al., 1997). Cells overexpressing activated rab7 stimulate vacuole formation (Papini et al., 1997). In contrast, vacuole formation is inhibited in cells expressing mutant rab7 suggesting that rab7 is required for vacuolation. A potential consequence of this altered subcellular trafficking includes the interruption of antigen presentation by newly synthesized major histocompatibility complex (MHC) class II molecules (Molinari et al., 1998).

Cytotoxin-associated gene product (CagA) and the cag Pathogenicity Island

The discovery that an H. pylori 128 kDa outer membrane protein CagA is not conserved between strains suggested that it may prove to be a marker of virulence (Tummuru et al., 1993). Initial studies identified an increased risk of peptic ulcer disease and gastric cancers in association with CagA+ strains (Atherton, 1997). However, subsequent studies in adults (Pan et al., 1997; Graham et al., 1996) and children (Loeb et al., 1998) have failed to support this correlation. These findings bring into question the importance of CagA as a virulence factor.
The identification of CagA led to the discovery of the cag pathogenicity island (Censini et al., 1996). The cagA gene maps to a 40 Kb region with a G+C content of 35% compared to 39% for the rest of the bacterial genome (Tomb et al., 1997). In addition, the region is inserted within a repetitive sequence of the glutamate racemase gene. Among strains that possess the pathogenicity island, variation exists within the coding regions such that not all strains contain the entire island. Comparisons of the sequences identified within the pathogenicity island with known genes suggests that the island encodes a novel type IV secretion system (Covacci et al., 1998). cagE (or picB), and potentially several other genes contained within the pathogenicity island are required for induction of the potent neutrophil chemokine IL-8, since mutation of the genes results in markedly reduced transcription of the chemokine (Tummuru et al., 1995; Censini et al., 1996).

The significance of the cag pathogenicity island in disease pathogenesis remains controversial. For example, the presence of the cag pathogenicity island is associated with more severe disease including peptic ulcers and gastric cancers in Western populations (Blaser et al., 1995; Parsonnett et al., 1997). In contrast, in Asian and Australian populations, infection with cag pathogenicity island + strains is not associated with an increased risk of the same complications (Pan et al., 1997; Mitchell et al., 1996). Additional studies are needed to define the role of the pathogenicity island in disease.

**Lipopolysaccharide and Molecular Mimicry**

Biochemical analysis of the lipopolysaccharide (LPS) produced by *Helicobacter pylori* strains identified the expression of carbohydrate structures similar to
human blood group antigens on the O side chains (Aspinall and Monteiro, 1996; Aspinall et al., 1996). Lewis blood group antigen expression in LPS extracted from clinical isolates is identified in up to 85% of clinical isolates (Wirth et al., 1996; Simoons-Smit et al., 1996), respectively. The frequent expression of Lewis-like carbohydrate determinants by *H. pylori* LPS indicates that this is a common feature.

The phenomenon of molecular mimicry involving these Lewis antigens could result in autoimmune-mediated destruction of gastric epithelial cells which also express these antigens (Appelmelk and Negrini, 1997). Both *H. pylori*-infected humans and mice develop autoantibodies that cross-react with human and murine gastric tissues (Appelmelk et al., 1996). Furthermore, gastritis develops in mice with a hybridoma which secretes anti-Lewis monoclonal antibodies. However, human serum samples obtained from *H. pylori*-infected individuals do not react with synthetic LeX or LeY (Appelmelk and Negrini, 1997). Instead, recent data suggest that the *H. pylori*-induced autoantibodies are directed against the protein constituents of parietal cell H⁺,K⁺-ATPase (Claeys et al., 1998). As such, the mechanisms by which *H. pylori* induces autoimmunity remain unclear.
Shigatoxin-producing *Escherichia coli*

Two decades ago the discovery of *E. coli* strains, mainly derived from children with diarrhea, which produce a toxin that is cytopathic to Vero cells heralded the emergence of a new category of pathogenic *E. coli*. These strains were originally termed Verotoxigenic *E. coli* (VTEC) (Konowalchuk et al., 1977) and are now known as Shigatoxin-producing *E. coli* (STEC). Subsequent studies have documented that infection with STEC causes both nonbloody diarrhea and hemorrhagic colitis (Riley et al., 1983). In addition, Karmali et al. (1983) was the first to identify the presence of the hemolytic uremic syndrome (HUS) in association with STEC infection. Further studies, including those documenting large outbreaks of disease (Bell et al., 1994), have confirmed that infection with STEC can result in more severe systemic sequelae, including both the hemolytic uremic syndrome and thrombocytopenic thrombotic purpura.

Generally, diarrhea in response to STEC infection resolves spontaneously. However, in children less than 10 years of age the risk for developing the hemolytic uremic syndrome (characterized by the triad of renal failure, hemolytic anemia and thrombocytopenia) is increased (Ostroff et al., 1989). Approximately 30 percent of children with HUS develop renal failure requiring supportive care and dialysis (Seigler et al., 1994). The mortality rate associated with HUS is between 3 and 5 percent (Martin et al., 1990; Rowe et al., 1991).

The major reservoir for STEC is the intestine of asymptotically colonized cattle and other ruminants (Wilson et al., 1996). Large foodborne outbreaks of STEC infection occur most frequently following ingestion of fecally contaminated undercooked ground meats including hamburger (Bell et al.,
The low infectious dose of STEC also contributes to human infection causing significant person-to-person spread and waterborne transmission of infection (Giannella, 1996).

Currently, there is no specific therapy for either STEC and its associated complications or for use in the prevention of sequelae following infection (Cohen, 1998). Thus, the elucidation of bacterial factors which contribute to disease pathogenesis remain a focus of intense investigation.

Pathogenesis of disease

Adhesion

For most enteric pathogens adherence to target cells is required for expression of full virulence (Finlay and Cossart, 1997). Animal studies indicate that this is also the case during STEC infection (Donnenberg et al., 1993). In animal models of infection, adherence of STEC is associated with the histopathologic findings of the attaching and effacing lesion similar to enteropathogenic E. coli (EPEC) (Tzipori et al., 1989; Donnenberg et al., 1993). Following intimate attachment of the bacterium, microvilli are effaced and cytoskeletal changes, including accumulation of actin, are observed underneath adherent bacteria (Ismaili et al., 1995). A pedestal is formed below the adherent organism in a dynamic process which allows motility of the microbe along the surface of the cell.

Similar to EPEC, STEC possesses an 94-97 kDa outer membrane protein called intimin which is encoded by the eae gene (Donnenberg et al., 1993;
McDaniel et al., 1995). The STEC intimin differs from EPEC intimin in the C terminal region, which codes for the proposed receptor binding site and is thought to confirm the colonization specificity of STEC to the large intestine (Yu and Kaper, 1992). In vitro and in vivo studies indicate that intimin is required but not sufficient for the formation of the attaching/effacing lesion (McKee et al., 1995; Foubister et al., 1994; Jarvis et al., 1995). In gnotobiotic piglets, infection with an eae mutant STEC strain abrogated the histopathologic features of the attaching/effacing lesion and resulted in attenuation of disease (Tzipori et al., 1995).

Not all STEC strains possess the eae gene (Dytoc et al., 1994). Therefore, additional adhesins have been considered. Our laboratory has identified an outer membrane protein, termed vintimin, that mediates adhesion to an epithelial cell line in vitro (Sherman et al., 1991). The lipopolysaccharide (LPS) of STEC initially was considered as a putative adherence factor. However, our laboratory (Cockerill et al., 1996) as well as other investigators (Bilge et al., 1996) were unable to substantiate a role for LPS in mediating bacterial binding since the loss of the O polysaccharide side-chain enhances bacterial binding to host epithelial cells.

Products encoded on a plasmid designated pO157, present in the majority of STEC strains, were also suggested as potential adhesins (Karch et al., 1987). Studies in our laboratory found no detectable difference in the induction of the attaching/effacing lesion between the parent strain and the plasmid-cured derivative (Dytoc et al., 1993). In addition, infection with pO157-deficient strains in an animal model produced equivalent diarrheal disease to that incurred by infection with the parent strain (Tzipori et al., 1987; Li et al., 1993). Taken
together, these findings do not support a specific role for the plasmid encoded genes in either adhesion or disease pathogenesis.

Signal transduction/ cytoskeletal changes

Bacterial binding initiates host cell signaling which is likely to play a role in disease pathophysiology (Finlay and Cossart, 1997). The related attaching/effacing pathogen EPEC activates a variety of signal transduction cascades within the target cell mediated by proteins encoded on the LEE (locus of enterocyte effacement) pathogenicity island (reviewed in Nataro and Kaper, 1998). The LEE pathogenicity island encodes intimin, a type III secretion system and the secreted proteins EspA, B and D.

EPEC infection is associated with an elevation in inositol trisphosphate and intracellular calcium (Baldwin et al., 1993; Dytoc et al., 1994). Phosphorylation of host cell proteins including myosin light chain is also detected (Manjarrez et al., 1992). Interruption of these signaling events by mutation of key mediators within the pathogenicity island abrogates the attaching/effacing phenotype (Foubister et al., 1994; Kenny et al., 1996). Signal transduction events also mediate bacterial binding. Tyrosine phosphorylation of a bacterial product termed tir (translocated intimin receptor) inserted into the eukaryotic cell membrane may serve as the receptor for EPEC intimin (Kenny et al., 1997).

Host cell signaling also is altered during STEC infection. STEC possesses a pathogenicity island coding for gene products similar to those found in the EPEC LEE pathogenicity island including the secreted proteins EspA, EspB, EspD, EspE, a type III secretion system and intimin (coded for by eae)
(McDaniel et al., 1995; Jarvis and Kaper, 1996). Investigation of host cell responses during STEC infection identified both common and divergent signaling compared with EPEC. Our laboratory has detected an increase in calcium levels in an intestinal cell line following infection with STEC (Ismaili et al., 1995). In contrast to EPEC, STEC infection is not associated with detectable tyrosine phosphorylation of host cell proteins (Ismaili et al., 1995; Ismaili et al. 1998).

Toxins

Toxins produced by STEC were initially termed Verotoxin due to their cytopathic effect on an African green monkey kidney cell line (Konowalchuk et al., 1977). However, characterization of the toxin showed marked similarities to Shiga toxin produced by *Shigella dysenteriae* type 1 (O'Brien et al., 1982). Furthermore, the toxin is neutralized by antibodies to Shigatoxin (O'Brien et al., 1982). Thus, it was renamed Shiga-like toxin (O'Brien et al., 1982; O'Brien and La Veck, 1983). Antibody neutralization studies demonstrate that STEC produces an additional toxin now termed Stx2 (Scotland et al., 1985). Another toxin produced by STEC, which is isolated from pigs with edema disease, is now termed Stx2e (Marques et al., 1987). In order to alleviate confusion, a new nomenclature has been proposed for the description of these toxins (Table 1-4).

Members of the Shigatoxin family produced by STEC include Stx1 and Stx2. STEC can produce Stx1 or Stx2 either alone or together in combination (Scotland et al., 1985; Stockbine et al., 1986). These bacteriophage-encoded toxins are characteristic A-B subunit toxins (reviewed in Lingwood, 1993). The A
Table 1-4: Proposed Nomenclature for Shigatoxins produced by *Escherichia coli* (Taken from ASM News, 1996; 62: 118)

<table>
<thead>
<tr>
<th>Current Name</th>
<th>Proposed Nomenclature</th>
<th>Gene</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shiga-like toxin I (SLT-I) or verotoxin I (VT1)</td>
<td><em>stx</em>1</td>
<td><em>Stx</em>1</td>
<td></td>
</tr>
<tr>
<td>SLT-II/ VT2</td>
<td><em>stx</em>2</td>
<td><em>Stx</em>2</td>
<td></td>
</tr>
<tr>
<td>SLT-IIC/ VT2c</td>
<td><em>stx</em>2c</td>
<td><em>Stx</em>2c</td>
<td></td>
</tr>
<tr>
<td>SLT-IIE/ VT2e</td>
<td><em>stx</em>2e</td>
<td><em>Stx</em>2e</td>
<td></td>
</tr>
</tbody>
</table>
subunit contains the enzymatic activity, while the B pentamer allows binding to the host cell membrane (O'Brien and Holmes, 1987). The B subunit binds to specific glycolipid receptors, globotriaosylceramide serves as the receptor for Stx1 and most forms of Stx2 (Lingwood et al., 1987) and Gb4 as the Stx2e receptor (Lingwood, 1993). The holotoxin then undergoes specific retrograde transport to the Golgi and the endoplasmic reticulum (Sandvig and van Deurs, 1996). Once translocated to the cytoplasm, the A subunit inhibits protein synthesis by inactivating the 60S ribosomal subunit thereby causing cytotoxicity in sensitive cells (Saxena et al., 1989).

Since the initial report by Karmali et al. (1983) describing the association of the hemolytic uremic syndrome with the presence of cytotoxin-producing E. coli strains in stools, many additional epidemiologic studies have linked the presence of these toxins with the complications associated with STEC infection (Karmali et al., 1985). In addition, the expression of Gb-3 in human renal tissue correlates with the age-related incidence of the hemolytic uremic syndrome (Lingwood, 1994). Some studies in animal models of infection also support a role for the toxins in mediating disease. For example, infant rabbits inoculated orally with STEC develop bloody diarrhea with histologic evidence of neutrophilic infiltration and epithelial damage in the colon (Pai et al., 1986). Infection of rabbits with an attaching and effacing lapine pathogen RDEC-1 strain (O157:H-) expressing Stx1 results in a greater severity of disease than infection with the Stx1 negative parent strain (Sjogren et al., 1994). In contrast, however, the presence or absence of Stx does not alter disease in STEC infected piglets (Tzipori et al., 1987) and infected rabbits (Li et al., 1993).
Limitations of these animal models include differences in the tissue distribution of the toxin receptor Gb-3. For example, in contrast to human renal tissue, rabbit renal tissue lacks Gb-3 (Boyd and Lingwood, 1989). Lapine renal intestinal cells express Gb-3 whereas the human intestine is deficient in Gb-3. As such, current animal models do not sufficiently reproduce renal disease and may not adequately represent the mechanisms responsible for diarrheal disease in humans (Nataro and Kaper, 1998).
Summary

This overview has highlighted the process of programmed cell death including its role in normal physiology as well as the possible sequelae of deregulation of apoptosis in disease including the multistage processes of carcinogenesis and bacterial infection. In addition, the current understanding of putative mechanisms involved in disease pathogenesis during infection with the gastrointestinal pathogens STEC and *H. pylori* are reviewed. Although the exact factors culminating in disease remain unclear, induction of programmed cell death by *H. pylori* and STEC or their products may well be involved. Investigation of the role of programmed cell death induced by these pathogens should improve our knowledge of disease pathophysiology as well as offer novel strategies to aid in the management of infection and resulting disease complications.
Chapter 2:

Hypothesis and Objectives
The hypotheses that formed the basis of the work in this thesis are:

(1) Bacterial pathogens induce apoptosis of target epithelial cells during infection.

(2) The induction of programmed cell death due to infection is involved in disease pathogenesis.

The overall objectives of my research were to determine whether there is an apoptotic response of epithelial cells to selected gastrointestinal bacterial pathogens and their products and to define the mechanisms that mediate programmed cell death.

The specific objectives of my research were to:

1. Determine if the gastric pathogen *Helicobacter pylori* induces apoptosis of infected gastric epithelial cells *in vivo* and to characterize the role of p53 (Chapter 3).

2. Elucidate the factors that stimulate cell death during *H. pylori* infection *in vitro*, including, in particular, the role of the Fas signaling cascade (Chapter 4).

3. Determine if Shigatoxins produced by *Escherichia coli* stimulate epithelial cells to undergo programmed cell death *in vitro* and to investigate the role of the toxin receptor, globotriaosylceramide, and the Bcl-2 family in the cell death cascade (Chapter 5).
Chapter 3:

Increase In Proliferation And Apoptosis Of Gastric Epithelial Cells Early
In The Natural History Of *Helicobacter pylori* Infection
Summary

Childhood acquisition of *Helicobacter pylori* is a critical risk factor for gastric cancer. Since tumorigenesis involves deregulation of proliferation and apoptosis, we examined gastric epithelial cell proliferation and apoptosis in *H. pylori*-infected children. Apoptosis and proliferation of gastric antral epithelial cells in biopsy specimens from patients with *H. pylori*-induced gastritis, secondary gastritis and noninflamed controls were compared. p53 protein expression was examined immunohistochemically. Apoptotic cells were identified in the surface epithelium in each group. The apoptotic index was higher in specimens from patients with *H. pylori* gastritis (120 ± 10) than secondary gastritis (50 ± 10) and noninflamed controls (40 ± 10, ANOVA p<0.005). Apoptosis decreased following *H. pylori* eradication and resolution of gastritis (p<0.02). An expanded proliferative compartment was identified in *H. pylori*-induced gastritis (32.4 ± 3.5; proliferative labeling index ± SE) compared to secondary gastritis (18.9 ± 2.8) and noninflamed controls (13.7 ± 3.1, ANOVA p<0.01). The accelerated cell turnover was associated with p53 overexpression (ANOVA p<0.005). Accumulation of p53 was not associated with expression of the cyclin-dependent kinase inhibitor p21. The occurrence of altered cell turnover early in the natural history of chronic infection provides an explanation for the increased risk of gastric cancer development associated with childhood acquisition of infection.
Introduction

The gastric pathogen *Helicobacter pylori* is now recognized as an important causal agent in both chronic-active gastritis and peptic ulcer disease (Veldhuyzen van Zanten and Sherman, 1994). In addition, epidemiologic studies have consistently identified an association between chronic infection with *H. pylori* and the subsequent development of gastric cancers (Correa, 1995) including carcinoma, lymphoma and MALT lymphomas. In 1994, a working group of the WHO International Agency for Research on Cancer concluded that *H. pylori* is a group 1 carcinogen in humans, and plays a causal role in the development of gastric cancer (International Agency for Research on Cancer, 1994).

It is widely accepted that tumor progression is a multistep process in which regulation of both cell proliferation and programmed cell death are disturbed (Schulte-Hermann et al., 1995). Programmed cell death, or apoptosis, is a distinct form of cell death which can be distinguished morphologically by condensation and margination of nuclear chromatin with the later formation of apoptotic bodies (Kerr et al., 1994). During apoptosis, DNA characteristically undergoes fragmentation into oligonucleosome fragments that can be detected by a variety of complementary techniques (Ben-Sasson et al., 1995). The identification of these fragments *in situ* is widely employed to distinguish cells undergoing apoptosis.

Acquisition of *H. pylori* infection during childhood appears to be a critical risk factor for the later development of gastric cancers (Blaser et al., 1995). The examination of alterations in cell turnover early in the natural history of infection
should further our understanding of the relationship between *H. pylori* and the development of gastric cancers. Therefore, both the degree of apoptosis and the proliferation of gastric antral epithelial cells were assessed in this study using paraffin-embedded biopsy samples obtained from pediatric patients infected with *H. pylori* and compared to specimens from patients with secondary gastritis and those with noninflamed gastric mucosa.

Mutation of the *p53* gene is the most frequent genetic abnormality encountered in human malignancies (Fritsche et al., 1993) including gastric cancers. A protective role for *p53* expression has been demonstrated in normal tissues with rapid cell turnover (Helander et al., 1993; Lahoti et al., 1996) or following exposure to DNA damaging agents or mutagens (Fritsche et al., 1993). Here, *p53*-mediated cell cycle arrest or apoptosis may protect the organism from propagation of cells with genetic damage (Donehower et al., 1996). We hypothesized that overexpression of wild-type *p53* in gastric epithelial cells may occur in response to infection with *H. pylori*. The *p53* protein mediates cell cycle arrest by transcriptionally activating the cyclin-dependent kinase inhibitor *p21\(^\text{waf1/cip1}\)*. Therefore, to investigate the role of the tumor suppressor *p53* protein during infection with *H. pylori*, an immunohistochemical analysis of both *p53* and *p21* expression was employed.
Methods

Tissue samples (Table 3-1)

Gastric biopsy specimens were obtained from the antrum of pediatric patients who underwent diagnostic upper gastrointestinal tract endoscopy at The Hospital for Sick Children, Toronto, Ontario, Canada between 1985 and 1996. The histopathology had previously identified four patient populations (Drumm et al., 1987; Drumm et al., 1990): 1) primary gastritis infected with *H. pylori* before (N=17) and after eradication therapy (N=6), 2) secondary gastritis (with inflammation due to other conditions including Crohn's disease, eosinophilic gastroenteritis and nonsteroidal anti-inflammatory medication, N=12), 3) non-inflamed mucosa (N=12) and 4) graft-versus-host disease (N=3). Eradication therapy consisted of a two to four week course of bismuth subsalicylate and amoxycillin, with or without metronidazole. Post eradication therapy gastric biopsies were obtained following a mean of 3.1 ± 0.9 months from the time of the initial endoscopies. *H. pylori* status was assessed as described previously by either a modified Steiner silver stain alone (N=6) or both a modified Steiner silver stain and culture (N=11) of gastric biopsy specimens (Drumm et al., 1990). Informed written consent was obtained for upper endoscopy and biopsy procedures.
Table 3-1. Clinical characteristics of the three patient groups evaluated in this study

<table>
<thead>
<tr>
<th></th>
<th>H. pylori + gastritis</th>
<th>H. pylori - gastritis</th>
<th>Non-inflamed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (yrs, range)</td>
<td>15 (8-18)</td>
<td>11.7 (1-17)</td>
<td>10.3 (2-16)</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>7/ 10</td>
<td>6/ 6</td>
<td>5/ 7</td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(No. of patients)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>abdominal pain</td>
<td>12</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>vomiting</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>hematemesis</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>anemia</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>melena</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>failure to thrive</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Biochemical Assessment of Apoptosis

The terminal transferase mediated biotinylated dUTP nick end-labeling (TUNEL) assay was performed as described previously (Lucassen et al., 1995) on sections of paraffin-embedded biopsy specimens, with minor modifications. Briefly, the sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol at room temperature. After blocking endogenous peroxidase by incubation in 1.2 % H2O2 in methanol for 30 minutes, sections were microwaved in 0.01 M citrate buffer for one minute (Strater et al., 1995). The sections were then incubated with proteinase K (20 μg/ml, Boehringer Mannheim, Laval, Quebec) for 15 minutes at room temperature and washed three times in distilled water. Sections were then preincubated with terminal transferase buffer containing 200 mM potassium cacodylate, 25 mM Tris-HCl pH 6.6, 0.2 mM ethylenediaminetetraacetic acid, and bovine serum albumin (0.25 mg/ml) for five minutes at room temperature. The sections were then incubated for sixty minutes at 37 °C with a reaction mixture containing the terminal transferase buffer with 1 mM cobalt chloride, 0.01 nM biotin 16-dUTP, and 0.5 U/μl of terminal transferase (Boehringer Mannheim, Laval, Quebec). The reaction was terminated using a solution of sodium chloride (300 mM) and sodium citrate (30 mM). The sections were incubated with avidin-conjugated peroxidase (Vector, Burlingame, CA) at a concentration of 1:1000 in PBS at 37 °C for thirty minutes followed by reaction in diaminobenzidine (Vector, Burlingame, CA) as described previously (Ben-Sasson, 1995). After light counterstaining with hematoxylin (Fisher Scientific, Fairlawn, NJ), the sections were mounted with Permount (Fisher Scientific, Fairlawn, NJ). The biopsy specimens obtained from patients with graft-versus-
host disease served as positive controls. For negative controls, terminal transferase was omitted from the reaction mixture.

Apoptotic cells identified by the TUNEL assay were quantitated under brightfield microscopy. The apoptotic index was determined by enumerating the number of positively labeled cells per crypt-epithelial cell unit in at least three well-oriented crypt epithelial cell units (Hall et al., 1994) and expressing the mean number multiplied by a factor of 100.

**Immunohistochemistry**

Serial sections from paraffin-embedded antral biopsy specimens were assessed immunohistochemically using the microwave antigen retrieval method, as reported by Shi et al., (Shi et al., 1991) with slight modifications noted below. Briefly, sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. After blocking endogenous peroxidase with H$_2$O$_2$, the sections were heated in 0.01 M citrate buffer in a microwave pressure cooker for 20 minutes (Miracco et al., 1995). The slides were allowed to cool to room temperature and nonspecific binding was blocked with normal horse serum (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 20 minutes at room temperature. The MIB-1 monoclonal antibody (Oncogene science, Cambridge, MA) was utilized for detection of nuclear Ki-67, a marker of proliferating cells (dilution 1:40) (Gerdes et al., 1991).

For p53 detection, the monoclonal antibody NCL-p53-DO7 (Novocastra, Newcastle, UK), which recognizes both wild type and mutant p53 (Kawamura et al., 1996), was employed (dilution 1:50). The optimal concentration for p53
detection was determined by employing a series of two fold antibody dilutions on consecutive sections of gastric mucosa. For detection of p21waf1/cip1 the WAF1 (Ab-1) antibody (Oncogene Research Products, Cambridge, MA) was employed (dilution 1:20). The antibodies were incubated overnight at 4 °C in a humidified chamber. The specimens were then stained using the avidin-biotin complex (ABC) immunoperoxidase technique employing commercially available reagents (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). The sections were counterstained with hematoxylin (Fisher Scientific, Fairlawn, NJ) and mounted with Permount (Fisher Scientific, Fairlawn, NJ). Sections from human tonsil and skin served as positive controls for the MIB-1 (Gerdes et al., 1991) and p53 (Helander et al., 1993) antibodies, respectively. Colonic tissue sections served as a positive control for p21 (El-Deiry et al., 1995). Confirmation of p53 expression was obtained using an additional monoclonal antibody NCL-p53-BP (dilution 1:50) (Novocastra, Newcastle, UK), which recognizes both wild-type and mutant p53 but recognizes a different epitope of the p53 protein (Bartek et al., 1993).

p53 and Ki-67 expression were evaluated under light microscopy. The proliferative labeling index was determined by enumerating the number of MIB-1 positive cells in at least three well-oriented crypt epithelial cell units and expressing the mean percentage of the total number of cells counted in each crypt epithelial cell unit (Brenes et al., 1993). Similarly, the p53 expression index was determined by enumerating p53-positive epithelial cells in at least three well-oriented crypt epithelial units and expressing the mean percentage of the total number of cells counted in each crypt epithelial unit.
Gastritis Score

Sections stained with hematoxylin and eosin for light microscopy were graded for the severity of gastritis as we have done previously (Drumm et al., 1988). The presence of an increased number of mononuclear cells, the presence and severity of mucus depletion, and the presence of polymorphonuclear leukocytes were each assessed separately and graded from 0 to 3+ (Marshall et al., 1987). The presence and number of H. pylori present in sections were excluded from this analysis.

Statistics

Results are expressed as means ± SEM. Comparison of results between groups was performed using ANOVA followed by post-hoc comparisons with the Newman-Keuls test. For comparisons between two groups, an unpaired Student's t test was performed. Correlation between data was determined by the linear least-squares regression method.
Results

Effect of *H. pylori* infection on gastric epithelial cell apoptosis

Apoptotic epithelial cells, identified by the presence of characteristic DNA fragmentation demonstrated using the TUNEL assay, were observed primarily in the superficial gastric mucosa (Figure 3-1) in all groups. In addition, apoptotic cells were present within lymphoid follicles present only in the lamina propria of *H. pylori*-infected mucosa. For epithelial cells, the apoptotic index was highest in gastric biopsy specimens obtained from patients with graft-versus-host disease (280 ± 40, mean ± SE). Patients infected with *H. pylori* (120 ± 10) had a higher apoptotic index compared to both biopsies from patients with secondary gastritis (50 ± 10) and histologically normal controls (40 ± 10; ANOVA, p<0.005) (Figure 3-2). The apoptotic index decreased following therapy for *H. pylori* only when the bacterium was successfully eradicated and gastritis resolved (13 ± 13, N=3; p<0.02).

There was no correlation between the degree of gastric epithelial cell apoptosis in *H. pylori*-infected patients and the severity of gastroduodenal disease identified during endoscopy. The presence of both duodenal or gastric ulceration and gastritis (100 ± 20, N=7) was not associated with a higher degree of apoptosis compared to the presence of gastritis alone (150 ± 20, N=9; p=0.07). In addition, the degree of gastritis did not correlate with the apoptotic index (Figure 3-3).
Figure 3-1: Photomicrographs demonstrating apoptotic cells identified by the TUNEL assay in gastric antral biopsy specimens from A) graft-versus-host disease, B) noninflamed mucosa, C) H. pylori-associated gastritis and D) secondary gastritis. Numerous brown stained apoptotic cells (arrows) are identified in A) and C). Occasional apoptotic cells (arrows) are seen in B) and D). Original approximate magnifications X 400.
Figure 3-2: Quantitation of apoptotic epithelial cells in patients with graft-versus-host disease (GVHD, N=3), *H. pylori* induced gastritis (HP+ gastritis, N=16), secondary gastritis (HP- gastritis, N=12) and noninflamed mucosa (normal, N=12). Results are expressed as the mean apoptotic index (100 times the mean number of apoptotic cells per crypt epithelial unit). Error bars represent standard error. † p<0.005, ANOVA; * p<0.005, ANOVA
Figure 3-3: Lack of correlation between gastritis score and A: apoptotic index in secondary gastritis (open squares, r=0.221) and *H. pylori*-induced gastritis (closed squares, r=0.095) and B: proliferative index in secondary gastritis (open squares, r=0.308) and *H. pylori*-induced gastritis (closed squares, r=0.073).
A

Gastritis Score

Apoptotic Index

B

Gastritis Score

Proliferation Index
Proliferative activity determined by Ki-67 immunohistochemistry

In gastric antral sections obtained from noninflamed mucosa and secondary gastritis (Figure 3-4), proliferating epithelial cells were limited to the neck region of the crypts. The proliferative compartment extended beyond the neck region towards the surface epithelium in antral sections obtained from children infected with *H. pylori*. As shown in Figure 3-5, the proliferative index increased approximately two-fold in patients with *H. pylori*-induced gastritis (33.1 ± 3.4) compared to both secondary gastritis (18.9 ± 2.8) and noninflamed controls (13.7 ± 3.1; ANOVA p<0.01). No correlation was found between the proliferative index and the degree of gastritis (Figure 3-3). The proliferative index did not differ between patients with peptic ulceration (32.1 ± 5.5) compared to those with gastritis alone (31.5 ± 4.8; p=0.9). Following anti-helicobacter eradication therapy the proliferative index returned towards normal values (16.3 ± 0.7, N=4; p=0.08).

*P53 expression during H. pylori infection*

In noninflamed antral mucosa, nuclear p53 expression was identified in occasional epithelial cells concentrated in the neck region (Figure 3-6). Sections from patients with secondary gastritis showed similar epithelial cell expression of p53. In contrast, sections from subjects with *H. pylori* infection showed increased nuclear expression of p53 corresponding to the proliferative regions identified by the Ki-67 immunohistochemistry in serial sections (Figure 3-6). As shown in figure 3-7, quantitation of p53 expression demonstrated comparable results in sections from patients with noninflamed mucosa (3.7 ± 0.9, N=10) and secondary gastritis (2.4 ± 0.9, N=11) whereas sections from
Figure 3-4: Photomicrograph of MIB-1 labeling of proliferating cells in A: noninflamed controls and B: *H. pylori*-induced gastritis. An increase in brown stained proliferating cells are identified in B (arrows). Original approximate magnification X 400.
Figure 3-5: Comparison of MIB-1 positive epithelial cells between noninflamed control mucosa (N=9), secondary gastritis (N=9), and H. pylori-induced gastritis (N=10). Results are expressed as the mean proliferative index (mean percentage of positively labeled cells per crypt epithelial unit). Error bars represent the standard error. * p<0.01, ANOVA
Proliferative Index

Control  HP- gastritis  HP+ gastritis

*
Figure 3-6: Photomicrograph of p53 in A) noninflamed mucosa and B) *H. pylori* induced gastritis. An increase in p53 expression seen as brown staining cells is evident in B (arrows). Original approximate magnification X 400.
Figure 3-7: Quantitation of p53 expression in noninflamed antral mucosa (N=10), secondary gastritis (N=11) and H. pylori-induced gastritis (N=16). Results are expressed as the mean p53 index (mean percentage of positively labeled cells per crypt epithelial unit). Error bars represent the standard error. *p< 0.005, ANOVA
patients infected with *H. pylori* demonstrated overexpression of p53 (19.9 ± 3.7; N=16, ANOVA, p<0.005).

*p21 expression during H. pylori infection*

Wild type p53 can transcriptionally transactivate genes involved in cell cycle arrest including the cyclin-dependent kinase inhibitor p21 (Gotlieb et al., 1996). Therefore, to investigate the relationship between p53-mediated cell cycle arrest and apoptosis during infection with *H. pylori*, we examined p21 expression. Staining for p21 was observed in the superficial epithelium and the gastric pits, as previously identified in normal gastric tissues (El-Deiry et al., 1995). In serial sections of *H. pylori*-infected gastric biopsies p21 staining was localized to regions where there was no evidence of p53 expression (Figure 3-8).
Figure 3-8: Immunohistochemical staining for p21 and p53 in *H. pylori*-infected gastric mucosa. (A) p21 staining is evident in the surface epithelium (arrows). (B) Expression of p53 in same area as panel A. Accumulation of p53 (arrows) is evident in epithelial cells in the neck region of the mucosa and not the surface epithelium. Original approximate magnification X 400.
Discussion

The pathogenic mechanisms responsible for the association of chronic infection with *H. pylori* and gastric cancers are yet to be determined. During the multistep process of carcinogenesis the regulation of cell proliferation and programmed cell death are disturbed (Schulte-Hermann et al., 1995). Therefore, we sought to investigate whether there were alterations in gastric epithelial cell turnover during infection with *H. pylori*. This is the first study which examines the balance between proliferation and apoptosis in the same patients during infection with *H. pylori*, the importance of which has been emphasized (Khosraviani and Williamson, 1996). In addition, this study investigates these effects among children in whom acquisition of infection with *H. pylori* is reported to be associated with a higher risk of developing neoplastic complications (Bourke et al., 1996).

Our results demonstrate that infection with *H. pylori* induces apoptosis and increased proliferation of gastric epithelial cells in children. The findings of increased epithelial cell apoptosis during *H. pylori* infection are supported by two recent reports which examined gastric antral biopsy specimens from adults before and after eradication therapy for *H. pylori* (Moss et al., 1996; Mannick et al., 1996). The presence of a comparison group of subjects with gastritis but without *H. pylori* infection enhances the specificity of the findings in our study. The lack of increased apoptosis or proliferation among patients with secondary gastritis compared to noninflamed mucosa in our study provides evidence for a specific effect of the bacterial infection in stimulating cell turnover. If persistent, these effects on cell turnover could increase the probability that genetic alterations are acquired in the target stem cell population and hence select cells
with a survival advantage (Schulte-Hermann et al., 1995). Such a scenario would explain the epidemiologic association observed between chronic infection and the initiation of gastric cancers.

The induction of apoptosis during infection in childhood was reversible, as demonstrated among patients with successful eradication of *H. pylori* infection. This finding provides further evidence for the specificity of involvement of the bacterium and allows for speculation regarding the merits of eradication of *H. pylori* infection. These data imply that eradication of the organism may well result in a reduction in the risk of gastric cancers. Indeed, preliminary studies indicate that eradication of *H. pylori* infection in patients undergoing endoscopic resection for gastric cancer results in a decreased risk for the subsequent development of metachronous gastric cancers (Uemura et al., 1996).

The degree of induction of apoptosis or proliferation during infection with the bacterium was not associated with disease severity. No correlation could be demonstrated between the presence of peptic ulcer disease and gastritis or gastritis alone and the apoptotic or proliferative indices. These results suggest that increased cell turnover may not be directly involved in ulcerogenesis. Alternatively, since the biopsy specimens were not obtained at the sight of the ulcer crater they may not directly represent cell turnover at this location (Stachura et al., 1993).

The mechanism for the induction of apoptosis and resultant increased proliferation during infection with *H. pylori* is unknown. Infection with strains possessing the cytotoxin associated gene (*cagA*) has been associated with more severe disease and with an increased risk for the development of gastric
cancers (Blaser et al., 1995). One study in adult patients with *H. pylori* suggests that infection with CagA positive strains results in enhanced proliferation of gastric epithelial cells but is not associated with increased induction of apoptosis (Peek et al., 1997). During the natural history of chronic infection to adulthood with CagA positive strains, a selection of cells resistant to apoptosis may occur and thereby provide a potential explanation for the association of these strains with gastric malignancies. However, the presence of the *cagA* gene in 33 of 33 *H. pylori* isolates tested from our institution (Loeb et al., 1998) suggests that *cagA* status likely is not an important determinant for the induction of apoptosis, at least in children. Among a subgroup of patients in which the expression of vacuolating cytotoxin activity in infecting strains has been determined (Loeb et al., 1998), the degree of epithelial cell apoptosis did not correlate with the presence or absence of the cytotoxin (data not shown).

A lack of correlation between the degree of gastritis and alterations in cell turnover suggest that mucosal inflammation does not play a significant role in the induction of apoptosis and proliferation during infection with *H. pylori*. Fan et al. (1996) demonstrated that the rate of gastric cell proliferation *in vitro* increases in response to both *H. pylori* infection and the immune/inflammatory responses to the bacterium. In addition, T cell-mediated epithelial cell proliferation (Ferreira et al., 1990) and apoptosis (de Lajarte et al., 1995) have been described. An increase in the population of CD8+ T cells has been demonstrated both within the epithelium (Hood et al., 1993) and the lamina propria (Fan et al., 1994) in *H. pylori* positive gastritis. Moreover, granzyme B-expressing T-cell lymphomas are predominantly localized in mucosa-associated lymphoid tissue (MALT) (de Bruin et al., 1994). This is of considerable interest since MALTomas are associated with infection with *H.
H. pylori (Wotherspoon et al., 1993). Ongoing studies aimed at specifically examining the involvement of cytotoxic T cells in the induction of gastric epithelial programmed cell death and proliferation, should clarify the potential role of these T lymphocytes during infection with the bacterium.

In this study, an increase in the expression of p53 was demonstrated in gastric antral epithelial cells in children infected with H. pylori. This may appear in contrast to previous studies in which an increase in gastric epithelial cell expression of p53 has been demonstrated only in areas of intestinal metaplasia or dysplasia (Ochiai et al., 1996; Imatani et al., 1996). However, previous studies have not routinely employed the microwave antigen retrieval technique which is necessary for optimal p53 staining (Shi et al., 1991). Several lines of evidence suggest that the overexpression of p53 demonstrated in these studies is of wild-type. A low level of p53 expression was evident in proliferating gastric epithelial cells in biopsy specimens obtained from noninflamed mucosa. Prior studies examining p53 mutations in gastric tissues have demonstrated enhanced expression of mutant p53 in advanced precancerous lesions such as intestinal metaplasia or dysplasia (Ochiai et al., 1996; Imatani et al., 1996). In the present study, precancerous lesions were not identified histologically in any of the biopsy specimens obtained from any of the children studied. Expression of the cell cycle dependent kinase inhibitor p21 in cells of the non-proliferative compartment of the gastric epithelium is also indicative of wild-type p53 status (El-Deiry et al., 1995). An inverse relationship between proliferating cells and p21 was found in gastric biopsies from each of the patient groups. Taken together, these findings indicate that the p53 expression identified in our study is of the wild-type.
The tumor suppressor protein p53 accumulates in normal tissues in response to DNA damage or increased proliferation since it is involved in both cell cycle arrest for DNA repair and the induction of apoptosis (Fritsche et al., 1993; Donehower et al., 1996). The ability of p53 to induce cell cycle arrest is due to the transcriptional activation of the cyclin-dependent kinase inhibitor p21waf1/cip1 (Gotlieb and Oren, 1996). To investigate the relationship between p53 expression and either cell cycle arrest or apoptosis during H. pylori infection in children we assessed the expression of p21waf1/cip1 immunohistochemically. Staining for p21waf1/cip1 was identified in the superficial epithelium and gastric pits. The accumulation of p53 was not associated with expression of p21waf1/cip1 suggesting that p53 does not cause cell cycle arrest during H. pylori infection. Therefore, these findings support the contention that the p53 protein could function to induce apoptosis during infection with H. pylori.

The mechanism for the stimulation of p53 accumulation is not known. H. pylori stimulates the production of antral mucosal reactive oxygen species (Davies et al., 1994). Recent findings demonstrate increased oxidative DNA damage in H. pylori-infected mucosa from children (Baik et al., 1996). H. pylori infection is also associated with enhanced nitric oxide synthase activity both in vitro (Shapiro et al., 1996) and in vivo (Mannick et al., 1996). Nitric oxide induces DNA strand breakage in human cells (Nguyen et al., 1992). The accumulation of p53 and resultant induction of apoptosis could occur in response to DNA damage induced by bacterial infection.

Evidence from a variety of experimental systems suggests that p53 mutation and inactivation is firmly associated with tumor progression in many human
malignancies (Cordon-Cardo, 1995) including gastric cancers (Ochiai et al., 1996; Hongyo et al., 1995). Mutations in p53 have been demonstrated in gastric precancerous lesions such as intestinal metaplasia (Ochiai et al., 1996). Loss of p53-mediated cell cycle checkpoints could result in enhanced genomic instability. In addition, loss of p53-mediated apoptosis during *H. pylori* infection would be expected to provide enhanced cell survival effects (Donehower, 1996). Indeed, although frank neoplasia did not develop, the loss of one copy of the *p53* gene in an animal model of *Helicobacter* infection conferred a growth advantage to gastric epithelial cells, as demonstrated by an enhanced epithelial cell proliferation compared to infected wild-type animals (Fox et al., 1996).

In summary, this study demonstrates a reversible alteration in gastric epithelial cell turnover, including increased apoptosis and proliferation, associated with accumulation of p53 induced specifically by infection with *Helicobacter pylori* in children. This alteration in cell turnover occurring early in the natural history of chronic infection provides an explanation for the association between early acquisition of *H. pylori* infection and the initiation of gastric cancers. The reversibility of these changes implies the potential for a reduction in the risk of gastric cancers following eradication of *H. pylori*. Additional epidemiologic studies should help to elucidate if prevention of gastric cancer is possible with the eradication of *H. pylori*.
Chapter 4:

*Helicobacter pylori* Induces Gastric Epithelial Cell Apoptosis In Association With Enhanced Fas Receptor Expression
Summary

The mechanisms involved in mediating enhanced gastric epithelial cell apoptosis observed during infection with *H. pylori in vivo* are unknown. To determine whether *H. pylori* directly induces apoptosis of gastric epithelial cells *in vitro* and to define the role of the Fas/ Fas ligand signal transduction cascade, human gastric epithelial cells were infected with *H. pylori* for up to 72 hours under microaerophilic conditions. As assessed both by transmission electron microscopy and fluorescent microscopy, incubation with a *cagA+/ cagE+/ VacA+* clinical *H. pylori* isolate stimulated an increase in apoptosis compared with untreated AGS cells (16% ± 3% vs. 6% ± 1%; p< 0.05) after 72 hours. In contrast, apoptosis was not detected following infection with *cagA-/ cagE-/ VacA-* clinical isolates or a *Campylobacter jejuni* strain. In addition to stimulating apoptosis, infection with *H. pylori* enhanced FAS receptor expression in AGS cells comparable to treatment with a positive control, interferon-gamma (12.5 ng/ml) (148 ± 24% and 167 ± 24% of control, respectively). The enhanced Fas receptor expression was associated with increased sensitivity to Fas-mediated cell death. Ligation of the Fas receptor with an agonistic monoclonal antibody resulted in an increase in apoptosis compared with cells infected with the bacterium alone (39%± 7% vs. 16% ± 3%, p<0.05). Incubation with neutralizing anti-Fas antibody did not prevent apoptosis of *H. pylori*-infected cells. Taken together, these findings demonstrate that the gastric pathogen *H. pylori* stimulates apoptosis of gastric epithelial cells *in vitro* in association with enhanced expression of the Fas receptor. These data indicate a role for Fas-mediated signaling in the programmed cell death occurring in response to *H. pylori* infection.
**Introduction**

Infection with the gastric pathogen *Helicobacter pylori* causes chronic-active gastritis and peptic ulcer disease (Tomb et al., 1997). In addition, *H. pylori* infection has been associated epidemiologically with the development of gastric cancers including adenocarcinoma (Huang and Hunt, 1998) and lymphoma (Parsonnet et al., 1994). The mechanisms by which *H. pylori* mediates these host responses, however, remain unknown.

Apoptosis is a genetically programmed form of cell death characterized by distinct morphologic and molecular features (Raff, 1998). Programmed cell death plays an important role in the regulation of epithelial cell numbers in the gastrointestinal tract (Jones and Gores, 1997). In addition, deregulation of the apoptotic pathway is implicated in a number of disease processes in the intestine including carcinogenesis (Merritt et al., 1995). Microbes have developed mechanisms to stimulate the apoptotic signal transduction cascade which likely influence disease pathogenesis (Zychlinsky and Sansonetti, 1997). Microbial pathogens, or their products, can directly activate the cell death signaling cascade. For example, invasive enteric pathogens such as Salmonella can directly induce apoptosis of intestinal epithelial cells (Kim et al., 1998). Alternatively, immune responses including infiltrating inflammatory cells and production of inflammatory mediators directed against the microbe can activate the pathway to cell death. For example, induction of the pro-inflammatory cytokine tumor necrosis factor alpha mediates, in part, the cell death of epithelial cells which occurs during Salmonella infection *in vitro*. Cytotoxic lymphocytes can induce apoptosis of hepatitis C virus-infected cells (Ando et al., 1997). One mechanism by which immune cells trigger apoptosis of
target cells occurs through binding of the Fas receptor to the Fas ligand (Raff, 1998).

Fas, or CD95, is a member of the tumor necrosis factor receptor family which when bound by its natural ligand stimulates an apoptotic signal through activation of the caspase cascade (Nagata and Golstein, 1995). Under physiologic conditions, the Fas system is involved in regulating the immune response by eliminating activated lymphocytes (Lenardo, 1996). Virus-infected cells are also eliminated through Fas/ Fas ligand interactions (Ando et al., 1997). In addition, current evidence links excessive activity of the Fas system with the pathogenic effects associated with infection with certain microbes. For example, the lymphocyte depletion observed in patients infected with the human immunodeficiency virus appears to be Fas-mediated (Bohler et al., 1997; Katsikis et al., 1995).

Alterations in the gastric epithelial cell cycle, including both enhanced proliferation and increased apoptosis of gastric cells, are identified during infection with H. pylori (Brenes et al., 1993; Moss et al., 1996). These changes in cell turnover are present in both H. pylori-infected children (Jones et al., 1997) and adults (Mannick et al., 1996). Investigations of the molecular determinants mediating apoptosis have identified both enhanced expression of the tumor suppressor p53 (Jones et al., 1997) and increased expression of the pro-apoptotic protein Bak in response to H. pylori infection (Chen et al., 1997). Among H. pylori-infected children, gastric epithelial cell apoptosis returns to baseline levels only following both eradication of the bacterium and resolution of the accompanying gastritis (Jones et al., 1997). These findings implicate immune-mediated apoptosis of gastric epithelial cells during H. pylori infection.
Therefore, the aims of this study were to determine if *H. pylori* can directly stimulate programmed cell death of gastric epithelial cells and to characterize the role of Fas-Fas ligand signaling in this cell death cascade.
Materials and Methods

Bacteria and growth conditions

*H. pylori* strain LC 11, a *cagA*-positive, *cagE*-positive and vacuolating cytotoxin (VacA)-producing *H. pylori* strain originally isolated from a child with duodenal ulcer disease and two *cagA*-negative, *cagE*-negative and VacA-negative strains, LC 3 and LC 20, were employed for these studies (Hemalatha et al., 1991). The presence of *cagA* and *cagE* was determined by isolation of genomic DNA and polymerase chain reaction (Loeb et al., 1998) using the primers GCCTACTGGGGAGGGATTG and GCCTGTAGTTGGTCTTTC for *cagA* and both AGACATGCAAAAAGGTAT and CAATCTAGTGGGTTGGA and TGCTGATACGATTAGAGA and TAGTCCCTTAGTGATGAT (kindly provided by Dr. Robin Beech, McGill University, Montreal, PQ) for *cagE*, respectively. The presence of the vacuolating cytotoxin was determined by the method of Cover et al (1992). Concentrated broth supernatants were incubated with HEp-2 cells for 24 hours at 37°C and vacuolation assessed under brightfield microscopy (Loeb et al., 1998).

*H. pylori* were grown under microaerophilic conditions on Columbia blood agar plates for 72 hours at 37 °C, harvested and resuspended in Brucella broth (Difco) supplemented with 10% heat-inactivated fetal calf serum, vancomycin and trimethoprim. Bacteria cells were grown overnight at 37 °C in an Erlenmeyer flask with shaking at 120 rpm, as described previously (Jones et al., 1997). Cells were then pelleted and resuspended in phosphate buffered saline (PBS, pH 7.4) at a concentration of 1 x 10^9 CFU/ml. *Campylobacter jejuni* strain TGH 9011 was kindly provided by Dr. V.L. Chan (University of Toronto).
Cell Culture

The human gastric adenocarcinoma cell line AGS was grown as a monolayer in tissue culture flasks at 37°C in 5% CO2. The tissue culture medium was Ham's F12 (Life Technologies Gibco BRL, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS, Cansera International, Rexdale, ON, Canada), and 0.1% sodium bicarbonate. The human gastric adenocarcinoma cell line KATO III was grown under similar conditions in RPMI 1640 media supplemented with 10% fetal calf serum. Cells were incubated for up to 72 hours with *H. pylori* (5 x 10^8 CFU/ml) under microaerophilic conditions to maintain *H. pylori* viability. Control cells were incubated under the same conditions in the absence of bacteria. In additional experiments, cells were incubated with *H. pylori* broth supernatant (0.5 ml) which was filtered through a 0.2 μM syringe filter (Gelman Sciences, Ann Arbor, MI).

Assessment of apoptosis

Transmission electron microscopy

For transmission electron microscopy, cells were grown to confluence in tissue culture flasks and incubated with *H. pylori* as described above. Cells incubated in the absence of *H. pylori* served as controls. Cells in suspension and trypsinized cells both were pelleted, fixed with 2% glutaraldehyde (vol/vol) in 0.1 M phosphate buffer, post-fixed in 2% osmium tetroxide, and dehydrated through a series of graded acetone washes (Dytoc et al., 1994). Samples were embedded in Epoxy resin and ultrathin sections were placed onto 300-mesh copper grids. The grids were then stained with uranyl acetate and lead salts, as
described previously (Dytoc et al., 1994). Grids were examined for the presence of apoptotic cells using a Philips 300 transmission electron microscope at an accelerating voltage of 60 kV (Kerr et al., 1995).

**Fluorescent dye staining**

Cells in suspension and trypsinized cells were pelleted and resuspended in 1 mL of phosphate-buffered saline. Acridine orange (100 µg/ml)/ ethidium bromide (100 µg/ml) in PBS was added to the suspension (Grossman et al., 1998). A drop of the suspension was applied to a microscope slide and apoptotic cells were assessed by fluorescence microscopy, as previously described (Grossman et al., 1998). The percentage of apoptotic cells was determined by enumerating 500 cells at multiple, randomly selected fields.

*Determination of Fas receptor expression*

**Fluorescent microscopy**

AGS cells were grown on Lab-Tek chamber slides (Nunc, Naperville, Illinois), as described above, until semi-confluent. Cells were then incubated in the presence or absence of interferon-gamma (IFN-γ) (12.5 ng/ml) for 24 hours at 37°C (Hayashi et al., 1997). Cells were washed with PBS and then fixed with 100% acetone for 5 minutes at room temperature. Cells were then incubated with an anti-Fas receptor monoclonal antibody (clone DX2, Oncogene Research Products, Cambridge, MA, USA) at a concentration of 2.5 µg/ml for 1 hour at 37 °C. Following washings, cells were incubated with FITC-conjugated anti-mouse IgG (1:100) for 1 hour at 37 °C. After washings, the slides were
mounted with coverslips and viewed by fluorescence microscopy using a Leitz Dialux 22 microscope (Grossman et al., 1998).

Imunoassay

A commercially available enzyme-linked immunosorbent assay (ELISA) (Oncogene Research Products, Cambridge, MA, USA) was employed to measure Fas receptor expression in tissue culture cells that had been incubated in the presence or absence of *H. pylori* strain LC 11 for 24 hours. Cells treated with IFN-γ (12.5 ng/ml) were used as positive control (Hayashi et al., 1997). Fas receptor expression was calculated according to the manufacturer's instructions per 10^6 cells enumerated. Briefly, cell extracts were obtained by incubating harvested cells in the supplied buffer with the supplied antigen extraction agent and then centrifuged to obtain a clear lysate. The lysates were diluted and added to the supplied microtiter plates. Following incubation with the detector antibody and streptavidin conjugate, absorbance of wells in the plates were read spectrophotometrically at 450/490 nm.

*Functional assessment of Fas receptor*

Untreated and *H. pylori*-infected AGS cells were exposed for 24 hours to activating anti-Fas monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) at a concentration which has previously been shown to induce apoptosis in Fas sensitive cells (100 ng/ml) (Hayashi et al., 1997). Apoptotic cells were enumerated by fluorescence microscopy following staining with acridine orange and ethidium bromide, as described above.
*H. pylori*-infected cells were incubated with a neutralizing anti-Fas antibody (Upstate Biotechnology, Lake Placid, NY) at 1000 ng/ml. Dose-response studies (concentrations ranging from 100 ng/ml to 2000 ng/ml) determined the maximal inhibition of apoptosis (50%) in AGS cells stimulated with the activating Fas antibody (100 ng/ml) was mediated at a dosage of 1 μg/ml. Apoptotic cells were enumerated as described above. Results are expressed as the mean of the data obtained from two independent experiments.

Statistical Analysis

Results are expressed as means ± SE. To test statistical significance between multiple groups, a one-way analysis of variance was used followed by post hoc comparisons with the Newman-Keuls test.
Results

Evaluation of apoptosis in H. pylori-infected gastric epithelial cells

To determine if infection with *H. pylori* alone could stimulate apoptosis of gastric epithelial cells *in vitro*, AGS and KATO III cells were incubated with the bacteria for up to 72 hours. AGS cells infected with a *cagA*/*cagE*/*VacA* isolate, strain LC 11, underwent apoptosis as assessed by fluorescence microscopy. As shown in Figure 4-1, apoptotic cells displayed the characteristic features of reduced size, cytoplasmic vacuolation and enhanced fluorescence of condensed and margined nuclear chromatin. These results were confirmed by transmission electron microscopy. In comparison with untreated cells, AGS cells infected with strain LC 11 demonstrated the ultrastructural features which characterize the process of programmed cell death including cytoplasmic vacuolation, condensed nuclear chromatin and formation of apoptotic bodies (Figure 4-2).

Quantitation of apoptotic AGS cells by fluorescence microscopy demonstrated that *H. pylori* LC 11-mediated cell death was time dependent. An increase in death of gastric epithelial cells was observed following 72 hours of infection with the bacterium compared with uninfected controls (16% ± 3% vs. 6% ± 1%; p< 0.05) (Figure 4-3). When AGS cells were infected for 72 hours with two clinical isolates which lack the putative virulence factors *cagE*, *cagA*, and vacuolating cytotoxin activity, apoptosis of gastric epithelial cells was not detected (7%± 1% vs. 7% ± 2%). Similarly, infection with the related enteric pathogen *C. jejuni* did not induce apoptosis of gastric cells (6% ± 3% vs. 6 ± 2%). In contrast to AGS cells, KATO III cells more readily underwent
Figure 4-1: Identification of apoptotic cells in untreated AGS cells (panel A) and *H. pylori*-infected cells (panel B) after 72 hours by acridine orange/ethidium bromide staining and fluorescent microscopy. (A) AGS cells demonstrate normal morphology. (B) *H. pylori*-infected AGS cells show morphologic features of apoptosis (arrows) including condensed and marginated chromatin with apoptotic body formation. Original approximate magnifications, x1,000.
Figure 4-2: Transmission electron photomicrographs of (A) uninfected and (B) *H. pylori*-infected AGS cells. (A) Control cells show normal cellular morphology. (B) *H. pylori*-infected AGS cells demonstrate the characteristic features of programmed cell death including cytoplasmic vacuolation (arrowhead), and apoptotic body formation (arrows). Approximate original magnifications x7,800.
Figure 4-3: Quantitation of apoptotic AGS cells infected with *H. pylori* for varying lengths of time (filled bars) in comparison to uninfected cells (hatched bars). Incubation with the bacterium resulted in an increase in apoptosis compared to untreated cells at 72 hours (ANOVA, p< 0.05). Results are expressed as the mean percentage of apoptotic cells per 500 cells enumerated. Variations are represented as the SE.
necrosis in response to infection with *H. pylori* strain LC11 for 72 hours as assessed by fluorescent microscopy, therefore the remaining studies were performed using AGS cells.

*Expression of the Fas receptor during H. pylori infection*

To determine if AGS cells had basal expression of Fas, which could be enhanced by gamma-interferon (IFN-γ) known to up regulate expression of the receptor in other cell lines (Hayashi et al., 1997), fluorescent microscopy utilising a monoclonal antibody to the Fas receptor was employed. As shown in Figure 4-4, AGS cells had a low level of Fas expression which was enhanced following stimulation by IFN-γ (12.5 ng/ml).

Compared to uninfected, control cells, infection with *H. pylori* strain LC 11 also enhanced expression of Fas as determined by ELISA *(p<0.05)(Figure 4-5).* *H. pylori*-stimulated Fas receptor expression was comparable to that mediated by IFN-γ *(148 ± 24% and 167 ± 24% of control, respectively, N=3, p>0.05).* The *H. pylori*-mediated Fas expression was not a result of cross reactivity with a bacterial product since assessment of bacterial extracts alone showed no detectable Fas expression.

*Sensitivity of H. pylori-infected cells to Fas stimulated cell death*

An agonistic monoclonal antibody to the Fas receptor, which stimulates Fas-sensitive cells to undergo apoptosis (Hayashi et al., 1997), was then employed to determine if the enhanced Fas expression was functional. As shown in Figure 4-6, incubation of AGS cells with the anti-Fas antibody mediated an
Figure 4-4: Fluorescent micrograph demonstrating Fas expression. (A) A low level of Fas expression is detected in untreated AGS cells. (B) Fas expression is enhanced following incubation of AGS with IFN-γ (12.5 ng/ml).
Figure 4-5: Effect of *H. pylori* on Fas receptor expression in AGS cells. Incubation with *H. pylori* enhanced Fas expression similar to treatment with 12.5 ng/ml of IFN-γ. Results are expressed as the percent increase in Fas receptor expression compared to untreated, control cells (± SE).
Effect of \textit{H. pylori} infection on Fas-stimulated apoptosis. Untreated (control) and \textit{H. pylori}-infected (strain LC 11) AGS cells were incubated in the presence or absence of an agonistic monoclonal antibody to the Fas receptor and apoptosis was assessed. Incubation with the Fas agonist enhanced programmed cell death in control cells. The apoptotic index in \textit{H. pylori}-infected cells incubated with anti-Fas increased compared to AGS cells incubated with the bacterium alone. Results are expressed as the percentage of apoptotic cells per 500 cells enumerated (± SE).

\textbf{Figure 4-6:} Effect of \textit{H. pylori} infection on Fas-stimulated apoptosis. Untreated (control) and \textit{H. pylori}-infected (strain LC 11) AGS cells were incubated in the presence or absence of an agonistic monoclonal antibody to the Fas receptor and apoptosis was assessed. Incubation with the Fas agonist enhanced programmed cell death in control cells. The apoptotic index in \textit{H. pylori}-infected cells incubated with anti-Fas increased compared to AGS cells incubated with the bacterium alone. Results are expressed as the percentage of apoptotic cells per 500 cells enumerated (± SE).
increase in apoptosis compared with untreated cells (21% ± 2% vs. 6% ± 1%, ANOVA, p<0.05). Furthermore, incubation of LC 11-infected gastric cells with the anti-Fas antibody resulted in a marked increase in programmed cell death compared with cells infected with the bacterium alone (39% ± 7% vs. 16% ± 6%, ANOVA, p<0.05). Incubation of LC 11-infected AGS cells with a neutralizing anti-Fas receptor antibody did not prevent cell death (8% vs. 10%) indicating that the induction of apoptosis observed following infection with the bacterium alone was not mediated by the enhanced Fas receptor expression.
Discussion

In vivo studies demonstrate that infection with *H. pylori* triggers apoptosis of gastric epithelial cells (Shirin and Moss, 1998; Jones et al., 1997). However, in this setting it is unclear whether immune factors or bacterial factors contribute to cell death. This study supports and extends recent evidence indicating that several mechanisms are involved in stimulating apoptosis of gastric epithelial cells during *H. pylori* infection (Rudi et al., 1998; Wagner et al., 1997). Our data demonstrate that *H. pylori* is capable of directly inducing cell death of gastric epithelial cells in vitro in the absence of immune cells. The mechanism of cell death differed between the two gastric cell lines. KATO III cells underwent necrosis in response to prolonged infection with the bacterium, while AGS cells underwent apoptosis. The underlying mechanism responsible for the observed difference in response of the cell lines to infection is not clear. However, the presence of disrupted apoptotic pathways in these transformed cell lines could provide one explanation (Hayashi et al., 1997). The response of AGS cells to infection with the bacterium mimics the *in vivo* setting suggesting that the AGS cell line serves as a better model system for investigating these apoptotic pathways.

The exact bacterial factors which directly mediate the death signal are not known. Fan et al. (1998) recently provided evidence that binding of *H. pylori* to the class II major histocompatibility complex expressed on gastric epithelial cells can transduce the cell death signal in vitro. In this study, the presence of factors considered to be associated with virulence including *cagE* and *cagA*, two genes found on the pathogenicity island, as well as vacuolating cytotoxin activity was associated with apoptosis. In contrast, apoptosis was not detected
results are in agreement with the recent findings of Rudi et al. (1998) who detected apoptosis of gastric epithelial cells following incubation with culture supernatants from a \textit{cagA+} \textit{H. pylori} isolate with cytotoxic activity but not with supernatants from a \textit{cagA-}, noncytotoxic strain. In contrast, another study detected apoptosis during infection with both \textit{cagA}-positive, \textit{vacA}-producing and \textit{cagA}-negative, \textit{VacA}-negative \textit{H. pylori} strains (Wagner et al., 1997). However, the \textit{cagE} status of the strains utilised in both of these studies was not determined. Taken together, these findings suggest that the induction of programmed cell death could mediate disease outcome. Of interest, a preliminary study demonstrated that infection with \textit{cagE+} strains is associated with peptic ulcer disease in children (Day et al., 1999).

In addition to cell death triggered directly by infection with \textit{H. pylori}, upregulation of the Fas receptor is observed in association with increased sensitivity to apoptosis upon ligation of the receptor. Wagner and colleagues (1997) also identified enhanced apoptosis in the HM02 gastric epithelial cell line following infection with \textit{H. pylori} and Fas ligation. However, the mechanism for the enhanced sensitivity to Fas signaling was not determined. This study suggests that \textit{H. pylori} infection enhances expression of the Fas receptor in gastric epithelial cells thereby resulting in an increased sensitivity to Fas-triggered cell death. \textit{H. pylori}-mediated enhanced Fas expression does not directly stimulate apoptosis since a neutralizing antibody did not prevent cell death. These findings indicate that immune-mediated cell death through the Fas/ Fas ligand pathway likely also contributes to the apoptosis that is observed during infection \textit{in vivo}. 
The factors mediating enhanced Fas receptor expression and Fas-mediated cell death during *H. pylori* infection are not known. A recent study demonstrated that IFN-γ, which is increased in the gastric mucosa during *H. pylori* infection (Katrunnen et al., 1995), augments apoptosis. IFN-γ also upregulates expression of the Fas death receptor (Ossina et al., 1997). Cytokines produced by inflammatory cells in the lamina propria in response to *H. pylori* infection could also modulate cell death. Further studies are required to determine the factors which increase the susceptibility of gastric cells to the Fas death cascade.

Our findings indicate that Fas-stimulated cell death could influence *H. pylori*-mediated disease pathogenesis *in vivo*. During infection with the bacterium, gastric epithelial cells exhibiting enhanced Fas receptor expression could be eliminated by infiltrating lymphocytes that express the Fas ligand. In support of this contention, a recent study identified an increase in Fas ligand mRNA expression in lymphocytes within the lamina propria and enhanced Fas receptor expression in both gastric epithelial cells and cells within the superficial lamina propria of *H. pylori*-infected gastric biopsy tissue (Rudi et al., 1998). Furthermore, the region of the gastric mucosa with enhanced Fas ligand mRNA corresponded to areas of enhanced apoptosis.

Animal models also suggest that Fas signaling plays a role in gastric injury. In a murine model of autoimmune gastritis generated either by thymectomy or adoptive transfer of a Th1 cell clone recognizing an epitope of the H⁺, K⁺-ATPase, enhanced Fas expression was detected on gastric parietal cells (Nishio et al., 1996). In contrast, gastric tissue from normal controls lacked detectable Fas. The topographic expression of this death receptor in parietal
cells correlated with induction of apoptosis as assessed by the terminal deoxynucleotidyl transferase-mediated nick end label (TUNEL) method (Nishio et al., 1996). This indicates that one mechanism by which autoimmune-mediated target cell destruction may be effected is through Fas- Fas ligand interactions. This is of particular interest since autoantibodies directed against gastric parietal H+,K+-ATPase are detected in sera from *H. pylori*-infected subjects and correlate with the presence of gastric atrophy (Claeys et al., 1998).

In summary, the present study shows that *H. pylori* infection is capable of activating the apoptotic cell death cascade in gastric epithelial cells by more than one mechanism. The bacterium can directly stimulate programmed cell death and also enhances both expression of the cell death receptor Fas and sensitivity to Fas mediated apoptosis. *In vivo* studies, including animal models of human disease (Lee et al., 1997), should now be undertaken to further delineate the role of Fas signaling in the pathogenesis of *H. pylori*-mediated disease.
Chapter 5:

*Escherichia coli* Shigatoxins Induce Apoptosis
In Epithelial Cells Via Reduction In Bcl-2 Expression
Summary

Human intestinal epithelial cells lack globotriaosylceramide (Gb-3), the receptor for Shigatoxin-1 (Stx1) and Shigatoxin-2 (Stx2). Therefore, the role of these toxins in mediating intestinal disease during infection with Shigatoxin-producing *Escherichia coli* is unclear. The aims of this study were to determine if Stx1 and Stx2 induce apoptosis in human epithelial cells expressing (HEp-2, CaCo2) or lacking (T84) Gb-3 and to characterize the role of the Bcl-2 family. Stx1 (12.5 ng/ml) induced apoptosis both in HEp-2 (22% ± 8% vs. 1% ± 0.3%, p=0.01) and CaCo2 (10% ± 1% vs. 3% ± 0.4%, p= 0.006) cells compared with uninfected controls but not in Gb-3 deficient T84 cells. The toxin-induced apoptosis of HEp-2 cells was dose- and time- dependent with the greatest effect observed at dosage of 12.5 ng/ml after incubation for 24 hours (21% ± 5% vs. 2% ± 1% for uninfected controls, p<0.05). Incubation with purified B subunit, the binding portion of Stx1, induced apoptosis in HEp-2 cells but to a much lesser degree than that mediated by the holotoxin. The addition of a protein synthesis inhibitor cycloheximide did not potentiate the apoptotic response induced by the B subunit. Toxin-mediated apoptosis of HEp-2 cells was associated with an enhanced expression of the proapoptotic protein Bax and decreased expression of the apoptotic antagonist Bcl-2. Inhibition of downstream caspase activation with the broad range caspase inhibitor Z-VAD-fmk prevented toxin-stimulated apoptosis (1% ± 0.4% vs. 25% ± 3%, p<0.001). In addition, overexpression of Bcl-2 blocked Stx1-stimulated cell death. These findings indicate that Shiga toxins produced by *E. coli* signal Gb-3 expressing epithelial cells to undergo apoptosis by both decreased Bcl-2 expression and enhanced Bax expression thereby resulting in activation of the caspase cascade.
Introduction

Infection with Shiga toxin-producing *Escherichia coli* (STEC) causes diarrhea and can result in the more severe sequelae of hemorrhagic colitis and the hemolytic uremic syndrome (Nataro and Kaper, 1998). The exact factors involved in disease pathogenesis are not known. However, bacteriophage-encoded Shiga-like toxins, called Stx1 and Stx2, are considered to be major virulence factors of STEC infection (Lingwood, 1996). Stx1 and Stx2 are A-B subunit toxins in which the B subunit binds as a pentamer to a specific glycolipid receptor, globotriaosylceramide (Gb3) (Lingwood et al., 1987).

Gb3 is variably expressed within different tissues. Within the hematopoietic system, Gb3 is present in erythrocytes, as the blood group Pk antigen, as well as in specific B cell populations, including Burkitt’s lymphoma cells and germinal center B cells (Taga et al., 1997). Therefore, Gb3 is also referred to as a B-cell differentiation antigen, CD77, and Burkitt’s lymphoma associated antigen.

Stx1 binds to the carbohydrate moiety of Gb3 (Gal α1-4 Gal) which protrudes from the plasma membrane while the ceramide portion of Gb3 is located within the membrane bilayer (Khine et al., 1998). Following binding to Gb3, the holotoxin is endocytosed and transported to the Golgi apparatus and then to the endoplasmic reticulum. The A subunit inhibits protein synthesis by acting on the 28S rRNA of the 60S ribosomal subunit thereby resulting in cell cytotoxicity (Lingwood, 1996). Ligation of Gb3 in Burkitt’s lymphoma cells also stimulates signal transduction events including increases in intracellular calcium and ceramide production (Taga et al., 1997).
The role of Stx1 and Stx2 in mediating intestinal disease is controversial (Nataro and Kaper, 1998). Studies in animal models of infection suggest that Stx can modulate disease severity including the production of diarrhea and hemorrhagic colitis. Purified toxin induces fluid accumulation in ligated rabbit intestinal loops in association with the presence of apoptotic intestinal villus epithelial cells (Keenan et al., 1986). Furthermore, infection of rabbits with a natural E. coli enteropathogen, strain RDEC-1 (serotype O15: H-) when expressing Stx1 results in more severe disease than that caused by infection with the isogenic toxin-negative strain (Sjogren et al., 1994). In contrast, in gnotobiotic piglets (Tzipori et al., 1987) and infant rabbits (Li et al., 1993) infection with Stx toxin positive or negative STEC strains results in no detectable difference in the diarrheal response.

The variation in disease in response to Stx in these animal models could be due to the presence or the absence of the receptor for Stx (Gb3) in the intestinal epithelium (Lingwood, 1996). discuss Gb-3- known ligand, role in lymphocyte differentiation, ceramide signaling Since the human intestine lacks detectable Gb3 (Holgresson et al., 1991), it has been suggested that Stx mediates only the vascular complications of disease including hemorrhagic colitis and the hemolytic uremic syndrome (Fontaine et al., 1988). However, previous studies have shown that Stx1 also undergoes directed translocation to the Golgi and endoplasmic reticulum in human intestinal cells deficient in Gb3 (Philpott et al., 1997).

Apoptosis, a distinct form of cell death, plays a crucial role in the development and maintainance of homeostasis of tissues (Jones and Gores, 1997).
Therefore, deregulation of the apoptotic pathway can result in pathologic processes (Jones and Gores, 1997; Jones et al., 1997; Zychlinsky and Sansonetti, 1997). A major molecular modulator of apoptosis is the Bcl-2 family (Adams and Cory, 1998). This family of proteins contains both pro-apoptotic members, such as Bax and Bak, and anti-apoptotic members such as Bcl-2 (Adams and Cory, 1998). Although the exact mechanisms by which these proteins regulate apoptosis are unclear, the ability of the different pro- and anti-apoptotic family members to form a dynamic equilibrium between hetero- and homodimers appears to regulate the apoptotic pathway (Kroemer, 1997). Recent evidence indicates that the ratio of suppressors to inducers determines the sensitivity of the target cell to an apoptotic stimulus by modulating mitochondrial cytochrome c release. For example, Bax induces the release of mitochondrial cytochrome c and triggers caspase activation. Bcl-2 or Bcl-xL are capable of preventing Bax-mediated cytochrome c release and cell death (Finucaine et al., 1999).

In human intestinal epithelium, the distribution of the death antagonist Bcl-2 is limited to the colonic crypts (Merritt et al., 1995) whereas Bax, an apoptotic agonist, is expressed near the gut lumen (Krajewski et al., 1994). This topographic expression of pro- and anti-apoptotic Bcl-2 family members correlates with the location of intestinal cells destined for apoptosis and proposed stem cells, respectively (Jones and Gores, 1997). In addition, aberrant Bcl-2 expression has been implicated in the pathogenesis of the adenoma to carcinoma sequence in colon carcinogenesis (Bronner et al., 1995; Merritt et al., 1995). Furthermore, enhanced Bak expression in association with apoptosis of gastric epithelial cells is observed during infection with the
pathogen *Helicobacter pylori* (Chen et al., 1997). These findings suggest that the Bcl-2 family plays a crucial role in regulating apoptosis in the intestine.

A growing list of bacterial pathogens is able to modulate apoptosis of eukaryotic cells (Zychlinsky and Sansonetti, 1997). For example, *Shigella flexneri* induces apoptosis of infected macrophages *in vitro* (Chen et al., 1996) and *in vivo* (Zychlinsky et al., 1996) mediated by the secreted bacterial product IpaB (Chen et al., 1996). In contrast, epithelial cells are resistant to *S. flexneri*-stimulated programmed cell death *in vitro* suggesting cell type specificity for the apoptotic signaling pathway (Mantis et al., 1996). Purified Stx1 is capable of causing apoptosis in Burkitt lymphoma cells (Manganey et al., 1993), Vero cells (Inward et al., 1995) and human renal tubular epithelial cells (Karpman et al., 1998). In Burkitt lymphoma cells, the susceptibility to apoptosis is related to expression of the receptor Gb3 (Manganey et al., 1993; Taga et al., 1997). Therefore, the aims of the present study were to determine if Stx1 and Stx2 induce apoptosis of mucosal epithelial cells, delineate the requirement for the Gb3 receptor and define the regulation of apoptosis by members of the Bcl-2 family following exposure to Stx.
Materials and Methods

Bacterial toxins

Stx1 and Stx2 were purified by methods, as described previously (Bielaszewska et al., 1997; Petric et al., 1987). Briefly, cell lysates were obtained from E. coli strain R82pJES 120 DH5a (for Stx2 purification) and strain JB28 (for Stx1 purification) by polymyxin B (0.1 mg/ml) treatment. Following precipitation by ultracentrifugation, the preparation underwent column chromatography purification by applications to hydroxylapatite column (Calbiochem, La Jolla, CA), chromatofocusing column of Polybuffer Exchanger 94 (Pharmacia, Baie d'Urfe, PQ) and Cibachron blue F3Ga column (Pierce, Rockford, IL), respectively. The purified B subunit of Stx1 was kindly provided by Dr. Clifford Lingwood (Hospital for Sick Children, Toronto).

Cell Culture

HEp-2 cells, a human laryngeal epithelial cell line expressing globotriaosylceramide (Gb-3) and previously demonstrated to serve as a good model system for investigating signal transduction responses to infection (Philpott et al., 1997), were grown as monolayers in tissue culture flasks at 37°C in 5% CO₂. The culture medium was Eagle Minimum Essential Media (MEM) (Life Technologies GIBCO BRL, Grand Island, NY) supplemented with 15% (vol/vol) heat-inactivated fetal calf serum (FCS, Cansera International, Rexdale, ON, Canada), 0.1% sodium bicarbonate, and 2% penicillin-streptomycin. Following overnight serum starvation which potentiated toxin-mediated cell death, cells were incubated for up to 48 hours with varying concentrations of
Stx1, Stx2 or the purified B subunit of Stx1 which were filtered through a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor MI). In some studies, HEp-2 cells were incubated with cycloheximide (10 μg/ml) to inhibit protein synthesis (Roger et al., 1998). In additional studies, cells were co-incubated with Stx-1 and varying concentrations of a monoclonal antibody to the Gb-3 receptor (38.13), kindly provided by Dr. C. Lingwood (Hospital for Sick Children, Toronto).

The human intestinal cell line CaCo2, which expresses Gb-3 (Jacewicz et al., 1995), was grown as a monolayer in tissue culture flasks. Cells were maintained in minimal essential medium (MEM) with Earle's salts, non-essential amino acids, 25 mM HEPES, 1 mM sodium pyruvate, (all from Life Technologies GIBCO BRL, Grand Island, NY) and 2% penicillin-streptomycin supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS, Cansera International, Rexdale, ON, Canada). Cells that had been incubated for 14 days were incubated with Stx1 (concentration range 12.5 ng/ml- 113 ng/ml) which was filtered through a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI).

T84 cells, a human intestinal cell line which lacks detectable globotriaosylceramide (Philpott et al., 1997), were grown as monolayers in tissue culture flasks. The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS, Cansera International, Rexdale, ON, Canada) and 2% (vol/vol) penicillin-streptomycin (GIBCO) and grown at 37°C in 5% CO2. Cells were treated for up to 48 hours with Stx1 (concentration range 12.5 ng/ml- 113 ng/ml).
Assessment of apoptosis

Transmission electron microscopy

For transmission electron microscopy, cells were grown to confluence in tissue culture flasks and incubated with Stx1 or Stx2 as described above. Cells incubated in the absence of toxin served as controls. Cells in suspension and trypsinized cells both were pelleted, fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (vol/vol), post-fixed in 2% osmium tetroxide, and dehydrated through a series of graded acetone washes (Dytoc et al., 1994). Samples were embedded in Epoxy resin, and ultrathin sections were placed onto 300-mesh copper grids. The grids were then stained with uranyl acetate and lead salts, as described previously. Grids were examined for the presence of apoptotic cells using a transmission electron microscope at an accelerating voltage of 60 kV (Kerr et al., 1995).

Fluorescent dye staining

Cells in suspension and trypsinized cells were pelleted and resuspended in 1 mL of phosphate-buffered saline. Acridine orange/ethidium bromide in phosphate-buffered saline (100 μg/ml) was added to the suspension. A drop of the suspension was applied to a microscope slide and apoptotic cells were assessed by fluorescent microscopy, as previously described (Grossman et al., 1998). The percentage of apoptotic cells was determined by counting 500 cells at multiple randomly selected fields.
Cell Death Detection by Immunoassay

To detect oligonucleosomes formed as a result of DNA fragmentation, the Cell Death Detection ELISAplus kit (Boehringer Mannheim, Indianapolis, IN) was utilized, as described by the manufacturer. Briefly, adherent control and Stx1 treated (12.5 ng/ml) HEp-2 cells were trypsinized and 5x10⁴ cells were lysed by incubation in the supplied lysis buffer. Supernatants of cell lysates were transferred to streptavidin-coated microtiter wells and cytoplasmic nucleosomes quantitated by using biotinylated anti-histone antibody and peroxidase-conjugated anti-DNA antibody following incubation with the peroxidase substrate 2,2'-azino-di[3-ethylbenzthiazolin-sulfonat]. An enrichment factor, to quantitate the relative increase in nucleosomes, was calculated as the ratio of specific absorbances in lysates from Stx1 treated cells compared with untreated cells. The histone-DNA complex provided by the manufacturer served as a positive control for the assay. HEp-2 cells were incubated with sorbitol (1M) for 2 hours at 37°C, which has previously been demonstrated to induce apoptosis (Koyama et al., 1997) and, therefore, served as an additional positive control. Cells that underwent freeze-thawing at -20°C for 1 hour served as a necrotic control in the assay.

Western blotting

Cells were lysed in 0.15 ml of ice-cold lysis buffer containing 150 mM NaCl, 1% Triton X 100, 10 mM Tris (ph 7.4), 5 mM EDTA, 1 mM phenylmethyl-sulfonylfluoride, apoprotinin (2 µg/ml), leupeptin (50 µg/ml), 1 mM benzamidine, pepstatin (1 µg/ml), 1mM sodium vanadate, 50 mM sodium fluoride, and 2 mM
sodium pyrophosphate for 30 minutes on ice (Guan et al., 1996). The lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C. Protein concentrations of the supernatants were measured by the Bradford method and samples were diluted with Laemmlı buffer. Samples with equivalent concentrations of protein were boiled for 2 minutes and subjected to 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto an Immobilon-P transfer membrane (Millipore, Bedford, Mass.). Blots were blocked with 5% skim milk in phosphate-buffered saline plus 0.5% Tween 20 overnight at 4°C. Separate blots were then incubated with anti-Bcl-2 (20 μl/ml; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bak (25 μl/ml; Oncogene Research Products, Cambridge, MA) and anti-Bax (25 μl/ml; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies for 1 hour at 37°C. Blots were washed in phosphate-buffered saline plus 0.5% Tween 20 and then incubated with peroxidase-conjugated anti-mouse immunoglobulin G antibodies (for Bcl-2 and Bak) or anti-rabbit antibodies (for Bax). Immunoblots were developed by using the ECL Western blotting detection system (Amersham Life Science; Buckinghamshire, England). Densitometry was performed to compare protein expression between groups by using the NIH Gel Plotting Macros program. Results are expressed as mean of 2-3 experiments.

Transfection of cultured cells

DNA used for transfection was purified by anion-exchange column (Qiagen, Valencia, CA). Subconfluent HEp-2 cells were transiently transfected in six well Costar cell culture dishes (Corning Incorporated, Corning, NY) using FuGENE transfection reagent (Boeringher-Mannheim, Laval, Quebec) with pcDNA3.Bcl-2 (4 μg) (Zanke et al., 1998) and co-transfected with a green fluorescent protein.
GFP marker plasmid (pEGFP C2) (Clontech Laboratories, Palo Alto, CA) at a molar ratio of 1:4. Control cells were transfected with the vector pCDNA3 (4 µg) (Invitrogen, San Diego, CA) and pEGFP C2. After culturing for 48 h, the cells were incubated in the presence or absence of Stx1 (12.5 ng/ml) for an additional 24 h at 37°C. Floating and trypsinized adherent cells were then stained with Hoescht (5 µg/ml) and the percentage of apoptotic nuclei among the GFP-positive cells enumerated by fluorescence microscopy (Tamm et al., 1998).

**Caspase Inhibition**

HEp-2 cells were preincubated with the broad spectrum caspase inhibitor benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (Z-VAD-fmk) or the CPP-32 family-specific inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-DEVD-fmk) (Calbiochem, San Diego, CA) at varying concentrations (1 µM-200 µM) for 1 h at 37°C prior to Stx1 treatment (Grossman et al., 1998). Inhibition of apoptosis was determined by fluorescence microscopy of cells stained with acridine orange and ethidium bromide, as outlined above.

**Statistical Analysis**

Results are expressed as the mean ± SE. To test statistical significance between multiple groups, a one-way analysis of variance was used followed by post hoc comparisons with the Newman-Keuls test. The two-tailed unpaired Student's t-test was employed where indicated.
Results

Effect of Stx1 on Gb-3 expressing cells

HEp-2 cells incubated with Stx1 underwent apoptosis as assessed both by fluorescent microscopy and by transmission electron microscopy, as shown in Figure 5-1. Apoptotic cells displayed the characteristic ultrastructural features of cell shrinkage, condensed and margined chromatin and fragmented nuclei.

DNA fragmentation and release of oligonucleosomes was detected in toxin-treated cells utilizing a cell death immunoassay with an apoptotic enrichment factor (4.9 ± 1.9) comparable to both the provided positive control, histone-DNA complex (7.7 ± 3.5), and in cells treated with sorbitol to induce apoptosis (5.2, N=1). In contrast, the apoptotic enrichment factor did not increase in cells undergoing necrosis following freeze-thawing.

HEp-2 cells incubated with Stx1 at varying concentrations for 24 hours induced a dose dependent increase in apoptosis (Figure 5-1E) with a maximal effect detected at a dose of 12.5 ng/ml (22% ± 8%, ANOVA, p<0.05). The Stx1-mediated apoptosis of HEp-2 cells was time dependent with the largest increase in cell death observed after 24 hours of incubation with the toxin (21% ± 5%, ANOVA p<0.05) (Figure 5-1F). Following Stx1 treatment of HEp-2 cells for 48 hours, a large percentage of apoptotic cells undergoing secondary necrosis was observed (68% ± 6%). At prior times, a minimal number of necrotic cells (<5%) comparable to that of controls was detected. Treatment of HEp-2 cells with boiled Stx1 (100 °C x 30 min) did not result in enhanced cell death.
Figure 5-1: Stx1 induces apoptosis in Gb-3-expressing HEp-2 cells. Identification of apoptotic cells in untreated HEp-2 cells (panels A,C) and Stx1-treated HEp-2 cells (12.5 ng/ml) (panels B, D) after 24 hours by acridine orange/ethidium bromide staining and fluorescence microscopy (panel A,B) and transmission electron microscopy (panels C,D). Untreated HEp-2 cells demonstrate normal morphology as assessed by fluorescence microscopy (panel A) and transmission electron microscopy (panel C). Stx1-treated HEp-2 cells show morphologic features of apoptosis including condensed and margined chromatin with enhanced fluorescence (arrows and arrowhead). (panel B) Nonviable apoptotic cells fluoresce orange (arrowhead). As assessed by transmission electron microscopy (panel D), Stx1 treated HEp-2 cells (arrows) showed the characteristic features of programmed cell death, including cytoplasmic vacuolation and condensed and margined nuclear chromatin. Original approximate magnifications of panels A and B, x400, panel C x7800 and panel D x6000.

Quantitation of dose (panel E) and time (panel F) dependent induction of apoptosis by Stx1. Incubation with varying concentrations of Stx1 (range 1.25-25.0 ng/ml) resulted in an increase in apoptosis compared with untreated cells at each concentration (ANOVA, p<0.05)(panel E). HEp-2 cells incubated with 12.5 ng/ml Stx1 (shaded bars) compared with untreated cells (stippled bars) demonstrated an increase in apoptosis at each time point (ANOVA, p<0.05)(panel F). Results are expressed as the mean percentage of apoptotic cells per 500 cells enumerated from three separate experiments. Error bars represent the SE.
compared to controls, indicating that the observed effects were not due to the presence of contaminating small amounts of lipopolysaccharide.

To determine if Stx1 would induce programmed cell death in other Gb-3 expressing epithelial cells, the human intestinal CaCo2 cell line was employed. Similar to the findings with HEP-2 cells, incubation with 12.5 ng/ml of Stx1 for 24 hours stimulated CaCo2 cells to undergo programmed cell death (10% ± 1% vs. 3% ± 0.4%, p= 0.006, Student's t test).

**Evaluation of apoptosis in Stx1-treated Gb-3 deficient T84 cells**

To determine if Stx1 functioned as a pro-apoptotic stimulus in an epithelial cell line lacking Gb-3, T84 cells were incubated with the toxin. In contrast to HEP-2 and CaCo2 cells, T84 cells were resistant to Stx1-mediated apoptosis, as measured by fluorescent microscopy following staining with acridine orange and ethidium bromide. At Stx1 concentrations (113 ng/ml) ten times as high as the dose required to achieve a maximal effect in HEP-2 cells, the percent of apoptotic T84 cells was not increased compared with untreated, control cells (0.4% ± 0.6%).

**Comparison of Stx1 and Stx2 mediated apoptosis in HEP-2 cells**

The induction of apoptosis in Gb-3 expressing HEP-2 cells and the resistance to toxin-mediated apoptosis in T84 cells is consistent with the idea that Gb-3 was necessary for the activation of the cell death program by the toxin. Therefore, we investigated the effects of the related Shigatoxin Stx2, which is also synthesized by Shigatoxin-producing *E. coli* but which binds less avidly to the Gb-3 receptor.
(Head et al., 1991). Stx2-induced programmed cell death of HEp-2 cells was demonstrated by typical morphologic features of cell blebbing and condensed and margined nuclear chromatin (Figure 5-2A). HEp-2 cells treated with equivalent concentrations of Stx1 and Stx2 both underwent apoptosis (Figure 5-2B). However, incubation with Stx1 resulted in a greater increase in cell death compared to treatment with an equivalent concentration of Stx2 (21% ± 4% and 13% ± 4%, respectively; ANOVA, p<0.05).

*Induction of apoptosis with the B subunit of Stx1*

To determine if binding to Gb-3 was sufficient to trigger apoptosis, HEp-2 cells were incubated with the purified B subunit of Stx1. Compared to incubation with the holotoxin, a 100-fold higher concentration of the B subunit was required to induce apoptosis (6% ± 1% vs. 4% ± 0.3% for the untreated control cells; p<0.05, ANOVA). These findings indicated that the holotoxin was more effective in transducing the cell death signal.

To determine if protein synthesis inhibition could enhance the B subunit-stimulated cell death, we compared the degree of apoptosis in B subunit treated cells in the presence or absence of cycloheximide (10 μg/ml). Compared to cells incubated with the B subunit alone, the addition of cycloheximide did not enhance apoptosis (6% ± 1% vs. 7% ± 1%).
Figure 5-2: Apoptotic response of HEP-2 cells to the related toxin Stx2.

(panel A) Transmission electron photomicrograph of HEP-2 cells incubated with Stx2 (12.5 ng/ml). The typical morphologic features of apoptosis including cell membrane blebbing, marginated and condensed chromatin are demonstrated. Approximate magnification x46,000.

(panel B) Comparison of apoptotic response in HEP-2 cells exposed to equivalent concentrations of Stx1 and Stx2 (12.5 ng/ml for 24 hours at 37°C). Apoptosis was induced in HEP-2 cells treated with Stx1 or Stx2 with a greater induction of cell death mediated by Stx1. Results are expressed as the mean percentage of apoptotic cells per 500 cells enumerated from five separate experiments. Error bars represent the SE. ANOVA, p<0.05.
Effect of Gb-3 receptor antibody on apoptosis

Incubation of HEp-2 cells with a Gb-3 monoclonal antibody (38.13) (300 μg/ml) for 24 hours did not result in enhanced apoptosis (3% ± 1%) compared to untreated cells (2% ± 1%). In the same experiments, incubation of cells with Stx-1 (12.5 ng/ml) resulted in increased apoptosis (44% ± 4%; ANOVA p<0.05).

To determine if the antibody stimulated receptor clustering without signaling an apoptotic response, cells were coincubated with Stx-1 and 38.13. Coincubation of Stx-1 (12.5 ng/ml) and 38.13 for 24 hours at 37 °C resulted in apoptosis (37% ± 7%) comparable to levels observed in cells incubated with the toxin alone (32% ± 8%, p=NS). A reduction in the concentration of Stx-1 (2.5 ng/ml) coincubated with 38.13 did not result in a detectable inhibition of the holotoxin-triggered apoptotic response. Similarly, preincubation of the antibody for one hour prior to treatment with Stx-1 (2.5 ng/ml) did not inhibit Stx-1-mediated apoptosis (10% ± 0.2%) compared to toxin-treated cells in the absence of Gb3 monoclonal antibody (12% ± 3%, p=NS).

Expression of Bcl-2, Bax, and Bak after treatment with Stx1 and Stx2

To delineate the role of Bcl-2 and the related family members in the toxin-mediated apoptotic cascade, expression of Bcl-2 and the proapoptotic homologues Bax and Bak were measured by Western blotting. As demonstrated in Figure 5-3A, following incubation with Stx1 or Stx2 for 24 hours, HEp-2 cells showed an increase in expression of the pro-apoptotic family member Bax compared to untreated cells (2.5± 0.5 fold and 2.1± 0.5 fold
Figure 5-3: Immunoblot analysis of Bcl-2 family member expression in response to toxin treatment.

(panel A) Bax, Bak and Bcl-2 expression in untreated HEp-2 cells and tissue culture cells incubated with 12.5 ng/ml of either Stx1 or Stx2 for 24 hours. Both Stx1 and Stx2 enhanced expression of Bax compared to untreated, control cells (Stx1: 2.5± 0.5-fold increase, Stx2: 2.1± 0.5-fold increase by densitometry).

(panel B) Bax and Bcl-2 expression in HEp-2 cells incubated in the presence or absence of Stx1 or Stx2 for 6, 24 and 48 hours. Densitometry showed an increase in Bax expression in cells incubated with Stx1 or Stx2 for 24 hours (2.1-fold and 2.8-fold increase) and for 48 hours, (2.6-fold and 1.8 fold increase, respectively). Equivalent protein concentrations, as determined by the Bradford method, were loaded into each lane.
A

Control  Stx1  Stx2

Bax

Bak

Bcl-2

B

Control  Stx1  Stx2

Bax

Bcl-2
increases by densitometry, respectively). In contrast to Bax, expression of the pro-apoptotic family member Bak did not differ from untreated cells following toxin treatment. Expression of the apoptotic antagonist Bcl-2 was reduced by 3.5-fold following incubation with Stx1 for 24 hours. A similar reduction was observed when HEp-2 cells were treated with Stx2 (1.5-fold decrease in densitometry values).

To determine the relationship between alterations in Bax and Bcl-2 expression and induction of programmed cell death in HEp-2 cells over time, immunoblotting was performed on cell extracts treated with the toxins for up to 48 hours. As shown in Figure 5-3B, incubation with Stx1 or Stx2 stimulated an enhanced expression of Bax at both 24 hours (2.1-fold and 2.8-fold increase compared to untreated cells, as determined by densitometry) and 48 hours (2.6-fold and 1.8-fold increases, respectively). Compared to control cells, expression of Bcl-2 was reduced by 1.3-fold and 1.5-fold in HEp-2 cells incubated with Stx1 and Stx2 for 24 hours. Reduced Bcl-2 expression was also observed after 48 hours of Stx1 or Stx2 treatment (4.8-fold and 2.6 fold reductions, respectively).

Prevention of Toxin-induced Cell Death by Inhibition of Caspases

To determine whether inhibition of caspase activity effects toxin-triggered apoptosis the caspase inhibitors Z-VAD-fmk and Z-DEVD-fmk were utilized. As shown in figure 5-4, preincubation of HEp-2 cells with the general caspase inhibitor, Z-VAD-fmk, blocked cell death in a dose dependent manner. The maximum inhibitory effect was observed at a dose of 50 μM (0.9% ± 0.4% vs. 25% ± 3%, p< 0.001). Higher doses of the specific caspase 3 inhibitor Z-DEVD
Figure 5-4: Caspase inhibition prevents Stx1 stimulated cell death of HEp-2 cells. Cells pre-incubated with the general caspase inhibitor Z-VAD-fmk showed a dose dependent reduction in apoptosis in response to Stx1 (12.5 ng/ml) (p<0.001). Pre-incubation with the specific caspase 3 inhibitor Z-DEVD-fmk also blocked cell death but a higher concentration (200 μM) was required to achieve the same effect. Results are expressed as the mean percentage of apoptotic cells per 500 cells enumerated from three to four separate experiments. Error bars represent the SE. ANOVA, p<0.05.
fmk (200 μM) was required to prevent toxin-mediated apoptosis (5% ± 0.6, p<0.05).

_Overexpression of Bcl-2 inhibits Stx1-mediated apoptosis._

To confirm the biologic importance of a reduction in Bcl-2 during toxin-triggered cell death, HEp-2 cells were transiently transfected with constructs for overexpression of Bcl-2 together with green fluorescent protein. Toxin-treated GFP-positive cells containing the empty vector displayed characteristic features of apoptosis, including condensed brightly stained chromatin with nuclear fragmentation. In contrast, the morphologic features of apoptosis were not detected in GFP-positive cells expressing the Bcl-2 construct. As shown in **Figure 5-5**, compared to cells transfected with the empty vector, transient transfection of HEp-2 cells with Bcl-2 prevented Stx1-induced apoptosis (48% ± 3% vs. 6% ± 2%, p<0.05).
Figure 5-5: Inhibition of Stx1-mediated apoptosis by overexpression of Bcl-2 in HEP-2 cells. Cells were transiently cotransfected at a molar ratio of 1:4 with GFP and either Bcl-2 (4 μg) or control vector (4 μg) and incubated with Stx1 (12.5 ng/ml) for 24 hr at 37°C. Apoptosis of GFP-positive cells was assessed by fluorescent microscopy following Hoescht staining. Cells transfected with the control vector underwent apoptosis in response to Stx1 (48% ± 3%). In contrast, apoptosis was inhibited in cells transfected with Bcl-2 (6% ± 2%). Results are expressed as the mean percentage of apoptotic cells per 100 GFP-positive cells enumerated in three separate experiments. Error bars represent the SE. ANOVA, p<0.05.
Bar chart showing apoptosis (%) for different treatments:

- Vector
- Vector + Stx1
- Bcl-2
- Bcl-2 + Stx1

Apoptosis values: Vector + Stx1 shows the highest level, followed by Vector, while Bcl-2 and Bcl-2 + Stx1 show the lowest levels.
Discussion

This study demonstrates that Stx1 and Stx2 produced by *E. coli* induce apoptosis of human mucosal epithelial cells which express the Gb3 receptor for the toxins. The B subunit of Stx1 also triggers cell death of epithelial cells but to a lesser degree than the holotoxin. In contrast, intestinal epithelial cells lacking the glycolipid receptor did not undergo apoptosis. In addition, evaluating the role of the Bcl-2 family of pro- and anti-apoptotic proteins in mediating programmed cell death showed that toxin treatment of Gb3 expressing cells was associated both with an enhanced expression of the pro-apoptotic homologue Bax and reduced expression of the apoptotic antagonist Bcl-2.

In previous studies, using immunogold labeling, we showed that Stx1 undergoes transcellular translocation in Gb3-negative T84 intestinal cells and is localized to organelles that are involved in retrograde transport of the toxin to both the endoplasmic reticulum and the Golgi network (Philpott et al., 1997). Similar subcellular organellar targeting of Shiga toxin from *Shigella dysenteriae* type 1 is dependent on the Gb3 receptor (Sandvig et al., 1994). These findings suggest that in T84 cells there exists an alternative receptor for Stx1 binding. Indeed, a recent study demonstrated that Stx1 and Stx2 bind to an uncharacterised protein receptor on Vero cells in addition to the previously identified glycolipid receptor, Gb3 (Devenish et al., 1998).

T84 cells were resistant to Shigatoxin-stimulated programmed cell death at doses up to ten times required to mediate a maximal apoptotic effect in HEp-2 cells, despite the ability of the toxin to undergo directed retrograde transport. Taken together, these findings suggest that Shigatoxins produced by *E. coli* are
capable of mediating apoptosis and that the Gb-3 receptor is required to activate the death signal transduction cascade. In support of this contention, the B subunit binding portion of the toxin lacking the enzymatic activity necessary to inhibit protein synthesis also was capable of inducing apoptosis in Gb3-expressing epithelial cells, albeit to a lesser degree than the holotoxin. These findings in epithelial cells are in agreement with previous studies which reported a reduced degree of apoptosis in Gb-3 expressing lymphocytes treated with the B subunit compared to those cells exposed to the holotoxin (Manganey et al., 1993).

Incubation with a specific monoclonal antibody to the receptor did not result in an induction of apoptosis in Gb3 positive cells. As suggested previously (Taga et al., 1997), the inability of the antibody to trigger cell death likely is due to the low binding affinity of the antibody and lack of receptor clustering. In support of this contention, the monoclonal antibody did not competitively inhibit the apoptotic signal transduced by Stx1.

The mechanism by which the holotoxin enhances the cell death signal triggered by Gb-3 binding alone remains unclear. Inhibition of protein synthesis can potentiate an apoptotic stimulus likely through the reduction of short-lived anti-apoptotic proteins (Schulze-Osthoff et al., 1998). Therefore, inhibition of protein synthesis by the A subunit could enhance the apoptotic cascade signaled by binding to Gb-3. However, treatment of cells with the protein synthesis inhibitor cycloheximide did not enhance the apoptotic effect of the B subunit alone, a finding which suggests that additional mechanism(s) may well be involved.
Our findings suggest that intestinal epithelial cells are resistant to the cell death cascade activated by Stx1. Whether or not cytokines, such as tumor necrosis factor-α, which are produced in response to infection with Shigatoxin-producing E. coli (Lopez et al., 1995), alter the expression of Gb-3 in intestinal cells and thereby enhance the sensitivity to apoptosis has yet to be determined. Previous in vitro studies of endothelial cells derived from human umbilical vein (Obrig et al., 1993) and saphenous vein (Keusch et al., 1996) demonstrated that Stx1 mediated cytotoxicity is potentiated by pre-treatment with lipopolysaccharide, butyrate and cytokines, including TNF-α and interleukin-1. TNF-α and IL-1 increase galactosyl transferase activity resulting in enhanced Gb-3 expression by endothelial cells (van der Kar et al., 1995). Similarly, butyrate pretreatment of CaCo-2A or HT-29 intestinal epithelial cells promotes Stx1 binding and cytotoxicity (Jacewicz et al., 1995). In contrast, butyrate treatment of T84 cells does not enhance sensitivity to Stx1 (Jacewicz et al., 1995). In these studies, however, apoptosis was not measured; instead, cytotoxicity was assessed as inhibition of protein synthesis. A more recent study specifically measured the apoptotic response of isolated renal tubular cells following exposure to Stx1 (Karpman et al., 1998). Toxin treatment induced apoptosis of tubular cells and this effect was enhanced by pre-stimulation with TNF-α. Thus, in vivo disease pathophysiology could be altered during infection with Shigatoxin-producing E. coli by both bacterial factors, including lipopolysaccharide, and by host factors such as the release of pro-inflammatory mediators.

A study of renal tissue obtained from three children with post-enteropathic hemolytic uremic syndrome detected apoptotic cells within renal tubuli and cortices (Karpman et al., 1998). Isolated human renal tubular cells underwent programmed cell death upon exposure to Stx1. Shigatoxins produced by E. coli
could contribute to the tissue injury observed in the hemolytic uremic syndrome. However, the mechanism(s) by which Shigatoxins mediate programmed cell death have not been elucidated. This is the first study to document the molecular determinants involved in the death signal transduced by Stx1 and Stx2 by demonstrating that induction of apoptosis by these toxins is associated with an enhanced expression of Bax, a pro-apoptotic homologue, and a reduced expression of Bcl-2, an inhibitor of apoptosis. Furthermore, both overexpression of Bcl-2 and caspase inhibition prevented toxin-mediated cell death.

The mechanism by which Bax stimulates apoptosis or Bcl-2 blocks cell death in sensitive cells remains unclear. Through competitive dimerization the ratio of pro- to anti-apoptotic Bcl-2 family members determines the fate of a cell to apoptotic stimuli (Kroemer, 1997). However, recent evidence suggests that the cell death signal transmitted by these proteins is more complex (Adams and Cory, 1998). A feature common to cells undergoing some forms of apoptosis is the disruption of the mitochondrial transmembrane potential through the opening of mitochondrial permeability transition pores (Kroemer, 1997). The breakdown of the transmembrane potential results in the release of cytochrome c into the cytoplasm. Cytoplasmic cytochrome c complexes with the molecule Apaf-1 and the proform of caspase 9. In the presence of ATP, caspase 9 is activated thereby triggering the caspase cascade resulting in membrane blebbing and DNA fragmentation— the pre-eminent features characteristic of programmed cell death (Zhivotovsky et al., 1998). Bax, which like Bcl-2 is localized to mitochondria, mediates the release of cytochrome c (Rosse et al., 1998). In cells overexpressing Bax, Bcl-2 overexpression prevents both the release of cytochrome c and apoptosis (Fontaine et al., 1999; Rosse et al., 1998).
In contrast, caspase inhibition prevents some features of apoptosis, such as nuclear fragmentation but does not block Bax-mediated release of cytochrome c (Rosse et al., 1998). Thus, the apoptotic cross-talk mediated by Bax and Bcl-2 likely involves factors in addition to the release of cytochrome c.

In summary, this study demonstrates that binding to the Gb3 receptor in human mucosal epithelial cells by the Shiga toxins produced by STEC mediates an apoptotic signal transduction cascade associated with enhanced expression of Bax and a reduction in Bcl-2 ultimately resulting in the activation of caspases as outlined in Figure 5-6. We speculate that the development of therapies designed to inhibit this cell death cascade could lead to improved treatment options to prevent the complications of infection due to enteric bacterial pathogens which secrete Shiga toxins.
Figure 5-6: Proposed model of pathways mediating apoptosis in epithelial cells following exposure to Stx1. Following binding of the holotoxin to the Gb-3 receptor in sensitive cells both a reduction in Bcl-2 and an increase in Bax expression result in activation of the caspase cascade, thereby, signaling the cell to undergo apoptosis. Both overexpression of Bcl-2 and inhibition of caspase activation block toxin-mediated programmed cell death.
Chapter 6:

Conclusions/ Future Directions
In addition to transport and absorptive functions, epithelial cells lining the gastrointestinal mucosa provide an important barrier function and are often the first cells to interact with microorganisms (Kim et al., 1998). Current evidence indicates that epithelial cells initiate a variety of signal transduction cascades in response to enteric pathogens (Finlay and Cossart, 1997). The induction of apoptosis could be an additional mechanism by which enteric bacterial pathogens interact with host epithelial cells. The findings from the studies outlined in this thesis support this contention since infection both with the gastric pathogen *H. pylori* and Shigatoxins produced by the unrelated intestinal pathogen enterohemorrhagic *E. coli* activate cell death cascades in epithelial cells. Furthermore, recent studies provide evidence that invasive enteric pathogens such as *Salmonellae* also are capable of causing programmed cell death of epithelial cells (Kim et al., 1998).

The role of apoptosis in disease pathogenesis during infection with bacterial pathogens remains unclear and requires further investigation. The induction of apoptosis could serve to protect the host by eliminating infected epithelial cells and thereby allowing the maintenance of tissue homeostasis. Alternatively, apoptosis is associated with less inflammation than necrotic cell death. Therefore, induction of apoptosis could create a less hostile environment for the microbe.
Our studies investigating the pathogenesis of *H. pylori* infection demonstrate that infection with the bacterium specifically stimulates an alteration in gastric epithelial cell turnover including both enhanced apoptosis and proliferation. During chronic infection this alteration in cell turnover could increase the target cell population for further tumorigenic events and hence may explain the association between chronic infection and the subsequent development of gastric cancers.

Our findings indicate that the bacterium can directly stimulate cell death of gastric epithelial cells. In addition, immune-mediated cell death may also be involved in disease pathogenesis. *In vivo*, the observed increase in apoptosis returns to baseline levels only if both the bacterium is eradicated and inflammation resolves. Our *in vitro* results demonstrate that *H. pylori* infection enhances Fas receptor expression in association with increased sensitivity to Fas triggered apoptosis. Thus during infection *in vivo*, in addition to cell death induced by *H. pylori* directly, infiltrating cytotoxic lymphocytes which express the Fas ligand may trigger apoptosis in Fas sensitive gastric epithelial cells. Indeed, a recent study demonstrated an increase in Fas receptor-expressing gastric epithelial cells and Fas ligand expressing infiltrating cells during *H. pylori* infection in adults (Rudi et al., 1998).
Shigatoxins produced by *Escherichia coli*

Studies presented in this thesis indicate that Shigatoxins produced by *Escherichia coli*, including Stx-1 and Stx-2, induce apoptosis of mucosal epithelial cells and delineate the underlying mechanisms involved in triggering the cell death cascade. The findings demonstrate that the induction of programmed cell death by Stx-1 and Stx-2 is mediated in part through binding to Gb-3 and suggest that the holotoxin is more effective than the B subunit in transducing the cell death signal. The lack of effect of protein synthesis inhibition on cell death stimulated by the B subunit of Stx-1 indicates that the enzymatic activity of the A subunit does not contribute to the enhanced apoptosis mediated by the holotoxin. In addition, these studies demonstrate that Stx-1 and Stx-2 trigger cell death through altered expression of members of the Bcl-2 family including both a reduction in the anti-apoptotic homologue Bcl-2 and an increase in the pro-apoptotic homologue Bax. Furthermore, overexpression of Bcl-2 or inhibition of downstream caspase activation blocks toxin-mediated cell death.

Understanding the effectors involved in the cell death signal triggered by these toxins should lead to the development of novel therapeutic agents aimed at inhibiting this signal transduction pathway. These agents should aid in the management of the severe and currently untreatable complications associated with infection with STEC.
Future Directions

The studies outlined in this thesis demonstrate that induction of apoptosis in mucosal epithelial cells occurs in response to gastrointestinal pathogens or their products. The bacterial mediated cell death is likely involved in disease pathogenesis and therefore warrants further investigation.

The bacterial factors which transmit the cell death signal during H. pylori infection remain unknown. Therefore, the in vitro model system developed in our studies can be utilized to determine the importance of specific bacterial virulence factors using both selected components of the bacterium, such as LPS, and by using mutagenesis studies. The system can also be exploited to determine if other Helicobacters, such as H. hepaticus, H. felis and H. mustelae, stimulate apoptosis.

The in vitro model can also be utilized to confirm the enhanced sensitivity of H. pylori-infected epithelial cells to Fas-mediated cell death by investigating the effects of co-culture with T cells expressing the Fas ligand. Furthermore, the development of the murine model of H. pylori infection in mice with disruptions in Fas signaling can be utilized to determine if the in vitro findings also occur in the in vivo setting and to define their importance in mediating disease.

Our studies utilizing Stx-1 and Stx-2 document that factors in addition to receptor binding are required to stimulate cell death in Gb-3-expressing cells. Further studies are now required to delineate the mechanism by which the holotoxin potentiates the cell death signal initiated by Gb-3 binding. In addition, the effect of inflammatory mediators in regulating the cell death cascade in
mucosal epithelial cells requires elucidation. Furthermore, the ability of specific inhibitors of caspase activation to prevent apoptosis *in vitro* suggests that these inhibitors may be beneficial in the *in vivo* setting. Investigation in an animal model of STEC infection is now required to determine whether caspase inhibition can alter disease severity.

It is clear that the interactions between host cells and infecting microbes influence disease pathogenesis. Apoptosis is one signaling pathway that bacteria or their products can mediate within host cells. Therefore, an increased knowledge of both the host and bacterial factors effecting the cell death signal should lead to a better understanding of mechanisms of disease and improved management of the complications associated with infection.
Chapter 7:

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