STUDIES ON THE MOLECULAR BASIS OF SHIGA TOXIN 1 INDUCED MAMMALIAN CELL DEATH

PhD THESIS

DEPT. OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
UNIVERSITY OF DHAKA
DHAKA, BANGLADESH
JANUARY, 2007

SUBMITTED BY:
REGISTRATION NO:48
SESSION: 2002-2003

STUDIES ON THE MOLECULAR BASIS OF SHIGA TOXIN 1 INDUCED MAMMALIAN CELL DEATH

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF DHAKA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

SUBMITTED BY:
REGISTRATION NO. 48
SESSION: 2002-2003
January, 2008

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
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CERTIFICATE

This is to certify that the thesis entitled "Studies on the molecular basis of Shiga toxin 1 induced mammalian cell death" was carried out by Md. Sabir Hossain, Registration No. 48, Session: 2002-03 for the fulfillment of the degree of Doctor of Philosophy from the Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh.

This work was carried out under our supervision and the style and contents of the thesis have been approved and recommended for the award of PhD degree.

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ABSTRACT

Culture supernatant obtained from the Shiga toxin (STX) gene containing bacteria *Shigella dysenteriae* 1 isolated from patients at ICDDR, B was found to induce fluid accumulation in rabbit ileal loop and kill HeLa cells. It was found that purified Shiga toxin1 (STX1) exhibited cytotoxic activity in different mammalian cells such as HeLa cells, mouse embryo fibroblasts, and Caco-2 cells (a human intestinal primary fibroblast cell line). The effects of STX1 on cell proliferation, chromatin condensation were analyzed using MTT cell viability assay and DAPI staining respectively. Cell cycle regulation and specific cellular expression were measured by flow cytometric analysis and western blot analysis respectively. STX1 was found to induce apoptosis in mammalian cells as determined by fragmentation of chromosomal DNA. STX1 was also found to induce apoptosis in mammalian cells as determined by the release of Cytochrome C from the mitochondria and induction of nuclear condensation. Flow cytometric analysis indicated that STX1 induced G1 cell cycle arrest and apoptosis. It was also found that STX1 activated DNA damage signaling as determined by induction of H2AX phosphorylation and cleavage of PARP. Inhibition of caspase 3 significantly reduced STX-1-induced phosphorylation of H2AX and nuclear condensation. It was also found that STX1 induces p53 expression, and activates ATM in mammalian cells. Interestingly, STX1-induced chromatin condensation was found to be significantly reduced in p53- and ATM-knockout cells. These results suggest that ATM/p53 signaling pathway play a key role in transducing STX1-induced apoptosis signals in mammalian cells. This is the first demonstration of STX1 induced G1 cell cycle arrest and involvement of ATM/p53 in STX1-induced apoptosis.

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Abbreviations

AMP	Adenosine mono phosphate
ATM	Ataxia Telangiectasia mutated
BSA	Bovine Serum Albumin
Cdk	Cycline dependent kinase
DAPI	4'-6'-Diamidino-2-phenylindole
DMEM	Dulbucco's Modified Eagles Medium
EIEC	Enteroinvasive *Escherichia coli*
FBS	Fetal Bovine Serum
Ipa	Invasive plasmid antigen
KDa	Kilo Dalton
MDa	Mega Dalton
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffer saline
ShET	Shigella Enterotoxin
Spp.	Species
STX	Shiga Toxin
TSB	Tryoticase soy broth
rRNA	Ribosomal RNA
WHO	World Health Organization

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

1.1.1 Overview

Shiga toxin (STX), a family of structurally and functionally related exotoxins produced by the enteric pathogens *Shigella dysenteriae* type 1 and STX-producing *Escherichia coli* (STEC). A severity of Shigella-associated tissue damage has been claimed to be correlated with the toxin producing ability of *Shigella* strains. *S dysenteriae* type 1 has been implicated in large-scale epidemics worldwide particularly in developing countries with many thousands of deaths (Talukder *et al.*, 2004). Molecular mechanism for the development of clinical manifestation has remained unknown. Although toxin producing gene have been discovered in *Shigella* spp and EIEC (O'Brien *et al.*, 1992; Sansonetti, 1998), a clear understanding of the role of toxin in Shigellosis has remained to be studied. In this study, I attempted to explore the molecular insight into STX1 induced mammalian cells death. I have demonstrated that *Shigella dysenteriae* 1 producing STX1 and STX1 causes cell death in different cell lines such as MEF, Caco-2, and HeLa. Purified STX1 was found to cause apoptotic DNA damage and G1S cell cycle arrest in HeLa cell line. Purified STX1 was found to activate ATM/p53DNA damage signaling pathway and induce apoptosis in mammalian cells. Interestingly, a significant reduction of apoptosis was found in cells deficient of either ATM or p53. These results for the first time demonstrate an involvement of ATM/p53 in STX-induced mammalian cells death.

1.2 Review of Literature

1.2.1 Shigellosis

Shigellosis is one of the major diarrheal diseases afflicting humans in the developing and underdeveloped parts of the world, especially Bangladesh. Shigellosis produces inflammatory reactions and ulceration on the intestinal epithelium followed by bloody diarrhea and mucus in the stool. Shigellosis is endemic in Bangladesh, and it is estimated that dysentery accounts for 20% of deaths related to diarrhea among children (Victora *et al.*, 1993). The genus *Shigella*, the causative agent of shigellosis, is comprised of four species, namely, *S. flexneri*, *S. dysenteriae*, *S. boydii*, and *S. sonnei*, and each of these species is further classified into 15 (including subtypes), 13, 18, and 1 serotypes, respectively, based on the O antigen component of lipopolysaccharide present on the outer membrane of the cell wall. *S. flexneri* and *S. dysenteriae* are the major concerns for developing countries. The manifestation of *S. dysenteriae* type 1 infection is more severe because of its capacity to produce Shiga toxin, which is exclusively produced by this type. Clinical infection can be transmitted by as few as 10 *Shigella* organisms even without neutralization of gastric acid (Dupoint *et al.*, 1989).

At least three periods of epidemic outbreaks of dysentery due to *S. dysenteriae* 1 have been recorded previously in the Indian subcontinent, in 1972-1973, 1983-1984, and 1993-1994 (Bennish and Wojtyniak, 1991; Hossain *et al.*, 1998; Rahaman *et al.*, 1975; Shahid *et al.*, 1985). Despite improvement of municipal water supplies and sanitation, shigellosis still occurs frequently. This raises important questions about the causes of shigellosis, its transmission, epidemiology, and the effectiveness of public health measures in overcoming this illness. Indiscriminate use of antibiotics in this region has resulted in the *Shigella* strains becoming resistant to multiple antibiotics. At present, most of the *Shigella* strains isolated from patients are resistant to ampicillin (AMP), sulfamethoxazole-trimethoprim (SXT), and nalidixic acid (Bennish *et al.*, 1992; Hossain *et al.*, 1998; Mamun *et al.*, 1997).

Diarrhoeal disease continues to be a leading cause of morbidity and mortality worldwide and is ranked fourth as a cause of death (WHO, 1999). It had been estimated that each year 163.2

million episodes of epidemic shigellosis occur in developing countries (3.5% of the population) and 1.5 million in industrialized countries (0.15 of the population). Approximately 1.1 million episodes (0.7%) result in death. Under 5- year-olds comprise the majority of cases (69%) and of fatal outcomes (61%) (WHO, 1999). While death from *Shigella* infection is a rare outcome in industrialized countries, for example, the case-fatality rate during the 1980s was reported to be 0.4% in the U.S.A.

Table 1.1: Estimated annual mortality from shigellosis in developing countries, by age group (WHO, 1999)

	0-11 months	1-4 months	>5 years
No. Of hospitalized patients	475315	311850	741850
No. of shigellosis cases that die (%)	66070(13.9)	29315 (9.4)	60830 (8.2)
No. of shigellosis cases that die outside the hospital	462490	205205	42580
Total *Shigella* death	1093505		

1.3 Toxins of *Shigella*

1.3.1 *Shigella* Enterotoxin

Enteroinvasive E. coli and *Shigella* infection are often manifested by watery diarrhoea. In contrast, surprisingly little is known of the precise mechanism by which *Shigella* cause watery diarrhoea. It has previously been hypothesized, that *Shigella* produces an enterotoxin. However, except for the cytotoxin /neurotoxin /enterotoxin elaborated by *Shigella dysenteriae* (Fontaine ***et al.***, 1988); little convincing proof has been generated to substantiate the contention that these organisms in fact produce enterotoxins in *S. flexneri*. Recently, two novel enterotoxins elaborated by *Shigella* species have been reported that may be responsible for the watery phase of shigellosis (Fasano ***et al.***, 1995; Nataro ***et al.***, 1995).

1.3.1.1 *Shigella* Enterotoxin 1 (ShET-1)

ShET-1 is a chromosomally encoded, 55 KDa complex protein that is universally elaborated by *Shigella flexneri* 2a strains but only rarely other serotypes (Noriega *et al.*, 1995). Sequencing analysis of the genes encoding this toxin disclosed the presence of two contiguous open reading frame (ORFs) of 534 (set1A) and 186 (Set1B) bp respectively, governed by the same promoter and separated by only 3 bp. set1 gene product having a size of ~20 KD and another gene product being 7 KD in size. The ShET-1 holotoxin has an apparent size of 55 KD, it is tantalizing to speculate that A1 –B5 configuration of one 20 –KD subunit (typical of the size of A subunits, other enterotoxins) bonded to five B subunits, each 7 KD in size (typical of B subunits of other enterotoxin). ShET-1 was found in 45% of S. *flexneri* strain but was not in either *S. sonnei* or *S. dysenteriae* (Vargas ***et al.***, 1999).

1.3.1.2 *Shigella* Enterotoxin 2 (ShET-2)

ShET-2 is a 62.8 KDa single protein that was originally detected in enteroinvasive *Escherichia coli* (EIEC) and referred to as EIEC enterotoxin is encoded in the *sen* gene (Fasano *et al.*, 1991). The gene encoding this toxin is located on the 140 MDa invasive plasmid of *Shigella* and seem to be present in more than 80% of a wide array of *Shigella* serotype examined (Narato *et al.*, 1995). Narato *et al.*, 1995 found that the *sen* gene in 73% of *S. flexneri* strains using a DNA probe and Vargas *et al.*, 1999 found ShET-2 together with ShET-1 in 36% of *S. flexneri* strains, 57% of *S. sonnei* strains and *S. dysenteriae* strains. Gene sequence analysis showed that both ShET-1 and ShET-2 are genetically unrelated to shiga toxin elaborated by *Shigella* dysenteriae type 1. Furthermore, the three toxins are also seem to be immunologically unrelated (Fasano *et al.*, 1997)

1.3.1.3 Shiga Toxins (Stxs)

Secreted bacterial proteins have been defined as exotoxins based on the ability to cause cell death, mediate cell shape change or disrupt normal cellular function. It has become clear, however, that many bacterial toxins are multifunctional proteins. This is particularly apparent when the actions of toxins are examined using different cell types. Bacterial toxins may activate cell signaling pathways leading to increased gene expression and/or arrest cell survival pathways. Thus, depending on the cell type utilized, toxins may cause necrotic or apoptotic cell death, may alter cell signaling pathways without causing cell death, or induce the expression of pro- or anti-inflammatory cytokines which in turn exacerbate local inflammation or the development of systemic disease (Schmitt, *et al.*, 1999; Henderson *et al.*, 1996). While *S. dysenteriae*1 .and STEC differ in their epidemiology and expression of virulence determinants, they share the property of producing one or more Stxs (alternatively called verotoxins or verocytotoxins). The toxins belong to a group of structurally and functionally related cytotoxins. The prototype toxin of the Stx family is Stx produced by *S. dysenteriae* serotype 1. STEC may produce one or more related toxins designated Stx1, Stx1c, Stx1d, Stx2, Stx2c, Stx2d, Stx2e or Stx2f (O' Brien *et al.*, 1992; Melton-Celsa *et al.*, 1998). Stx1 is most closely related to Stx, differing by only a single amino acid in the A-

subunit of the toxin. Stx1 and Stx2are approximately 56% homologous at the deduced amino acid sequence level, while the variants of Stx2 are 84-99% homologous to Stx2 (Melton-Celsa *et al.*, 1998). The genes for Stx1, Stx2 and the Stx2 variants are encoded by lambdoid bacteriophages which lysogenize STEC (Schmidt, 2001). The bacteria may express more than one Stx because they may harbor more than one Stx-encoding bacteriophage. The toxin-converting phages are important for horizontal transmission of stx genes and Stx-expressing Citrobacter freundii and Enterobacter cloacae strains have been described (Paton and Paton, 1998). Although phage sequences can be detected adjacent to the genes encoding Stx in *S. dysenteriae* serotype 1, it appears that insertional sequences have rendered the prophage defective for excision (McDonough and Butterton, 1999).

Shiga toxin belongs to the large family of ribosome-inactivating proteins (RIPs). All the Stxs are AB5 toxins consisting of a single ~32 kDa A-subunit in non-covalent association with a pentamer of identical B-subunits. The molecular mass of each globotriaosylceramide (Gb3 or CD77), while Stx2e, a toxin of veterinary importance, B-subunit is ~7.7 kDa. X-ray crystallographic analysis demonstrated that the B-subunit pentamers form a doughnut- shaped structure with the carboxy-terminus of the A subunit inserted into the central pore of B-subunits (Fraser *et al.*, 1994). Toxin binding to cells is mediated by the B-subunits through interaction with membrane neutral glycolipids of the globo-series. For the Stxs causing disease in humans, the toxin receptor is binds globotetraosylceramide (Gb4). Gb3 is expressed on epithelial and endothelial cells derived from a variety of sites in humans and animals (Lingwood, 1996). Once Stxs bind their glycolipid receptors, the toxins are internalized via clathrin-coated pits and transported through the trans- Golgi network and Golgi apparatus to the endoplasmic reticulum (ER) and nuclear membrane. This pattern of intracellular tracking is referred to as retrograde transport (Sandvig and van Deurs, 2002). During retrograde transport, the A-subunit is cleaved by furin, a calcium-sensitive serine protease localized to the Golgi network. The resultant A-subunit fragments, A1+A2, remain associated by a disulfide bond. An alternative mechanism of A-subunit processing involving the action of the protease calpain has also been described (O' Loughlin and Robins-Browne, 2001). Once in the ER, the disulfide bond linking A1+A2 is reduced, and the A1 fragment is translocated across the ER membrane into the cytoplasm via a mechanism which remains to be fully

characterized. The A1 fragments of Stxs possess N-glycosidase activity and act to catalytically cleave a single adenine residue from the 28S rRNA component of the eukaryotic ribosomal 60S subunit. Following depurination, elongation factor 1-dependent aminoacyl-tRNA binding is inhibited and peptide elongation ceases (O'Brien *et al.*, 1992). The invasiveness of *Shigella* is critical for the development of bacillary dysentery. However, orally administered either a toxigenic or atoxigenic *S. dysenteriae* isogenic strain to rhesus monkeys and showed that the Stx-producing strain caused more damage to intestinal capillaries, suggesting that the toxin mediates vascular tissue damage caused by the invasive organisms (Fontaine *et al.*, 1988). The characteristic intestinal damage caused by STEC includes hemorrhage and edema in the lamina propria with focal necrosis and neutrophil infiltration. Recently, it was shown that Stx1 and Stx2are translocated across polarized intestinal epithelial monolayers via transcellular and paracellular mechanisms (Hurley *et al.*, 1999; Philpott *et al.*, 1997). In this manner, Stxs may gain access to and damage colonic blood vessels. Stxs have also been shown to induce the expression of the neutrophil- specific chemokine interleukin-8 by human intestinal epithelial cells in vitro (Thorpe *et al.*, 1999), suggesting that Stxs may participate in the process of gut inflammation. Incubation of fluoresceinated Stx1 with human whole blood resulted in almost exclusive binding of the toxin to neutrophils (te Loo *et al.*, 2000), and Stx2was detected in the circulation of HUS patients associated with neutrophils (te Loo *et al.*, 2001). Scatchard analysis using radioiodinated Stx1 showed that human neutrophils possess approximately 2.1U105 toxin binding sites per cell with a Kd =1038 mol l31. Interestingly, Stx1 could be transferred from neutrophils to the surface of tumor necrosis factor-K (TNF-K)-treated human glomerular microvascular endothelial cells in vitro (te Loo *et al.*, 2000). Collectively, these data suggest that the toxins (i) possess mechanisms to cross the intestinal epithelial barrier (ii) induce a neutrophil- rich inflammatory infiltrate in the gut and (iii) circulate in the bloodstream by 'piggy-backing' on neutrophils. The presence of Stxs in the circulation appears to be a critical determinant in the development of HUS and CNS complications, as much of the vascular histopathology can be reproduced in animals intravenously administered purified Stxs (Moxley and Francis, 1998). The toxins appear to target glomerular and CNS microvascular endothelial cells for damage.

It has been reported that RIPs act on 28S rRNA and catalyze removal of specific adenine residue (Endo and Tsurugi, 1987; Endo *et al.*, 1988b). However, recently RIP have also been demonstrated to remove several adenine residues from naked RNA and DNA in vitro (Barbieri *et al.*, 1994; Barbieri *et al.*, 1997; Barbieri *et al.*, 2000; Nicolas *et al.*, 2000). Shiga toxin has also been shown to induce nuclear DNA damage in human endothelial cells and releases single-stranded DNA (Brigotti *et al.*, 2002). Extensive removal of adenine by STX has been proposed to weaken sugar-phosphate back-bone of DNA/RNA leading to generation of apurinic/apyrimidinic (AP) sites (Brigotti *et al.*, 2001). Accumulation of AP sites in the genome may produce a DNA damage response leading to the activation of signaling pathways involved in cell cycle arrest, DNA repair and apoptosis. It has been shown that 28S rRNA is one of the major target for RIPs (O'Brien *et al.*, 1992). Inhibition of protein synthesis by RIPs has been proposed to be responsible for induction of apoptosis in mammalian cells (Obrig *et al.*, 1987; Endo *et al.*, 1988a; Endo *et al.*, 1988b). However, cytotoxic effect due to inhibition of protein synthesis inhibitor has been controversial (Sandvig and van Deurs, 1992; Sakamoto *et al.*, 2003). It is possible that RIP activates yet an unidentified signaling pathway(s) leading to cell death.

1.4 Shiga Toxin Producing Organisms

S. dysenteriae serotype 1 and STEC are etiologic agents of the bloody diarrheal diseases bacillary dysentery and hemorrhagic colitis, respectively. Shigellae are invasive bacteria capable of replication within the cytoplasm of colonic epithelial cells. STEC, in contrast, are non-invasive. Some, but not all, STEC attach to the intestinal epithelium in such a manner as to disrupt normal brush border. Filamentous actin and other cytoskeletal elements accumulate adjacent to adherent bacteria so that the organisms appear to be raised on pedestals (Knutton *et al.*, 1989). These unique adherence structures are called attaching and effacing (A/E) lesions and STEC capable of causing A/E lesions are referred to as enterohemorrhagic E. coli (EHEC). The bacterial proteins necessary for A/E lesion formation, as well as proteins for the type III secretion apparatus to secrete the adherence proteins, are encoded on a V35 kb DNA insert called the locus of enterocyte effacement (LEE) pathogenicity island (reviewed in Kaper, 1998)). Thus, EHEC have acquired a large DNA fragment encoding the proteins

mediating intimate adherence and cytoskeletal rearrangements. Patients with bacillary dysentery or hemorrhagic colitis are at increased risk for developing life-threatening systemic complications, primarily acute renal failure and central nervous system (CNS) abnormalities. The acute renal failure that may follow infection with Stx-producing bacteria is called the hemolytic uremic syndrome (HUS) and is characterized by non-immune anemia, thrombocytopenia, and glomerular thrombotic microangiopathy with fibrin-enriched thrombi deposited within glomerular capillaries (Proulx *et al.*, 2001). CNS abnormalities include lethargy, disorientation, seizures, paralysis and coma (Siegler, 1992). Children and the elderly are more likely to develop renal and neurological complications following infection with STEC. *Shigella* are distributed worldwide but the highest incidence of bacillary dysentery occurs in regions of overcrowding, malnutrition, and inadequate waste and drinking water supplies. Bacillary dysentery is primarily a disease of childhood with most patients being 65 years old. *Shigella* cause endemic dysentery in Africa, Southeast Asia and the Indian subcontinent, where estimated incidences are 750-2000 cases per 1000 children per year (Acheson and Keusch, 1995). Although both *S. dysenteriae* serotype 1 and STEC may be transmitted by ingestion of contaminated water or person-to-person spread, outbreaks of hemorrhagic colitis often occur after the ingestion of STEC-contaminated foods. Undercooked ground beef, unwashed vegetables, and unpasteurized fruit juices have been implicated in major outbreaks of bloody diarrhea. Thus, STEC infections are more prevalent in developed countries with widespread distribution systems for meats and vegetables. The most prevalent E. coli serotype associated with outbreaks of hemorrhagic colitis in the USA, Canada, UK and Japan is E. coli O157:H7. In the USA, it is estimated that there are ~73,000 cases/year of bloody diarrhea caused by E. coli O157:H7 and ~37 000 cases per year associated with non-O157: H7 serotypes (Mead *et al.*, 1999).

1.5 Apoptosis

Apoptosis (programmed cell death) and necrosis are currently defined as the two major modes of cell death. In some instances, necrosis is a passive process characterized by the loss of plasma membrane integrity, cell swelling and inflammation due to the release of cellular contents into the periphery. In contrast, apoptosis is an active process generally characterized

by morphological cell changes including cell shrinkage associated with cytoplasmic condensation and vacuolation, membrane blebbing, apoptotic body formation, chromatin condensation, nuclear fragmentation, and loss of cell adhesion (Zimmermann *et al.*, 2001). However, as has been recently noted, when describing apoptotic versus necrotic changes, it is extremely important to discriminate between antemortem biochemical changes versus postmortem morphological changes in cells (Kanduc *et al.*, 2002). Apoptosis is initiated in response to stimuli which activate genetically programmed signaling cascades. Cytoplasmic constituents are not spilled into the extracellular milieu so that inflammation is not a characteristic of apoptotic cell death. Apoptotic bodies are generally internalized and processed by phagocytic cells.

A major biochemical feature of apoptosis is the internucleosomal fragmentation of genomic DNA, although it should be noted that DNA fragmentation is not a universal characteristic of apoptosis (Nagata, 2000). DNA fragmentation appears to be a multi-step process with DNA first cleaved into large fragments (50-200 kb) and subsequently degraded to nucleosomal units of multiples of 180-200 bp. The presence of fragmented DNA (DNA laddering) is readily assessed using agarose gel electrophoresis. DNA fragmentation also forms the basis of the TUNEL staining assay in which the fragments are used as substrates for the enzyme terminal deoxyribonucleotidyl transferase. Although many stimuli may activate programmed cell death, most stimuli appear to signal through a common, tightly regulated pathway involving the sequential activation of proteases called caspases. Caspases are cysteinedependent aspartate-specific proteases that exist as inactive precursors or pro-caspases. In general, caspase activation requires a single proteolytic cleavage of a latent singlechain zymogen to produce a heterodimer. The caspase heterodimers then self pair to form the active tetrameric molecule (Stennicke *et al.*, 2000). Caspases are unusual proteases in that aspartate specificity is rare, and many caspases possess the capacity to mediate auto-proteolysis. Programmed cell death takes place through two pathways categorized on whether apoptotic signals originate outside of or within cells. The extrinsic or extracellular pathway of programmed cell death generally requires the ligation of receptors of the tumor necrosis factor receptor (TNFR) superfamily containing cytoplasmic death domains. Caspase 8 is activated following its recruitment to the cytosolic face of the receptor-ligand complex. The intrinsic or

mitochondrial pathway involves the disruption of mitochondrial membrane potential, the release of Cytochrome C, and the activation of caspase 9. The intrinsic pathway of caspase activation is regulated by members of the Bcl-2 protein family, which include proapoptotic protein BH-3 interacting domain (Bid), Bcl-2-associated X protein (Bax) and Bcl-2antagonist/killer (Bak), and anti-apoptotic proteins such as Bcl-2and myeloid cell leukemia-1 (Mcl-1). The intrinsic pathway is responsible for apoptosis in response to ionizing radiation and cytotoxic chemotherapeutic drugs. Activation of caspase 8 or caspase 9 by the extrinsic or intrinsic pathways leads to the activation of a common downstream caspase, caspase 3. Caspase 3 activates the DNase that executes the cleavage of DNA. Additional molecular details of caspase activation can be found in recent reviews (Zimmermann *et al.*, 2001; Kim *et al.*, 2003). Recently, a third mechanism of signaling for programmed cell death was described which requires the activation of caspase 12. In mice, caspase 12 appears to be localized to the ER and is activated in response to stimuli which induce ER stress (such as brefeldin A or tunicamycin) and release Ca2þ stores. Mice lacking caspase 12are resistant to apoptosis induced by ER stress but are sensitive to other death stimuli (Nakagawa *et al.*, 2000). The human caspase 12 homolog has been cloned but contains loss of function mutations which render the protein inactive, suggesting that caspase 12m ay not play a role in apoptosis induction in human cells (Fischer *et al.*, 2002).

1.5.1 Shiga Toxin and Apoptosis

Numerous studies have examined the mechanism of cell death mediated by purified Stxs. Cultured cells or cell lines used in these studies include epithelial cells, endothelial cells, B-lymphoma cell lines, astrocytoma cells, monocytic cell lines, neutrophils and amniotic cell lines. These cell types respond differently to Stxs. For example, T84 cells, a human intestinal epithelial cell line, lack the toxin glycolipid receptors and are not killed by Stxs in vitro, yet are capable of toxin translocation from apical to basolateral surfaces (Hurley *et al.*, 1999). Bovine crypt intestinal epithelial cells, in contrast, express Gb3 but escape killing by routing the toxins to lysosomes rather than to the ER (Hoey *et al.*, 2003). Purified Stx2 has been shown to inhibit cell death of neutrophils, cells that normally undergo spontaneous apoptosis, suggesting that the toxins may increase the life span of cells capable of mediating extensive

vascular damage (Liu *et al.*, 1999). While Stxs may not induce apoptosis in all cell types, there is ample evidence suggesting that apoptosis is critical in the development of vascular lesions and tissue damage following translocation of the toxins into the bloodstream. Based on the current available literature, a model for the progression of disease is shown in Fig. 1. In contrast to the studies using T84 cells (Smith *et al.*, 2003) treated the human intestinal epithelial cell line, HCT8, with Stx1 and demonstrated the activation of the stressactivated kinase cascades JNK/SAPK and p38, and the activation of caspase 3 leading to apoptosis with DNA fragmentation. Toxin enzymatic activity was required for the activation of stress-activated kinases and cytotoxicity. Caspase 3 activation and cytotoxicity were blocked by the p38 kinase inhibitor SB202190. The signaling proteins necessary to link toxin enzymatic activity with activation of kinase and caspase cascades remain to be characterized. Suzuki *et al.*, 2000 reported that the Stx2A-subunit, but not Stx1 A-subunit, possesses a pentapeptide sequence (NWGRI) similar to Bcl-2homology domain BH1. Thus, the Stx2A 1-subunit may directly interact with mitochondrial Bcl-2leading to activation of caspase 3 in Bcl-2expressing human hepatoma (HepG2) cells.

Many studies have documented that purified Stxs induce apoptosis in cultured primary epithelial cells, including human renal proximal tubule and renal cortical epithelial cells (Kodama *et al.*, 1999); Karpman *et al.*, 1998). Apoptosis of renal cortical cells in vivo was demonstrated in biopsies taken from three children with HUS (Karpman *et al.*, 1998). The biopsies showed extensive cortical necrosis and thrombotic microangiopathy, and TUNEL staining revealed apoptotic nuclei in tubules and glomeruli. A similar pattern of TUNEL staining was noted in the kidney tissues of mice fed Stx2-producing E. coli. The mechanisms of Stx-induced apoptosis have been extensively studied using the human laryngeal epithelial cell line HEp-2and the human cervical epithelial cell line HeLa. In HEp-2cells, Stx1, Stx2 and Stx2c induce apoptosis by activating caspases 8, 9 and 3 (Ching *et al.*, 2002). Treatment of HEp-2cells with the toxins resulted in increased poly- (adenosine diphosphate (ADP)-ribose) polymerase (PARP) cleavage. PARP is a caspase 3 substrate, and the cleavage of PARP is thought to act in a proapoptotic manner by inhibiting DNA repair. Bid, a proapoptotic member of the Bcl-2 family is cleaved to its active 15 kDa form by the action of caspase 8. Activated Bid (truncated Bid or tBid) is known to promote the oligomerization of the

proapoptotic proteins Bak and Bax (Degli and Dive, 2003) that, in turn, trigger the release of cytochrome c from mitochondria and activate the downstream caspase 3. These data suggest that Bid acts as a link between caspase 8 and the intrinsic or mitochondrial pathway in Stx-mediated apoptosis. Purified Stx B-subunits triggered HEp-2cell apoptosis, although higher doses were necessary compared to the holotoxins.

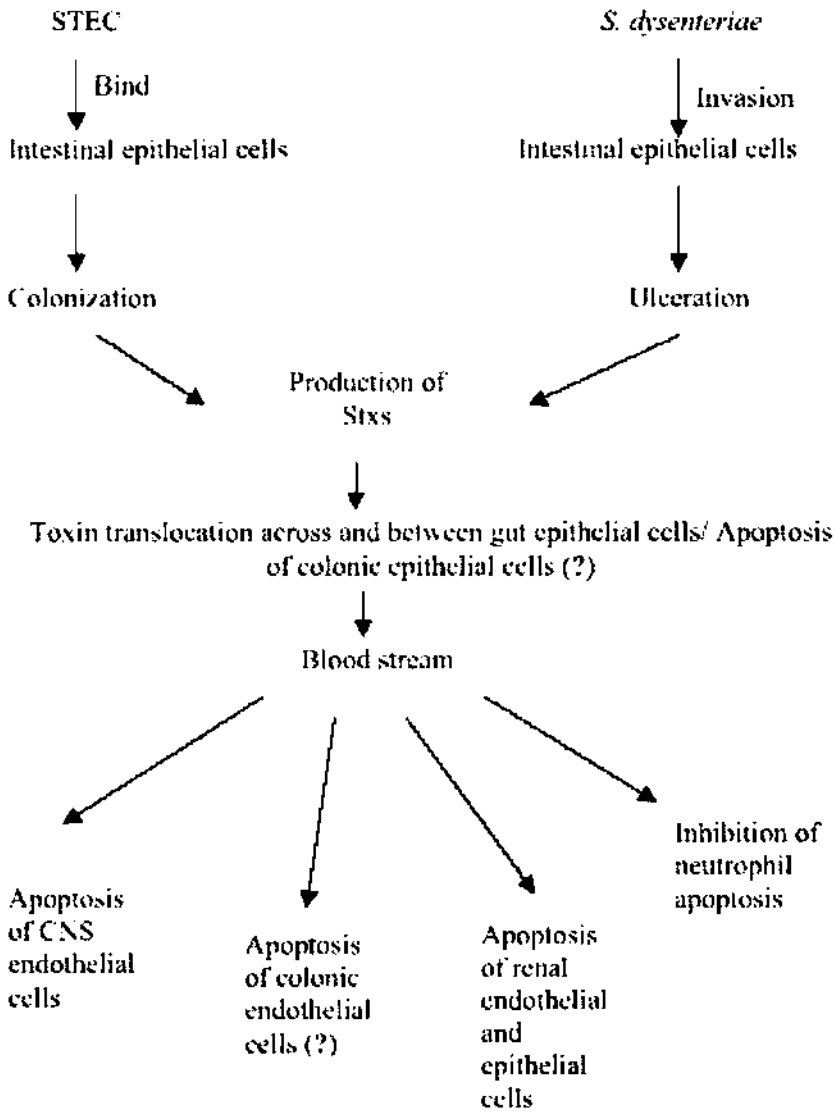


Fig. 1.1: Model of disease progression of bacillary dysentery/hemorrhagic colitis to extraintestinal complications.

Stx1 or Stx2 treatment of HEp-2cells resulted in the upregulated expression of the proapoptotic Bax protein, and overexpression of the Bcl-2 protein protected cells from Stx-induced apoptosis (Jones *et al.*, 2000). Nakagawa*et et al.*, 1999 transfected HeLa cells with plasmids expressing either Stx1 A- or B-subunits. Stx1 A-subunit-transfected cells died via necrosis as assessed by lactate dehydrogenase release and inactivation of reduced nicotinamide adenine dinucleotide (NADH)-generating dehydrogenases without DNA fragmentation. Stx1 B-subunit-transfected cells became apoptotic as evidenced by DNA fragmentation and caspase 1 and 3 activation. Fujii *et al.*, 2003 reported that Stx1 treatment of HeLa cells results in the activation of caspases 3, 6, 8, and 9, and pre-treatment of cells with caspase 3, 6, and 8 inhibitors, but not caspase 9 inhibitors, protected cells from apoptosis. Caspase 6 induces nuclear disassembly and chromatin condensation by activating lamin A.

Bid activation and cytochrome c release were demonstrated in Stx1-treated HeLa cells. However, Stx1 treatment also upregulated the expression of the caspase 9 inhibitor X-linked inhibitor of apoptosis protein (XIAP) which suggests that the intrinsic or mitochondrial pathway and caspase 9 activation may not play a major role in apoptosis induction in this cell type. In contrast to the studies of Nakagawa *et al.*, 1999 Stx1 B-subunits did not induce HeLa cell apoptosis, and retrograde transport of the holotoxin to the ER appeared to be essential for apoptosis induction (Fujii *et al.*, 2003). There are a number of reports indicating that Stxs induce apoptosis in endothelial cells isolated from various anatomical sites, but the mechanistic aspects of Stx-induced endothelial cell apoptosis are just emerging. Pijpers *et al.*,, 2001) showed that human foreskin microvascular endothelial cells (FMVEC) are sensitive to apoptosis induced by Stxs at concentrations as low as 10-100 fM. In nanomolar concentrations, purified Stx B-subunits also induced FMVEC apoptosis. A peptide aldehyde inhibitor of caspase 3 protected cells from apoptosis. In studies using the human dermal microvascular endothelial cell line HMEC- 1, Stx1 and Stx2 were shown to rapidly (within 4 h) induce apoptosis and inhibit the expression of the anti-apoptotic Bcl-2 family member Mcl-1 (Erwert, 2003). Mcl-1 is normally constitutively expressed by endothelial cells. The decrease in detectable Mcl-1 levels was toxin dose and time dependent. A pan-caspase inhibitor did not prevent the decrease in the Mcl-1 protein levels, suggesting that caspases do not contribute to this phenomenon. The proteasome inhibitor lactacystin prevented the

diminution of Mcl-1 levels and protected the cells from apoptosis. Using the same cells, another anti-apoptotic protein, FLICE-like inhibitory protein (FLIP), was also shown to be down-modulated by Stx1 (Erwert *et al.*, 2002). FLIP is rapidly degraded by proteasomes and needs to be continuously synthesized for cell survival. These studies suggest a crucial role for proteasomes in the regulation of apoptosis. In the face of inhibition of de novo protein synthesis mediated by Stxs, labile antiapoptotic factors are rapidly degraded, triggering the onset of apoptosis. FLIP is homologous to caspase 8, but is enzymatically inactive, thereby serving in a dominant negative fashion to prevent apoptosis via inhibition of caspase 8 activation (Irmler *et al.*, 2003). Thus, the role of FLIP in protecting against Stx-induced apoptosis is consistent with previous findings implicating a caspase 8-dependent mechanism for Stx-mediated apoptosis.

It has become increasingly clear that Stxs induce apoptosis in some, but not all, cell types. The capacity of the toxins to trigger programmed cell death pathways may contribute to the development of bloody diarrhea and extraintestinal complications. An overall model of apoptosis induction mechanisms by Stxs derived from recently described studies is shown in Fig. 2. Apoptosis induction is complex, and may involve extracellular and intracellular signaling pathways. Although Stx-mediated activation of the upstream caspase, caspase 8, has been reported, it is not clear whether caspase 8 is activated by the extrinsic pathway acting through engagement of membrane-associated receptors, or by intrinsic signaling proteins through the ribotoxic stress response. Receptor-ligand complexes directly interacting with caspase 8 following treatment of cells with toxins or purified B-subunits remain to be identified. If apoptosis induction requires retrograde transport, the precise points in the trafficking process initiating apoptotic signals are currently unknown. Studies using HeLa and HMEC-1 cells have suggested that toxin enzymatic activity is necessary to induce apoptosis through a mechanism completely independent of caspase activation. Such a mechanism may involve protein synthesis inhibition and proteasome-mediated degradation of anti-apoptotic proteins.

Finally, it should be noted that Stxs may not trigger a uniform apoptotic signaling cascade in all cell types. Thus, the differential signaling events caused by the toxins in different cell

types await further clarification. A better understanding of the mechanisms of apoptosis induction by Stxs may lead to the development of effective therapeutic strategies to interrupt the progression of disease from the bloody diarrheal phase to life-threatening complications.

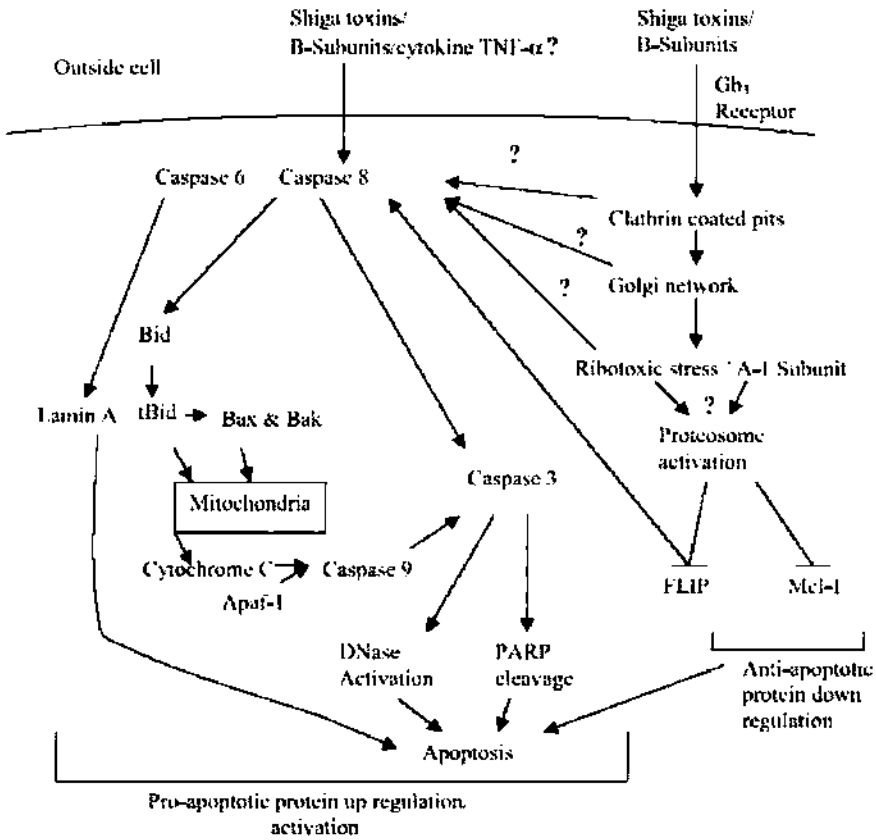


Fig. 1.2: Putative mechanisms of apoptosis triggered by Stxs. Question marks denote steps in programmed cell death pathways requiring further experimental

1.5.2 Mitochondria in Apoptosis

Increases in cytosolic Ca^{2+} levels due to activation of ion channel-linked receptors, such as that for the excitatory amino acid neurotransmitter glutamic acid, can induce permeability transition (PT) of the mitochondrial membrane. PT constitutes the first rate-limiting event of the common pathway of apoptosis. Upon PT, apoptogenic factors leak into the cytoplasm from the mitochondrial intermembrane space. Two such factors, cytochrome c and apoptosis inducing factor (AIF), begin a cascade of proteolytic activity that ultimately leads to nuclear

damage (DNA fragmentation, DNA mutations) and cell death.. Release of cytochrome c from mitochondria is a major event during apoptosis. Released cytochrome c has been shown to activate caspase-dependent apoptotic signals. Parallel to nuclear accumulation of cytochrome c, acetylated histone H2A, but not unmodified H2A, was released from the nucleus to the cytoplasm. Cytochrome c was also found to induce chromatin condensation. The nuclear accumulation of cytochrome c may be directly involved in the remodeling of chromatin.

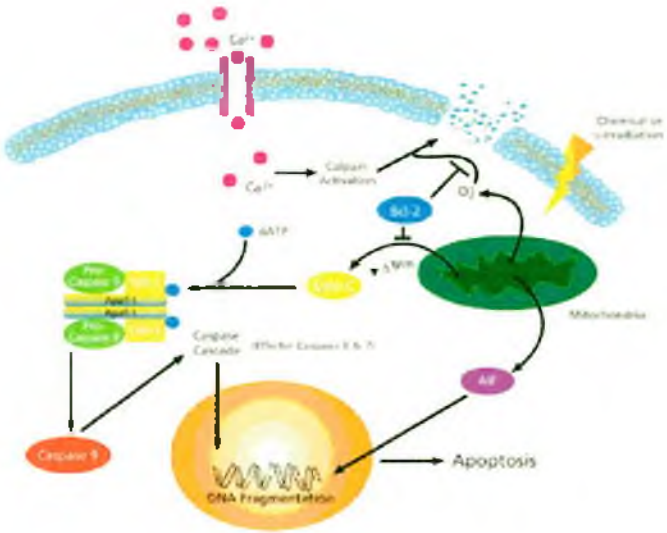


Fig. 1.3: Nuclear translocation of Cytochrome C during apoptosis.

1.5.3 DNA Damage and Apoptosis

DNA damage is a common event in life, as evident by the continuous requirement of DNA repair to maintain genome integrity. The machinery of apoptosis is also common, present in a latent form in most if not all cells of multicellular organisms. A connection between DNA damage and apoptosis is supported by a large body of literature.

What is very clear is that the tumor suppressor protein p53 provides an important link between DNA damage and apoptosis. p53-regulated genes that is relevant to apoptosis (Benchimol, 2001). Thus far, none of the p53-regulated and apoptosis-relevant genes, by

themselves, can account for p53-mediated apoptosis in mammalian cells (Benchimol, 2001). By contrast, the *Drosophila* p53 (Dmp53) has been connected to Reaper, which plays a central role in DNA damage-induced apoptosis in the fly (Nordstrom and Abram, 2000).

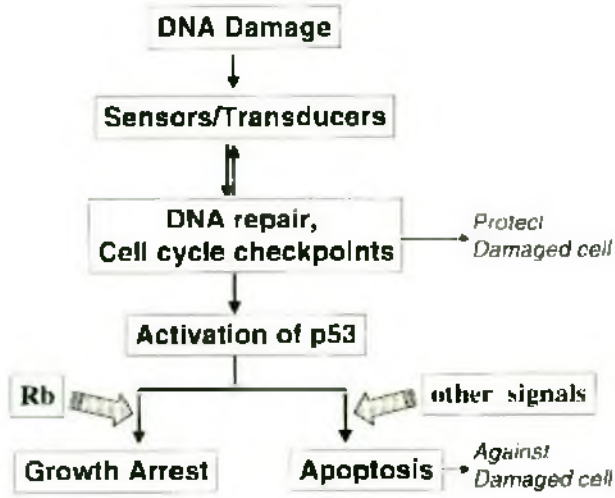


Fig. 1.4: Apoptosis is one of several integrated responses to DNA damage.

The activation of DNA repair and cell cycle checkpoints are conserved from unicellular to multicellular eukaryotes with the goal of protecting the damaged cell. The activation of p53 and related family members can either enforce the cell cycle arrest or induce apoptosis. The p53-dependent responses are directed against the damaged cell to protect the organism. The rules that govern the choice between growth arrest and apoptosis are likely to be enforced by other proteins that can antagonize or synergize with p53 to regulate apoptosis

The mechanisms that link DNA damage to the activation of p53 are also complex. The activation of p53 by ionizing radiation is better understood, and this is dependent on the ATM kinase (Khanna *et al.*, 2001). The ATM kinase functions as a transducer of DNA damage signal to p53 (Khanna *et al.*, 2001). Ionizing radiation can activate the ATM kinase, possibly because ATM can bind to broken ends of DNA (Khanna *et al.*, 2001). The ATM and its related ATR kinase can also be activated by other types of DNA lesions. Cisplatin adducts in DNA, for example, are recognized by the mismatch repair (MMR) proteins, which

appear to provide an essential link between platinated DNA and the ATM/ATR kinases (Bellacosa, 2001). The MMR proteins are important guardians of the genome, because they prevent the accumulation of spontaneous mutations generated during DNA replication (Bellacosa, 2001). Obviously, MMR proteins do not signal apoptosis during each round of DNA replication. However, MMR proteins do activate apoptosis in response to cisplatin and other alkylating agents (Bellacosa, 2001). Similarly, the ATM kinase can also stimulate DNA repair or apoptosis. In a majority of cells, ATM kinase offers important protection against ionizing radiation. However, with developing neurons, ATM kinase is required to activate apoptosis following ionizing radiation (Khanna *et al.*, 2001).

Exactly how MMR proteins and ATM-kinase distinguish between their repair functions *versus* their apoptosis signaling function is not understood. Because of their involvement in DNA repair, these proteins may 'sense' the efficiency or the status of DNA repair. Perhaps their function in signaling apoptosis is only switched on after they have 'sensed' failure in DNA repair. The notion that apoptosis is only activated after DNA repair fails is logically sound. However, there is no direct evidence supporting this hypothesis. Failure in DNA repair will cause cell death, even in yeast, which lacks the apoptosis program. Death from excessive DNA damage is passive, and should be experimentally distinguished from apoptosis, which is an active process.

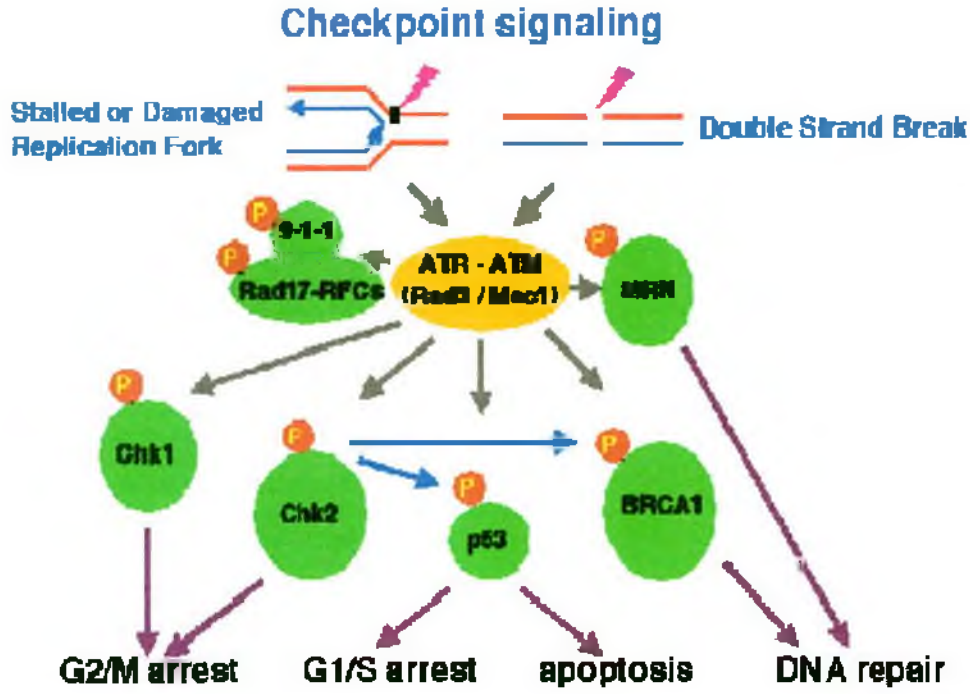


Fig. 1.5: DNA Damage Signaling, DNA Repair, Cell Cycle Arrest and Apoptosis

The rules that govern the connection between DNA damage and apoptosis should operate on the already identified platform of lesion-sensors (MMR), signal transducers (ATM), and transcription regulation (p53, p73). It should be noted that this platform does not solely regulate apoptosis. The primary response to DNA damage is the stimulation of DNA repair and the activation of cell cycle checkpoints (Figure 1.5). The biological goal of this primary response is to protect the damaged cell. Apoptosis is a secondary response to DNA damage, with the biological goal of protecting a multicellular organism against a damaged cell. The p53-dependent transcription response can also trigger another secondary response to protect the organism, and this is to enforce the growth arrest of a damaged cell. The rules that

govern DNA damage-induced apopotis, therefore, must be able to choose between p53-dependent growth arrests *versus* apoptosis (Figure 1.5).

The choice between p53-dependent arrest and apoptosis can be made by two distinct, albeit not mutually exclusive, mechanisms. The first mechanism is to regulate p53 itself. The p53 protein that activates the growth arrest genes, e.g., p21Cip1, might be differentially modified from the p53 protein that activates the apoptosis genes, e.g., Bax, Noxa and Puma. The second mechanism is to regulate other proteins that can constrain the function of p53. For example, the retinoblastoma tumor suppressor protein RB is required for DNA damage-induced growth arrest (Harrington et *al.*, 1998; Flatt *et al.*, 2000; Knudsen *et al.*, 2000). Thus, the functional status of RB might determine whether a damaged cell chooses to arrest or to commit suicide. The pro-apoptotic proteins that are upregulated by p53 are also subjected to functional modulation by post-transcriptional mechanisms, which may also constrain the apoptosis function of p53 (Korsmeyer *et al.*, 1999).

The conundrum that p53 can activate a large number of genes, but none of them seems to play a deterministic role in apoptosis, supports the idea that p53 alone cannot specify the fate of apoptosis upon DNA damage. In the coming years, we expect to learn more about parallel pathways that either antagonize or synergize with p53 to regulate apoptosis in DNA damaged cells. In a forward-looking review, El-Deiry discusses the interplay between p53- and TRAIL-regulated apoptosis pathways and their potential application to cancer therapy (El-Deiry, 2001). The lesson biologists have learned repeatedly in complex systems is combinatorial specificity. The time has come to look beyond p53 for answers in DNA damage-induced apoptosis.

1.6 Cell Cycle

1.6.1 G1 and S Phases of the Cell Cycle

In proliferating cells, the cell cycle consists of four phases. Gap 1 (G1) is the interval between mitosis and DNA replication that is characterized by cell growth. The transition that occurs at the restriction point (R) in G_1 commits the cell to the proliferative cycle. If the conditions that signal this transition are not present, the cell exits the cell cycle and enters G_0, a

nonproliferative phase during which growth, differentiation and apoptosis occur. Replication of DNA occurs during the synthesis (S) phase, which is followed by a second gap phase (G2) during which growth and preparation for cell division occurs. Mitosis and the production of two daughter cells occur in M phase. Passage through the four phases of the cell cycle is regulated by a family of cyclins that act as regulatory subunits for cyclin-dependent kinases (cdks). The activity of the various cyclin/cdk complexes that regulate the progression through G1-S-G2 phases of the cell cycle is controlled by the synthesis of the appropriate cyclins during a specific phase of the cell cycle. The cyclin/cdk complex is then activated by the sequential phosphorylation and dephosphorylation of the key residues of the complex, located principally on the cdk subunits. The cyclin cdk complex of early G1 is either cdk2, cdk4, or cdk6 bound to a cyclin D isoform. There are several proteins that can inhibit the cell cycle in G1. If DNA damage has occurred, p53 accumulates in the cell and induces the p21-mediated inhibition of cyclin D/cdk. Mdm2, by facilitating the nuclear export/inactivation of p53, becomes part of an inhibitory feedback loop that inactivates p21-mediated G1 arrest. Similarly, activation of TGF-b receptors induces the inhibition of cyclin D/cdk by p15, while cyclic AMP inhibits the cyclin D/cdk complex via p27. If the cyclin D/cdk complex is inhibited, retinoblastoma protein (Rb) is in a state of low phosphorylation and is tightly bound to the transcription factor E2F, inhibiting its activity. Passage through the restriction point and transition to S phase is triggered by the activation of the cyclin D/cdk complex, which phosphorylates Rb. Phosphorylated Rb dissociates from E2F, which is then free to initiate DNA replication. Cyclin E/cdk2 accumulates during late G phase and triggers the passage into S phase. The entire genome is replicated during S phase. The synthesis and accumulation of cyclin B/cdc2 also begins during S phase, but the complex is phosphorylated at Thr^{14} - Tyr^{15} and is inactive. Cyclin A/cdk2 accumulates during S phase and its activation triggers the transition to G2, a phase characterized by the accumulation of cyclin B/cdc2, the inhibition of DNA replication, cell growth and new protein synthesis.

1.6.2 G2 and M Phases of the Cell Cycle

The transition from G_2 phase to mitosis is triggered by the Cdc25-mediated activation (dephosphorylation) of the cyclin B/cdc2 complex (MPF). The activation of cyclin B/cdc2

that is necessary for G/M progression is currently the most well characterized step in the cell cycle. CyclinB/cdc2 is activated by phosphorylation of Thr^{160} and the dephosphorylation of Thr^{14} and Tyr^{15}. Thr^{160} is phosphorylated by cyclin activating kinase (CAK), following the activation of CAK by a cyclin activating kinase (CAKAK). However, the complex is kept in an inactive state due to the phosphorylation of Thr^{15}, which is catalyzed by the Wee1 kinase. Cyclin B/cdc2 activation is triggered when Cdc25, a phosphatase, dephosphorylates Thr^{15}. In turn, the activity of Cdc25 is regulated by both activating and inhibitory phosphorylations. Phosphorylation of Scr^{216} by Chk1 (a check point activated kinase that participates in the G_2-arrest of cells with damaged DNA) leads to the inactivation of Cdc25, while phosphorylation by an M-phase activated kinase creates a positive feedback loop leading to the rapid activation of the cyclin B/cdc2 complex. MPF catalyzes the phosphorylation of lamins and histone 1, and is involved in the regulation of events receding cell division, such as spindle formation, chromatin condensation, and fragmentation of the nuclear envelope and of organelles such as the Golgi and endoplasmic reticulum. The metaphase to anaphase transition is triggered by inactivation of MPF and the degradation of cyclin B. This induces the separation of chromatids and their movement to the poles of the mitotic spindle, after which the mitotic apparatus disappears, the nuclear membranes reform and the nucleoli reappear. During cytokinesis, the cytoplasm divides and the resulting daughter cells enter G_1.

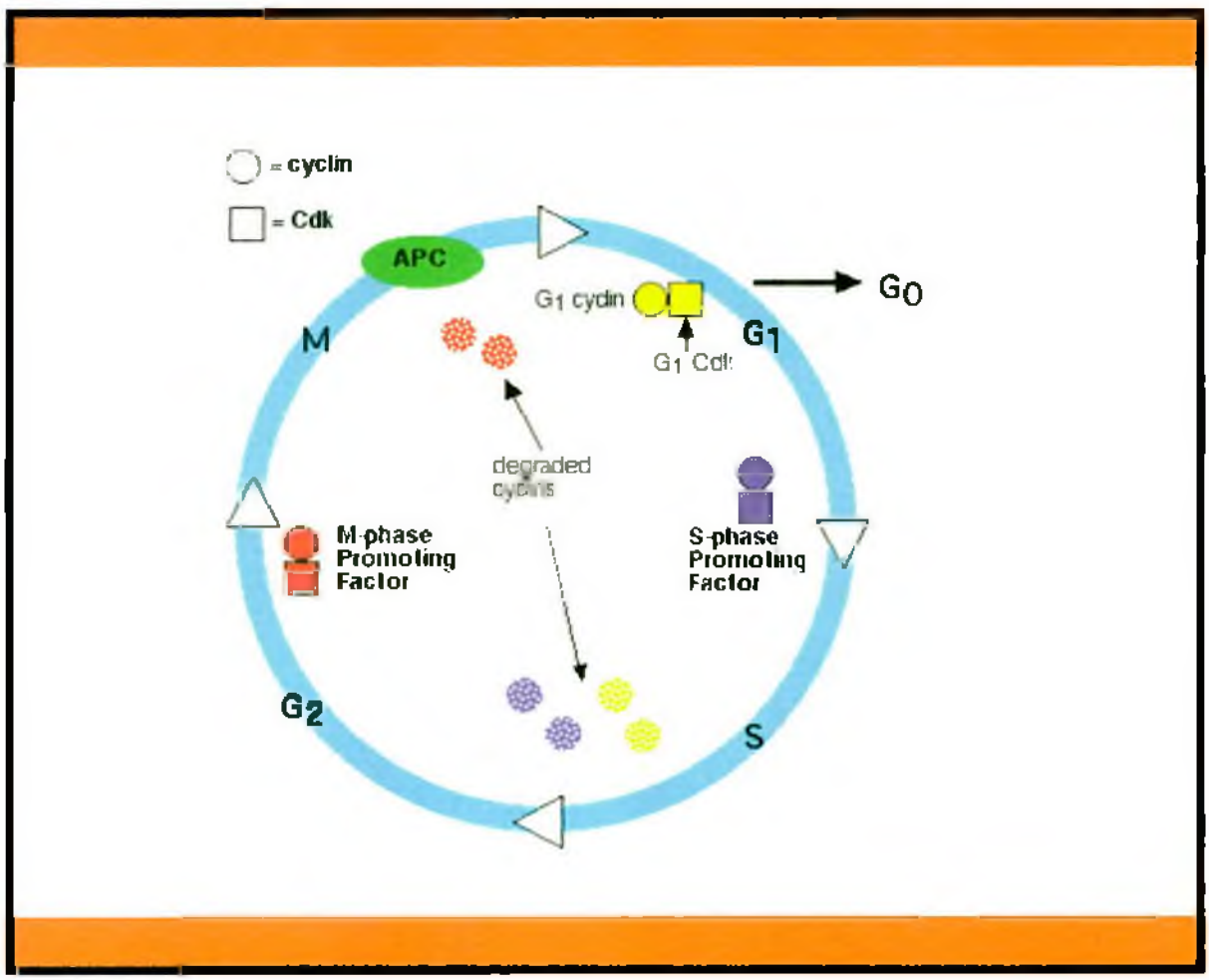


Fig. 1.6: Cell cycle progression through different phases.

1.6.3 Cell Cycle Arrest at Different Checkpoint

1.6.3.1 G1/ S Checkpoint

The G1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first "gap" phase (G1) into the DNA synthesis phase (S). Two cell cycle kinases, CDK4/6-cyclin D and CDK2-cyclin E, and the transcription complex that includes Rb and E2F are pivotal in controlling this checkpoint. During G1-phase, the Rb-HDAC repressor complex binds to the E2F-DP1 transcription factors, inhibiting downstream transcription. Phosphorylation of Rb by CDK4/6 and CDK2 dissociates the Rb-repressor complex, permitting transcription of S-phase-promoting genes including some that are required for DNA replication. Many different stimuli exert checkpoint control including TGFβ, DNA damage, contact inhibition, replicative senescence and growth factor withdrawal. The first four act by inducing members of the INK4 or Kip/Cip families of cyclin dependent kinase inhibitors (CKIs). TGFβ also inhibits the transcription of cdc25A, a phosphatase required for CDK activation. In response to DNA damage-induced activation of the ATM/ATR/Chk1/2 pathway, cdc25A is ubiquitinated and targeted for degradation via the SCF ubiquitin ligase complex. Targeted degradation of cdc25A in mitosis via the APC ubiquitin ligase complex allows progression through mitosis. Growth factor withdrawal activates GSK-3β, which phosphorylates cyclin D, leading to its rapid ubiquitination and proteosomal degradation. Ubiquitin/proteasome-dependent degradation and nuclear export are mechanisms commonly used to rapidly reduce the concentration of cell cycle control proteins. Some redundancy and tissue specific requirements exist as shown by animal models.

1.6.3.2 G2/M DNA Damage Check Point

The G2/M DNA damage checkpoint prevents the cell from entering mitosis (M-phase) if the genome is damaged. The cdc2-cyclin B complex is pivotal in regulating this transition. During G2-phase, cdc2 is maintained in an inactive state by the kinases Wee1 and Myt1. As cells approach M phase, the phosphatase cdc25 is activated by phosphorylation. Cdc25 then activates cdc2, establishing a feedback amplification loop that efficiently drives the cell into mitosis. DNA damage activates the DNA-PK/ATM/ATR kinases, initiating two parallel cascades that inactivate cdc2-cyclin B. The first cascade rapidly inhibits progression into mitosis: the Chk kinases phosphorylate and inactivate cdc25, which can no longer activate cdc2. The second cascade is slower. Phosphorylation of p53 dissociates it from MDM2,

activating its DNA binding activity. Acetylation by p300/PCAF further activates its transcriptional activity. The genes that are turned on by p53 constitute effectors of this second cascade. They include 14-3-3, which binds to the phosphorylated cdc2-cyclin B complex and exports it from the nucleus; GADD45, which apparently binds to and dissociates the cdc2-cyclin B complex; and p21/Cip1, an inhibitor of a subset of the cyclin-dependent kinases including cdc2 (CDK1).

1.6.4 DNA Damage and p53-mediated Cell Cycle Arrest:

Mammalian cells respond to DNA-damaging agents by activating cell cycle checkpoints. These control mechanisms determine a temporary arrest at a specific stage of the cell cycle to allow the cell to correct possible defects (Hartwell and Weinert, 1989; Hartwell and Kastan, 1994). At least two checkpoints monitor DNA damage: one at the G_1/S transition and the other at the G_2/M transition. The G_1 checkpoint prevents replication of damaged DNA, whereas the G_2/M transition is inhibited by damaged and/or incompletely replicated DNA (Hartwell and Weinert, 1989; Hartwell and Kastan, 1994). Several findings have demonstrated that the product of the *p53* tumor suppressor gene is responsible for the G_1 checkpoint (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992), In response to genotoxic stress, the levels of p53 protein increase and this increase determines a transient arrest of cell cycle progression in the G_1 phase (Kastan *et al.*, 1991, Kuerbitz *et al*, 1992), or triggers apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993). The arrest in G_1 is thought to give the cells time to repair critical damage before DNA replication occurs, thereby avoiding the propagation of genetic lesions to progeny cells. The cell cycle can resume once the damage has been repaired or, if the damage is too extensive, the cell will undergo apoptosis. In this scenario, in cells with no or mutated p53, DNA replication proceeds in the presence of a damaged template, thereby generating clones of genetically aberrant cells from which malignant clones may arise. Loss of the G_1 checkpoint also results in genomic instability, as seen by the increase in the frequency of gene amplification in p53-defective cells (Yin *et al.*, 1992; Livingstone *et al.*, 1992). Based on such findings it has been proposed, and it is now largely accepted, that the main function of normal p53 is to preserve genome integrity by acting as the "guardian of the genome" (Lane, 1992).

Recent observations suggest that p53 also plays a role in regulating the G_2/M transition (Agarwal *et al.*, 1995; Stewart *et al.*, 1995). However, it has also been documented that the G_2/M transition may be regulated independently from p53, since cells that are p53 nullizygous or with mutated p53 show a DNA damage-induced G_2 arrest (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992).

Wild-type p53 is a sequence-specific transcription factor that activates the transcription of downstream effector genes. Among these genes is $p21^{CIP1}$, the identification of which shed some light on the mechanisms by which p53 induction mediates cell cycle arrest. $p21^{CIP1}$ inhibits kinase activity of various cyclin/cyclin-dependent kinase complexes, which are key elements in cell cycle progression (Harper *et al.*, 1993; Xiong *et al.*, 1993; Gu *et al.*, 993), and by doing so it prevents the phosphorylation of the G_1 cyclin/cyclin-dependent kinase substrate pRB, thereby preventing the transition from the G_1 to the S phase of the cycle (Slebos *et al.*, 1994). Even though the role of p53 in mediating G_1 arrest has been widely accepted, some discrepancies have been reported in that wild-type p53 cells do not always display a G_1 arrest after radiation (Li C-Y *et al.*, 1995; Nagasawa *et al.*, 1995).

In contrast to the prediction based on the guardian of the genome role of wild-type p53, increased resistance to radiation has been reported in p53-defective cells (Lowe *et al.*, 1993; O'Connor *et al.*, 1993; McIlwrath *et al.*, 1994; Fan *et al.*, 1994). In contrast, other studies have shown no effect of p53 status on cell survival following radiation (Slichenmyer *et al.*, 1993).

The normally unstable p53 protein is stabilized by damage DNA, so its concentration increases. Acting as transcription factor, p53 induces expression of p21, a cyclin-kinase inhibitor that inhibits all Cdk-1, Cdk-2, Cdk-4 and Cdk6-cyclin complexes. Binding of p21 to these Cdk-cyclin complexes leads to cell cycle arrest in G1 and G2.

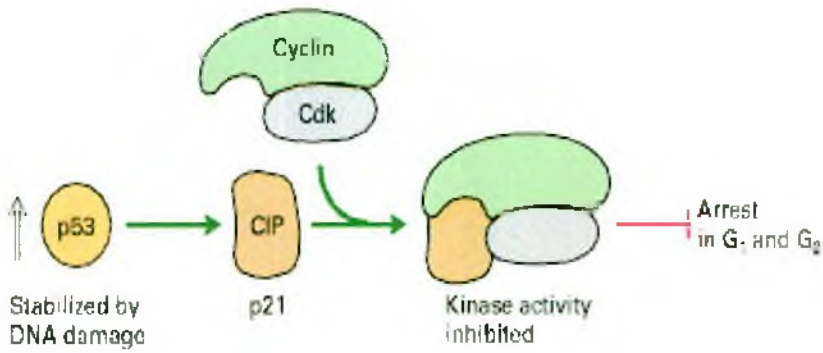


Fig. 1.7: p53 induced cell cycle arrest in response to DNA damage

1.6.5 p53 and Apoptosis

The p53 tumor suppressor limits cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stresses such as DNA damage, hypoxia, and oncogene activation. Many apoptosis-related genes that are transcriptionally regulated by p53 have been identified. These are candidates for implementing p53 effector functions. In response to oncogene activation, p53 mediates apoptosis through a linear pathway involving bax transactivation, Bax translocation from the cytosol to membranes, cytochrome c release from mitochondria, and caspase-9 activation, followed by the activation of caspase-3, -6, and -7. p53-mediated apoptosis can be blocked at multiple death checkpoints, by inhibiting p53 activity directly, by Bcl-2 family members regulating mitochondrial function, by E1B 19K blocking caspase-9 activation, and by caspase inhibitors. Understanding the mechanisms by which p53 induces apoptosis, and the reasons why cell death is bypassed in transformed cells,

is of fundamental importance in cancer research, and has great implications in the design of anticancer therapeutics.

1.6.6 p53 and Poly (ADP-ribose)polymerase (PARP) Cleavage and Apoptosis

PARP (poly ADP ribose polymerase) cleavage is downstream of ICE-like protease action in the induction of apoptosis. However, does PARP cleavage (as demonstrated by Western blot) necessarily imply apoptosis as the mechanism of cell death? Does a time course demonstration of the absence of PARP cleavage (spanning a period of time when cell death is morphologically observed) exclude apoptosis? Also, any tips on detecting PARP cleavage, especially using the new anti-PARP antibodies from Boehringer, would be greatly appreciated

Poly(ADP-ribose)polymerase (PARP) has long been known to play a role in the recognition of DNA damage and in DNA repair (Tong *et al.* 2001). One of the first events to take place after DNA strand breakage, caused either directly by genotoxic agents or indirectly by enzymatic incision of a DNA-base lesion, is the increased synthesis of poly(ADP-ribose) by PARP (Fig. 3). This is followed by poly(ADP-ribosyl)ation of proteins localized near the DNA strand breaks. The PARP protein detects DNA strand breaks and catalyzes the attachment of ADP-ribose units from NAD to itself and to other proteins (Lindahl *et al.*, 1995). The substrates of PARP can then influenze the architecture of chromatin or act in DNA metabolism.

PARP is known to be involved in the regulation of p53, although the relationship is not clear. There are studies showing that PARP upregulates p53. Cell lines derived from Chinese hamster V79 cells that are defective in poly(ADP-ribosyl) action have lower basal p53 levels and fail to activate p53 in response to etoposide (Whitacre *et al*,. 1995). Also, in lymphoblastoid cells with normal PARP activity, the PARP inhibitor 3-aminobenzamide (3AB, Purnell & Whish 1980) suppresses the accumulation of p53 after BPDE (Venkatachalam *et al.* 1997). On the other hand, contradictory results have also been published. X-ray-induced p53 accumulation was prolonged in mouse prostate cells treated with a PARP inhibitor 3AB (Lu & Lane 1993). Also, alkylating *N*-methyl-*N*-nitrosourea (MNU) caused increased accumulation of p53 in PARP-deficient splenocytes (Ménissier de

Murcia *et al.* 1997). It has been suggested that PARP plays a positive role in the activation and upregulation of p53 (Malanga *et al.*, 1998). Smulson *et al.*, 2000 have shown that, in human osteosarcoma cells, p53 is poly(ADP-ribosyl)ated by PARP. PARP has also been shown to activate DNA-dependent protein kinase (DNA-PK) activity *in vitro* and thus to regulate the activity of p53 by phosphorylation (Ruscetti *et al.* 1998). The PARP inhibitor 3AB also acts as an inhibitor of tumour promotion in mouse skin (Ludwig *et al.* 1990). On the other hand, it can act as a cocarcinogen for UV-induced carcinogenesis (Epstein & Cleaver 1992).

1.7 Signal Transduction of Apoptosis

Inducers of apoptosis are categorized into three groups (death factors, genotoxic anti-cancer drugs, and factor deprivation). The death receptor pathway or extrinsic pathway involves CD95 (also known as Apo-1 or Fas) and TNF-R (tumor necrosis factor) families of receptors. Mitochondria, on the other hand playcritical role in apoptosis by DNA damage induced manner. Fas ligand, a representative of death factors, binds to Fas receptor, and causes its trimerzation. The trimerized death domain in the Fas cytoplasmic region recruits pro-caspases 8 through a FADD/MORT1 adaptor, and forms a DISC. The pro-caspase 8 is auto-activated at DISC, and becomes a mature active enzyme. Two routes have been identified to activate caspase 3 by caspase 8. In one route caspase 8 directly processes procaspase 3 in the downstream, and caspase 3 cleaves various cellular proteins including ICAD. CAD is released from the ICAD, and degrades chromosomal DNA. In another route, caspase 8 cleaves Bid, a proapoptotic member of Bcl-2 family, which translocates to mitochondria to release cytochrome C into the cytosol. Bcl-2 or Bcl-xL, anti apoptotic members of the Bcl-2 family, inhibits the release of cytochrome C, the mechanism of which is not well understood. As soon as the cytochrome C is released it then activates caspase 9 together with Apaf-1, and caspase 9 in turn activates caspase 3. The signal seems to transfer into the mitochondria in a P53 dependent manner by as yet unidentified mechanism.

The Bcl-2 family of proteins regulate apoptosis by controlling mitochondrial permeability and the release of cytochrome c. The anti-apoptotic proteins Bcl-2 and Bcl-xL reside in the outer

mitochondrial wall and inhibit cytochrome c release. The proapoptotic Bcl-2 proteins Bad, Bid, Bax and Bim may reside in the cytosol but translocate to mitochondria following death signaling, where they promote the release of cytochrome c. Bad translocates to mitochondria and forms a pro-apoptotic complex with Bcl-xL. This translocation is inhibited by survival factors that induce the phosphorylation of Bad, leading to its cytosolic sequestration. Cytosolic Bid is cleaved by caspase-8 following signaling through Fas: its active fragment (tBid) translocates to mitochondria. Bax and Bim translocate to mitochondria in response to death stimuli, including survival factor withdrawal. p53, activated following DNA damage, induces the transcription of Bax, Noxa and PUMA. Upon release from mitochondria, cytochrome c binds Apaf1 and forms an activation complex with caspase-9. Although the mechanism(s) regulating mitochondrial permeability and the release of cytochrome c during apoptosis are not fully understood, Bcl-xL, Bcl-2 and Bax may influence the voltage-dependent anion channel (VDAC), which may play a role in regulating cytochrome c release.

1.7.1 ATM/p53 Signaling Pathway

The ataxia telangiectasia-mutated gene (ATM) encodes a protein kinase that acts as a tumor suppressor. ATM activation, via IR damage to DNA, stimulates DNA repair and blocks cell cycle progression. One mechanism through which this occurs is ATM dependent phosphorylation of p53. p53 can cause growth arrest of the cell at a checkpoint to allow for DNA damage repair or can cause the cell to undergo apoptosis if the damage cannot be repaired. The critical role of p53 is evident by the fact that it is mutated in over 50% of all human cancers.

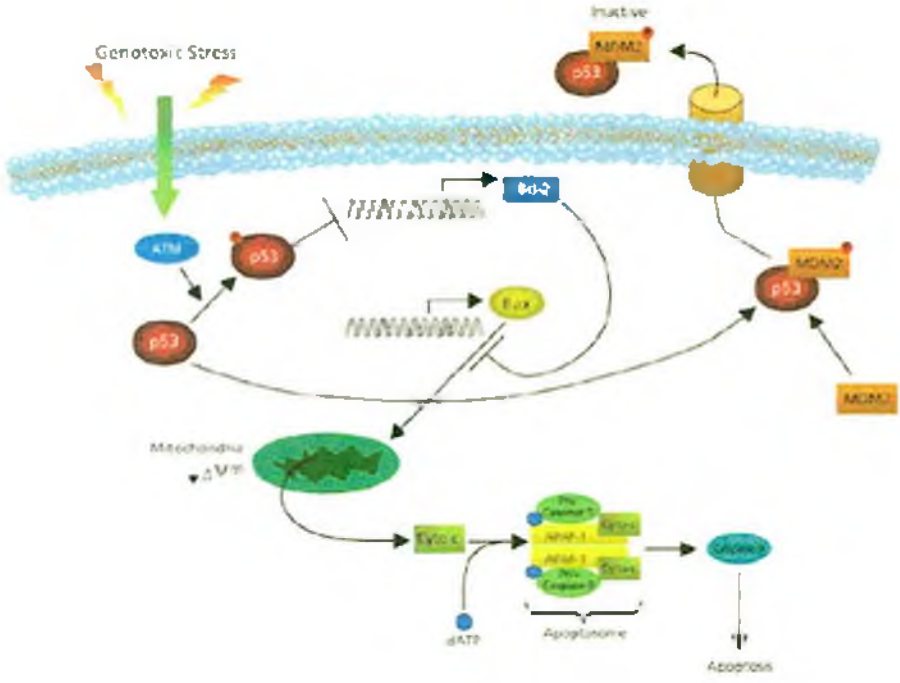


Fig. 1.8: ATM/p53-dependent DNA damage signaling and apoptosis

1.7.2 ATM Phosphorylates Histone H2AX in Response to DNA Double-strand Breaks.

A very early step in the response of mammalian cells to DNA double-strand breaks is the phosphorylation of histone H2AX at serine 139 at the sites of DNA damage. Although the phosphatidylinositol 3-kinases, DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangiectasia mutated), and ATR (ATM and Rad3-related), have all been implicated in H2AX phosphorylation, the specific kinase involved has not yet been identified. To definitively identify the specific kinase(s) that phosphorylates H2AX in vivo, we have utilized DNA-PKcs-/- and Atm-/- cell lines and mouse embryonic fibroblasts. We find that H2AX phosphorylation and nuclear focus formation are normal in DNA-PKcs-/- cells and severely compromised in Atm-/- cells. We also find that ATM can phosphorylate H2AX in vitro and that ectopic expression of ATM in Atm-/- fibroblasts restores H2AX phosphorylation in vivo.

The minimal H2AX phosphorylation in Atm-/- fibroblasts can be abolished by low concentrations of wortmamin suggesting that DNA-PK, rather than ATR, is responsible for low levels of H2AX phosphorylation in the absence of ATM. Our results clearly establish ATM as the major kinase involved in the phosphorylation of H2AX and suggest that ATM is one of the earliest kinases to be activated in the cellular response to double-strand breaks.

1.8 Advanced Molecular Technology for the Analysis of Apoptosis

1.8.1 Cellular Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular proliferation (cell growth).

The amount of yellow MTT reduced to purple formazan is measured spectrophotometrically by a spectrometer. This reduction takes place only when mitochondrial reductase enzymes are active, and thus conversion is directly related to the number of viable cells. The production of purple formazan in cells treated with an agent is measured relative to the production in control cells, and a dose-response curve can be generated.

1.8.2 Chromatin Condensation Assay

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters. Because of this property DAPI is a useful tool in various cytochemical investigations. When DAPI binds to DNA, its fluorescence is strongly enhanced, what has been interpreted in terms of a highly energetic and intercalative type of interaction, but there is also evidence that DAPI binds to the minor groove, stabilized by hydrogen bonds between DAPI and acceptor groups of AT, AU and IC base pairs.

1.8.3 Apoptotic DNA Fragmentation Assay

The detection of apoptosis in cultured cells relies heavily on techniques involving the extraction of nuclear DNA and characterization of such oligonucleosomal ladders by gel electrophoresis. Fragmented DNA remains in the supernatant and can be used directly for agarose gel electrophoresis. DNA fragments in the range of 20–300 kb, an early sign of cellular apoptosis (Cohen G.M. *et al.*,1992) that precedes the generation of low molecular weight oligonucleosomal fragments, are also retained in the supernatant following PEG precipitation. In addition, the percentage of DNA fragmentation in cultured cells detected by this assay exhibits a direct correlation with the percentage of apoptotic nuclei present in these cultures.

1.8.4 Flow Cytometry

Flow cytometry is a technology that has impacted both basic cell biology and clinical medicine in a very significant manner. The essential principle of a flow cytometry is that single particles suspended within a stream of liquid are interrogated individually in a very short time as they pass through a light source focused at a very small region. The optical signal generated are mostly spectral bands of light in the visible spectrum, which represent the detection of various chemical or biological components, mostly fluorescence. A key aspect of flow cytometers is that because they can analyze single particles/ cells into populations based upon a statistical difference.

1.8.4.1 Measuring Apoptotic Cells

Apoptosis consists of a cascade of events. Many of the changes during the progression of this cascade can be followed using flow cytometry (Ormerod and Ormerod, 2001). Only some of these changes are well suited to assays for the measurement of the number of apoptotic cells. Most assays either measure changes in the plasma membrane or are based on the extensive degradation of DNA in apoptotic cells. Certain proteins are cleaved during apoptosis and, recently, antibodies have been produced to the cleavage products. Immunofluorescent detection of these products could also form the basis of an assay for apoptotic cells. Methods utilizing changes in the plasma membrane, such as the annexin V assay, do not lend

themselves to a concomitant assay of the cell cycle. I have therefore restricted my comments to assays relying on DNA degradation and the products of protein cleavage. Most assays for apoptotic cells can, under some circumstances, give both false positive and false negative results. For example, two frequently used assays (estimation of a 'sub-G1' peak in the DNA histogram and the TUNEL assay, see below) rely on extensive degradation of DNA during apoptosis. However, some cultured cell lines do not show this phenomenon (Oberhammer *et al.*, 1993; Ormerod and Ormerod, 2001). Apoptotic cells were first recognized from the characteristic changes in their morphology, particularly changes in the nucleus. Before commencing any assay, the presence of apoptotic cells should be confirmed by observing the nuclear morphology; the simplest method being to use fluorescence microscopy. Cells can be stained by incubating them with either acridine orange or the *bis*-benzimidazole, Hoechst 33342, or by fixing in ethanol and staining with either PI or DAPI. (Darzynkiewicz *et al.*,1997) and Ormerod and Ormerod, 2001 have shown examples of the nuclear morphology of apoptotic cells revealed in this way.

At a late stage of apoptosis, cells in culture lose the integrity of their plasma membrane; sometimes called secondary necrosis. As the DNA degrades, the cells will lose DNA and eventually break up. This process results in the presence of fragments of cells and nuclei containing small amounts of DNA, which may interfere with assays for apoptotic cells. Darzynkiewicz *et al.*, 1994) and Ormerod, 2000a have described detailed methods for assaying apoptotic cells.

One of the features of apoptotic cells is extensive degradation of the DNA at the linkers between the nucleosomes. The result is a series of oligomers of DNA with a unit length of about 180 bp. If apoptotic cells,are fixed in ethanol, and then resuspended in a buffer, such as PBS, the smaller fragments of DNA are extracted so that the apoptotic cells have a lower DNA content compared to the normal cells. Normally, the cells are stained with PI; an argon-in laser producing blue light is used in the flow cytometer and red fluorescence from the PI/DNA is recorded (Ormerod *et al.*, 2000b). When the DNA histogram is measured, the apoptotic cells form a peak below the G1 peak (often referred to as the sub-G1 peak).

The amount of DNA extracted and, hence, the position of the sub-G1 peak in the DNA histogram depends on the buffer used (Gong *et al.*, 1994) and the cell type (Ormerod *et al.*, 2000a). Consequently, when working with a new cell line, preliminary work is needed to establish the best conditions for the assay. Apoptotic cells should be observed as a distinct peak. Necrotic cells, whose DNA is degraded randomly, will have a reduced DNA content and will be distributed across the same region of the histogram. Fragments of broken cells containing small amounts of DNA may also be present. For this reason, a linear amplifier should always be used to record the DNA histogram. There are examples in the literature of the use of a logarithmic amplifier. If the histograms are inspected, frequently, the so-called sub-G1 peak will be found to contain particles whose DNA content is a few percent or less of that of cells in G1. It is inappropriate to count particles containing such small amounts of DNA as apoptotic cells. The DNA histogram will show the cell cycle distribution of the nonapoptotic cells, but it is seldom possible to deduce the cell cycle phase from which apoptosis took place.

1.8.4.2 Measuring Cell Cycle Progression

The methods described above will give information about the cell cycle phases of the nonapoptotic and apoptotic cells. If apoptosis is induced within a few hours of the start of treatment, this information is all that is likely to be needed. However, if the time scale for the induction of apoptosis is more protracted, information about cell cycle progression before the onset of apoptosis may be required.

The DNA histogram yields the relative number of cells in G1/G0, S and G2/M phases of the cell cycle. Although some information about cell cycle progression can be deduced by following changes in the cell cycle phases with time, it gives basically static information. For example, although the percentage of cells in S phase can be estimated, the measurement does not tell you directly whether those cells are still moving through the S phase. The recording of a DNA histogram is straightforward and is easy to combine with an Immunofluorescent stain for a cell cycle or apoptosis related antigen (see above). It should always be carried out before

using a more sophisticated method. Methods for recording a DNA histogram have been discussed by (Ormerod *et al.*, 2000b).

1.8.5 Western Blotting

A widely used method for the detection of specific protein after electrophoresis. The sample is electrophoresed on a polyacrylamide gel then blotted to a membrane. The membrane is incubated with the antibody to the specific protein. Further, the secondary antibody labeled with an enzyme such as HRP (or ALP) is added and reacted. Chemiluminescence detection of the specific protein is done by applying chemiluminescent substrate of the enzyme.

1.9 Aims and Objectives

Shiga toxin (STX) belongs to the large family of ribosome-inactivating proteins (RIPs). There are two main subgroups of Shiga toxin: Shiga toxin 1 (STX1) and Shiga toxin 2 (STX 2). A large number of variants of STX 1 and STX2 have been reported (Gannon and Gyles, 1990; Lin *et al.*, 1993). Each molecule of shiga toxin contains one 'A' subunit and five 'B' subunits. In STXs, A subunit possesses the RNA-N-glycosidase activity and B subunits mediate binding of the toxin to the receptor on the target cells. It has been shown that 28S rRNA is one of the major targets for RIPs (O'Brien *et al.*,1992). Inhibition of protein synthesis by RIPs has been proposed to be responsible for induction of apoptosis in mammalian cells (Obrig *et al.*, 1987). However, cytotoxic effect due to inhibition of protein synthesis inhibitor has been controversial (Sandvig and van Deurs, 1992; Sakamoto *et al.*, 2003). Shiga toxins are potent cytotoxins and suggested to be associated with development of hemolytic uremic syndrome (HUS) and/or CNS complications (Proulx *et al.*, 2001). It is believed that STXs-induced apoptosis plays a critical role in acute renal failure, seizures, paralysis, coma, and death. However, molecular mechanism of STX-induced cell death remains to be understood. Therefore, present study will help better understand the molecular mechanism of STX-induced cell death. The major aims and objectives of this study are listed below:

1. To detect the toxin producing gene(s) in *Shigella* spp by PCR assay.
2. To investigate the enterotoxic activity of Shiga toxin1.
3. To investigate the cytotoxic activity of Shiga toxin1 in different mammalian cell line
4. To investigate the Shiga toxin1 induced apoptosis in HeLa Cell line.
5. To demonstrate the Shiga toxin1 induced cell cycle arrest in HeLa Cell line.
6. To demonstrate the Shiga toxin1 induced activation of DNA damage signaling pathway in HeLa Cell line.

Chapter 2

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Materials

Tissue culture cells used in the current study include HeLa, MEF, and Caco-2 cells. Antibodies against a synthetic peptide consisting of the phospho-Ser-139 of H2AX were purchased from Travigen Inc. Antibodies against histone 2A, cytochrome c, p53, ATM and actin were purchased from Santa Cruz Biotech, Inc. The caspase 3 inhibitor (Z-DEVD-FMK) was purchased from Calbiochem. Caco-2 cell line was obtained from Dr. P.J. Sinko (Rutgers University, New Jersey), HCT116 and HCT-116 $p53^{-/-}$ (obtained from Dr. Bert Vogelstein, Johns Hopkins Medical School, Baltimore, MD), ATM cell lines, YZ5 (ATM introduced) and FT169A (ATM deficient) were obtained from Dr L.F. Liu, Dept. of Pharmacology, RWJMS-UMDNJ. Shiga toxin 1 was purchased from List Biological laboratories, Inc (CA).

2.2 Bacterial Strains

A total of 15 clinical isolates of *S. dysenteriae* type 1 isolated from patients attending the Dhaka treatment center of ICDDR, B, Bangladesh and from abroad from 1984 to 2003 (Listed in table 2.1) were used in this study. The strains were isolated and characterized in the Clinical Microbiology Laboratory of ICDDR, B following standard microbiological and biochemical methods (World Health Organization, 1987). *E. coli* MC-1061 and VTEC-3 (a verotoxin producing *E. coli*) were used as shiga toxin genes (*stx* 1 and *stx* 2) negative and positive control strains respectively.

Table 2.1: *S. dysenteriae* 1 strains isolated from Bangladesh and different geographical region

Strain	Country/Year of Isolation	Reference
KO-220	Bangladesh, 1997	Enteric Microbiology Laboratory, ICDDR, B.
KO-234	Bombay, India, 1984	
K-573	Bangladesh, 2000	
BG-186	Bangladesh, 1984	Naheed *et al.*, 2004
BG-188	Bangladesh, 1984	
WB-234	India, 1984	
WB-253	India, 1984	
NP-33498	Nepal, 2002	
BG-15874	Bangladesh, 2003	
BG-15858	Bangladesh, 2003	
BG-836	Bangladesh, 2003	
WB-6	India, 2002	
WB-18	India, 2002	
NP-13500	Nepal, 2003	
NP-15464	Nepal, 2003	

2.3 Serological Typing

All the strains (n=15) were serologically confirmed by using commercially available antisera kit (Denka Saiken, Co. Ltd. Japan). Strains were subcultured on MacConkey agar (Difco, Becton Dickinson & Company Sparks, MD, USA) plates and after about 18 h of incubation, serological reactions was performed by the glass slide agglutination test as described previously by El-Gendy *et. al.*, 1999.

2.4 Detection of Enterotoxin Genes by PCR Assay

Materials:

i) 10X PCR buffer (GIBCO-BRL).

ii) 50 mM $MgCl_2$ (GIBCO-BRL).

iii) 2.5 mM dNTPs (GIBCO-BRL).

iv) Taq DNA polymerase (5U/µl, GIBCO-BRL).

v) Primers.

vi) Mineral oil (GIBCO-BRL).

vii) Filtered deionized water.

viii) Agarose.

ix) Ethidium bromide (10 mg/ml).

x) TBE (Tris-borate EDTA) buffer (GIBCO-BRL).

xi) 100bp DNA size standard (Bio-Rad).

Table 2.2: Primers used for detection of enterotoxin genes

Gene encoding virulence factor	Oligonucleotide sequence (5′ to 3′)	Size of amplified product (bp)	Reference
set 1A	TCACGCTACCATCAAAGA TATCCCCCTTTGGTGGTA	309	Fasano *et al.*, 1995
set 1B	GTGAACCTGCTGCCGAATTC ATTTGTGGATAAAAATGACG	147	Fasano *et al.*, 1995
sen	ATGTGCCTGCTATTATTTAT CATAATAATAAGCGGTCAGC	799	Nataro *et al.*, 1995
stx-1	ATCTCATGCGACTACTTGAC ACCCTGTAACGAAGTTTGCG	140	Bonnet *et al.*, 1998
stx-2	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATTACACAGGAGC	540	Bonnet *et al.*, 1998

Procedure:

The method, which was previously described by Vargas, *et al.*,1999 was followed to detect the enterotoxin genes. Representative strains were grown on MacConkey agar for overnight. A single colony of each isolate was suspended in 25 µl of reaction mixer containing 2.5 µl of 10x PCR, 1.5 µl of 50 mM $MgCl_2$, 2 µi of 2.5 mM dNTPs, 1 µl of

primer (forward and reverse) together with 1 unit of *Taq* DNA polymerase (5 U/μl). Volume of the reaction mixture was adjusted by adding filtered deionized water. PCR assays were performed in a DNA thermal cycler (model 480; perkin-Elmer Cetus, Emeryville, Calif.) with the following program: 30 cycles at 95o C for 50 s, 55o C for 1.5 min and 72o for 2 min with a final extension at 72o C for 10 min for *set1, sen* genes. 30 cycles at 94o C for 60 s, 55o C for 1.0 min and 72o C for 1.0 min with a final extension at 72o C for 10 min for *stx* genes. Amplification products were subjected to horizontal gel electrophoresis in 1% agarose gel in TBE (tris-borate EDTA) buffer at room temperature at 100 volt (50mA) for 1h. Briefly, 10 μl of amplified DNA for each sample was mixed with 1 μl of tracking dye and loaded into an individual well of the gel (5 mm thick). DNA bands were detected by staining the gel with ethidium bromide (0.5 μg/ml) for 30 minutes at room temperature and photographs were taken with the UV trans-illuminator. 100 bp DNA size standard (Bio-Rad) was used as marker to measure the molecular size of the amplified products.

2.5 Toxin Preparation and Concentration

Materials:

i. Trypticase soy broth (TSB)
ii. Sucrose
iii. PBS (P^H 7.2)
iv. Dialysis bags
v. 0.22 μm Millex filter (Millipore corp., USA)
vi. EDTA

Procedure:

Culture filtrates of all the strains were prepared following the method described elsewhere (Sanyal *et al.*, 1980) and concentrated by the method described by Nur-E-Kamal *et al.*, 1993. Briefly, the organisms were grown at 37° C for 4 hr in 5 ml TSB medium to develop inoculum. The primary inoculums was then added to 40 ml of sterile TSB medium and grown for overnight. Then the cells were pelleted by centrifugation (SORVALL RC-5B refrigerated centrifuge) at 12,000 g for 20 minutes at 4° C. After centrifugation the supernatant was collected in EDTA treated dialysis bag followed by concentration of the diluted supernatant as describes by (Nur-E-Kamal *et al.*, 1993). Briefly, the dialysis bag containing crude supernatant was placed in fresh and dried sugar at 4° C. After 1 hr of concentration, the proteins were collected out from the dialysis bag and filtered through 0.22 μm Millipore filter and stored at –20° C for further use.

2.6 BioRad Protein Assay:

Materials:

i. Bio-Rad dye reagent
ii. Bovine serum albumin (BSA)
iii. Whatman filter paper # 1
iv. Filtered distilled deionized water

Procedure

Dye reagent was diluted so that 1 part Dye Reagent was mixed with 4 parts distilled deionized water. The diluted dye reagent was then filtered through Whatman #1 filter (or

equivalent) to remove particulates. Five dilutions of a protein standard (BSA) was prepared for the preparation of standard curve. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml. 100 µl of each standard along with sample solution was pipetted into a clean, dry test tube. Protein solutions are assayed in duplicate. 5.0 ml of diluted dye reagent was added to each tube and was mixed vigorously by vortex. The mixture was incubated at room temperature for at least 5 minutes. Absorbance was measured at 595 nm. It should be noted that absorbance would increase over time so that samples should incubate at room temperature for no more than 1 hour.

2.7 Enterotoxicity Assay of STX1

Enterotoxicity assay of Shiga toxin were performed using animal model: rabbit ileal loop assay.

2.7.1 Rabbit Ileal Loop Assay

Reagents:

Pentobarbital Sodium (Sigma)
PBS (pH: 7.2)

Procedure:

Rabbit Ileal Loop Assay was performed according to the method described earlier (Singh and Sanayl, 1978). Briefly, adult albino Rabbits (New Zealand) of 9-10 weeks age of nearly 2 kg of body weight were starved for 48 hours allowing water *ad libutum.* After proper anesthesia with lower dose of Pentobarbital Sodium (0.5ml/kg body weight, intravenous), the intestine were exposed and loops of 5-7 cm in length with 3-5 cm intervals between each were tied beginning near the ileocaecal junction. One ml culture supernatant of each strain were inoculated in each loop and usually 6-7 loops were made in one rabbit. The animals were sacrificed after 18 hours with excess of Pentobarbital

Sodium. The length of each loop and the volume of fluid accumulated were measured to determine the amount of fluid accumulated per unit length of gut. Each test was done in two rabbits.

2.8 Cytotoxicity Analysis of STX1 in HeLa Cell Line

2.8.1 HeLa cell monolayer preparation from Stock

Materials:

i. Delbucco's Modified Eagles Medium (DMEM) ,
ii. Fetal Bovine Serum
iii. Penicillin, Streptomycin, Glutamine
iv. HeLa cell line culture medium (DMEM+ 10% FBS+ 1% (penicillin, streptomycin and glutamine)

Procedure:

HeLa cell line was prepared from stock vial stored previously at liquid N_2 (-80° C). The stock vial was thawed very rapidly at 37° C water bath. After thawing the cells were taken into a V-bottomed falcon tube and added with 1 ml of fresh medium (only DMEM). Cells were pelleted by centrifugation for 5 minute at 1500g. Supernatant was discarded and the cells were re-suspended in 3 ml of fresh medium by mixing well with sterile Pasteur pipette. The whole suspensions were then distributed into two 25 cm^2 flat bottomed flask containing 10 ml of cell line culture medium. The cells were then propagated in 25 cm^2 culture flask at 37° C in 5% CO_2 /95% air atmosphere.

2.8.2 HeLa Cell Maintenance

i. Cells were spitted at least three times before using them in experiments.

ii. Standardize propagation conditions was done..

iii. Cells were spitted as follows: Cells were counted after trypsinization and plated a measured number of cells rather than performing a 1:10, 1:20 etc.

iv. Cells were maintained in culture for no more than 20 consecutive passages.

v. New cells were grown up in a timely manner, i.e., at least three passages before the old ones reach the 20 passage limit. Old cells were maintained until it was shown that they behaved like the old cells.

2.8.3 HeLa Cell Counting

Materials:

i) 0.4% Trypan Blue solution

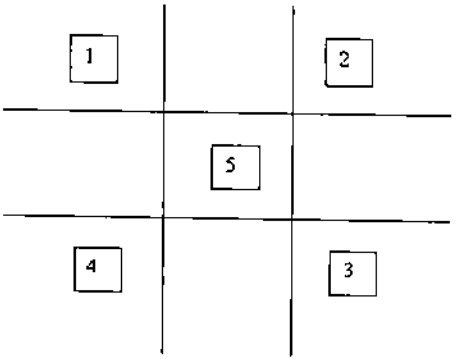
ii) Hemocytometer

Procedure:

Cell suspension were taken and diluted 1 : 2 (or higher, as needed to obtain a minimum count of 150 cells / 5 squares) into 0.4% Trypan Blue solution. Twelve µl were applied to one side of a hemocytometer. Hemocytometer holds 10 µl on each side. Cells were counted in five squares. Number of cells per milliliter and total number of cells were calculated.

Cell count x 1000 (factor converting µl to ml) x 2 (Trypan blue dilution) x 2 (two halves of hemocytometer) = cell count / ml (x total ml = total # of cells)

Hemocytometer Cells Counting:



2.8.4 Toxin Treatment and Incubation

HeLa Cell Monolayer were obtained by seeding 10^4-10^5 cells in 96 well tissue culture plates with ensuring similar distribution of cells at each well containing 200 µl of DMEM cell line culture medium. After 48 hrs of incubation Filtered Culture Supernatant of each strain were treated to the Cell Monolayer at different dilution (neat, 1:4, 1:10, and 1:40). The assay plates were incubated with toxins for overnight at 37° C in 5% CO_2 /95% air atmosphere.

2.8.5 Cytotoxicity Analysis with Dose in HeLa Cell Line

The cytotoxic activity of Shiga toxin 1 in HeLa cell line was determined by a previously described methods of Blanco *et al.*, 1990. Briefly, HeLa cell monolayer were obtained by seeding 10^4-10^5 cells in 96 well tissue culture plates with ensuring similar distribution of cells at each well. After 48 hrs of incubation Filtered Culture Supernatant of each strain were treated to the Cell Monolayer at different dilution (neat, 1:4, 1:10, and 1:40) and incubated for overnight at 37° C in 5% CO_2 /95% air atmosphere and the cytotoxic effects were observed under inverted microscope for each dilution.

2.9 Cytotoxicity Analysis of STX1 in Different Mammalian Cell Line

2.9.1 HeLa, MEF and Caco-2 Cells culture and Shiga Toxin 1 Treatment

Materials:

v. Delbucco's Modified Eagles Medium (DMEM)
vi. Fetal Bovine Serum
vii. Penicillin, Streptomycin, Glutamine
viii. HeLa cell line culture medium (DMEM+ 10% FBS+ 1% antibiotic and glutamine)

Procedure:

HeLa, MEF and Caco-2 Cells were cultured in DMEM containing FBS in six wells (corning) plate in an amount so that each of the well contains $3x10^6$ cells. Measured amount of cells were plated in each well. Equal distribution of cells through out the well was confirmed by gentle shake clockwise and anti-clockwise. The plates were grown into CO_2 injected special incubator for 24-48 hr. After semiconfluence growth of monolayer, the cells were then treated with STX1 to a final concentration of 5, 25, 100ng/ml and cultured for 24 hours. Cell viability was evaluated using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann T, 1983).

2.9.2 Cell Viability Assay (MTT Assay)

Materials:

i. MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
ii. DMEM
iii. DMSO (dimethyl sulfoxide)

Procedure:

Cell viability was evaluated using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described previously by Mosmann, 1983. Briefly, HeLa, MEF, and Caco-2 cells were cultured in DMEM containing 10% Fetal bovine serum to semiconfluency. STX1 was added to a final concentration of 5, 25 or 100 ng/ml. Cells were cultured for 24 hours. Cell viability was evaluated using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Briefly, a sterile solution of 0.1 mg/mL MTT in DMEM was added to each well in a multi-well plate, and incubated at 37°C for 4 hrs. . The MTT color complex was then solubilized in 100µl dimethyl sulfoxide (DMSO) to each well, the solution transferred to a 96-well plate and transmission evaluated at 570 nm by microplate reader. Data are expressed as percent of control (no oligo and no Cellfectin).

2.10 Chromatin Condensation Assay (DAPI Staining)

Materials:

i. DAPI (4'-6-Diamidino-2-phenylindole)
ii. 4% Para-formaldehyde
iii. PBS (P^h 7.2)
iv. PBS + 2% FBS
v. Triton-X 100

Procedure:

HeLa cells were stained with DAPI fluorescent dye by the methods described by (tayeb K *et. al*; 1998). Briefly, HeLa cells were grown in six wells (corning) plate in an amount so that each of the well contains $3x10^6$ cells. The plates were grown into CO_2 injected special incubator for 24-48 hr. After confluence growth of monolayer, STX1 was added to a final

concentration of 5, 25 or 100 ng/ml. After application the plate was gently shaken for the equal distribution of toxin so that the toxin equally affects all the cells. Cells were cultured for 24 hours. The cells were harvested by trypsinization with the addition of 400 µl of Trypsin-EDTA followed by incubation in 37° C temperature for 1-2 min. The harvested cells were washed twice with PBS (7.2). The cell wall porosity was increased to facilitate DAPI staining by treating with 0.1% Triton-X 100 in PBS followed by incubation in ice for 10 min. The treated cells were then centrifuged and re-suspended (5000 cells/µl) in 1ml of PBS buffered 4% Para-formaldehyde containing 10µg/ml DAPI with an incubation period of 30 minutes in dark room. The stained cells were observed under florescent microscope at the 350 nm of wavelength. The magnification was 100X. The images were captured by computational support added with the microscope and average count per field was done.

2.11 DNA Fragmentation Assay by Agarose Gel Electrophoresis

Materials:

i. Tris-HCl
ii. EDTA
iii. Triton-X100
iv. Phenol: Chloroform: Isoamylalcohol
v. 3M NaCl
vi. Ethanol
vii. TE buffer
viii. RNase A stock solution (10 mg/ml)

Procedure:

DNA Fragmentation Assay by Agarose Gel Electrophoresis was determined by the method described earlier by Tayeb and William, 1999. Briefly, HeLa cells were grown in six wells (corning) plate in an amount so that each of the well contains $3x10^6$ cells. The plates were grown into CO_2 injected special incubator for 24-48 hr. After confluence growth of monolayer, STX1 was added to a final concentration of 25 or 100 ng/ml. The media containing toxin was removed by using Pasteur pipette with negative pressure produced by suction pump. 4 ml of PBS (P^H-7.2) was added to each well and shaken gently. After that the PBS was also removed by Pasteur pipette. PBS (P^H-7.2) wash was repeated for one more time. When the washing was completed the wells was added with 400 µl of lysis buffer followed by 30 minutes incubation on ice. Lysed cells were then collected in 1.5 ml eppendorf tube and the cellular debris were separated from cellular cytoplasmic contents by centrifuging at 10000 g for 10 min. The DNA fragments were then extracted from the supernatant with phenol: chloroform: isoamylalcohol (25:24:1). Same volume of phenol: chloroform: isoamylalcohol was added to the supernatant collected from the previous step. Then the solution was mixed vigorously by inverting the eppendorf tubes repeatedly for 30 minutes. As soon as the milky white color of the solution develops the mixture was centrifuged at 13000g for 10 minutes. After centrifugation the mixture was separated into 3 distinct layers, the upper layer containing desired fragmented DNA, the middle containing protein complex and the bottom layer containing phenol. Thus the upper layer was taken very carefully by using cut-tips. DNA was precipitated by adding 1/10 volume of 5M NaCl and 2 volume of absolute ethanol. Following addition of both the salt and alcohol the mixture was incubated overnight at -20^0C. In the next day the DNA was pelleted by centrifugation at 10000g for 20 min. The pelleted DNA was then rinsed with 70 % ethanol followed by resuspending in TE (Tris- EDTA) buffer containing 100 µg/ml RNase A. After 2 h of incubation at 37 °C, DNA samples were run in 1.5% agarose gel by simple gel elcctrophoresis as above described method. After 2 hr run the gel was then stained with .02% ethidium bromide, and visualized under UV light.

2.12 Flow Cytometry

Reagents:

i. Cell suspension buffer: PBS (7.2) containing 2% FBS
ii. DNA Extraction buffer: 192 ml 0.2 M Na_2HPO_4, 8 ml 0.1 M citric acid: P^H-7.8
iii. Propidium iodide Staining Solution: 3.8 mM Na-citrate, 50 µg/ ml PI (Sigma) in PBS
iv. RNase A Stock Solution: 10 mg/ml RNase A (Sigma)

Procedure:

Flow cytometric analysis for cell cycle distribution was performed accordingly to the suppliers protocol (Becton Dickinson, CA, USA).with ModFit LT™ Software (Becton Dickinson, CA, USA). using FACScaliber (Becton Dickinson, CA, USA). Briefly, HeLa cells were grown in six wells (corning) plate in an amount so that each of the well contains $3x10^6$ cells. The plates were grown into CO_2 injected special incubator for 24-48 hr. After confluence growth of monolayer, the cells were then treated with culture supernatant of different each strain. For each well 400 µl of culture supernatant was applied. After application the plate was gently shaken for the equal distribution of toxin so that the toxin equally affects all the cells. Cells were cultured for 2, 4, 6, 8 and 24 hours. In each cases the cells were harvested by trypsinization. Briefly, cells in each well were treated with 400 µl of Trypsin- EDTA followed by incubation in 37^0 C for 1-2 minutes. As soon as the incubation is completed Trypsin is immediately neutralize by adding 500 µl of cell suspension buffer PBS containing 2% FBS to each well. The trypsinized cells were then collected into the v-bottomed falcon tube followed by 2 times washing with PBS. Therefore, the 1-2 X 10^6 cells were re-suspended in 1 ml PBS. After harvesting 1-2 X 10^6 cells were fixed with ice-cold absolute ethanol. Briefly, 3 ml of absolute ethanol was added

drop wise to 1 ml of cell suspension with vigorous agitation so that the cells were not allowed to form clump. Then the cells were incubated for 1 hr at 4° C. After incubation the fixed cells were pelleted at little higher speed (2000g for 5min) as the cells became flocculent. Fixed cells were washed twice with PBS followed by re-suspending in 500 µl of PBS. 0.2-1.0 ml of DNA extraction buffer was added to the cell suspension followed by incubation in room temperature for 5 min. After centrifugation the DNA was stained with 1 ml of Propidium iodide (Sigma) staining solution. The DNA materials were then added with 50 µl RNase A. Finally the stained DNA materials were kept in 4° C until analyzed by Flow cytometer (FACSciliber).

2.12.1 Cell Acquisition by FACScaliber

DNA materials were analyzed by FACScaliber with the help of Cell Quest software (Becton Dickinson) by taking FL2-A in the Y axis and FL2-W in the X axis.

2.12.2 Cell Distribution Analysis by ModFit LT™ Software

Percentage of cell population in different phase was analyzed by ModFitLT software. The synchronization assay was done to check whether the cells are in G_0/G_1, S or G_2M phase.

2.13 Subcellular Fractionation and Western Blotting

2.13.1 Subcellular Fractionation

Materials:

i. 2 x Lammelli SDS sample buffer
ii. hsp60 (a mitochondrial protein)
iii. Topoisomerase I (a nuclear protein)

Procedure:

HeLa Cells were grown in six-well plate in an amount so that each of the well contains $3x10^6$ cells. Measured amount of cells were plated in each well. Equal distribution of cells through out the well was confirmed by gentle shake clockwise and anti-clockwise. The plates were grown into CO_2 injected special incubator for 24-48 hr. After confluence growth of monolayer, the cells were then treated with STX1 (25 ng/ml) or BSA (25 ng/ml). After 6 hours of treatment, the cells were collected and processed. (a) A Nuclear fraction was prepared as described by (Nur-E-Kamal *et al.*, 2004). Briefly, proteins in nuclear fraction were dissolved in SDS Lammelli sample buffer and separated by SDS-PAGE (12%). (b) Cells were lysed in SDS Lammelli sample buffer to prepare total cellular protein extract. Proteins in total cellular extract were separated by SDS-PAGE (12%). The cytoplasmic fraction was separated from mitochondrial and nuclear fraction by centrifugation at 12,000 X g for 18 min at 4^0C. Equal volume of 2x Laemmli SDS sample buffer was added to nuclear, mitochondrial, and cytoplasmic fractions. Fractionation of the mitochondrial and nuclear proteins was confirmed by probing the membrane for hsp60 (a mitochondrial protein) or topoisomerase I (a nuclear protein) using their specific antibodies.

2.13.2 Resolving Proteins by SDS-PAGE

Materials:

i. Separating Gel: 12.5%
ii. Stacking Gel: 5%
iii. Running Buffer ph 8.3
iv. 10% SDS
v. 30% Acryl amide and Bis Acryl amide
vi. 10% APS
vii. 0.1% BMB (Tracking dye)
viii. Commassie Stain
ix. TEMED
x. Sample Loading Buffer

Procedure:

Prepare polyacrylamide gel according to standard protocol. Load samples and run gel at 25 mA (2 gels run at 50 mA) in 1x SDS Running Buffer. [At this point, the gel can either be transferred to a membrane (see Western protocol) or stained with Coomassie (see below)]. Place gel in a plastic container. Cover with isopropanol fixing solution and shake at room temperature. For 0.75 mm-thick gels, shake 10 to 15 min; for 1.5 mm thick gels, shake 30 to 60 min. Pour off fixing solution. Cover with Coomassie blue staining solution and shake at RT for 2 hr. Pour off staining solution. Wash gel with 10% acetic acid to destain, shaking at RT ON

2.13.3 Transfer to Nitrocellulose Membrane

Materials:

i. 50 mM sodium phosphate buffer (pH 7.5)
ii. Whatman no. 1 filter paper
iii. Scotch-Brite pad and
iv. cathodc grid

Procedure:

Proteins resolved by SDSPAGE were electrophorctically transferred to an NCM by using a modification of the procedure (Towbin et al.) for SDS-containing gels. A sandwich was constructed consisting sequentially of anode grid, Scotch-Brite pad, sheet of Whatman no. 1 filter paper, NCM, polyacrylamide slab gel, sheet of Whatman no. 1 filter paper, Scotch-Brite pad, and cathode grid. The sandwich was placed in an electrophoric destainer (E-C Apparatus Corp., St. Petersburg, Fla.) filled with 50 mM sodium phosphate buffer (pH 7.5) (2) that had been deaerated under reduced pressure for 12 h. Electrophoresis was at 6 V/cm for 2 hr at ambient room temperature.

2.13.4 Western Blotting:

Materials:

Transfer Buffer
1xSDS Running Buffer in 20% Methanol
1x PBS/0.1% Tween 20
Blotting Buffer, stored at 4^0C
5% milk in 1x PBS/0.1% Tween 20

Procedure:

Western blotting was performed accordingly to the ECL protocol provided by the suppliers (Amersham Biosciences) using specific antibodies. Briefly, Protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% (W/V) non-fat dry milk in Tris-buffered saline containing 0.05%(W/V) Tween 20. After O/N blocking the blot was washed 3 times with distilled water for 10 minutes. Ten ml of the primary antibody solution (anti-Cytochrome C, anti-p53, anti-phospho-ATM, anti-$H2AX^P$ and Anti-PARP) was then added to the membrane and kept in a 4^0C Shaker for O/N. After O/N incubation the blot was washed 3 times (10min/wash) with 1X TBST. Then 10 ml of secondary antibody solution was added to each blot and incubated at RT on a shaker. After that the blot was washed again for 3 times with 1X TBST (10min/wash). Proteins were visualized using the ECL system (Amersham).

Chapter 3

RESULTS

3.0 Results

3.1 Selection of Bacterial Strain by PCR Assay

All the 15 *S. dysenteriae* 1 strains were analyzed for the presence of toxin genes such as *set*, *sen*, *stx*-1 and *stx*-2 by PCR. All *S. dysenteriae* 1 contained only *stx* 1 gene. Of 15, only two *S. dysenteriae* 1 strains were negative for *sen* gene, since these strains did not harbor the 140 MDa plasmid. Both *set*1A and *set*1B genes were absent in all *S. dysenteriae* 1 strain. Therefore, purified STX1 and *S. dysenteriae* 1 strains bearing only STX1 gene (negative for *set* and *sen* genes) were used as a source of STX1 in this study. VTEC-3 (Verotoxin producing *E. coli*) was used as a positive (*stx*-1 and *stx*-2 gene) control and *E coli* MC-1061 as negative control.

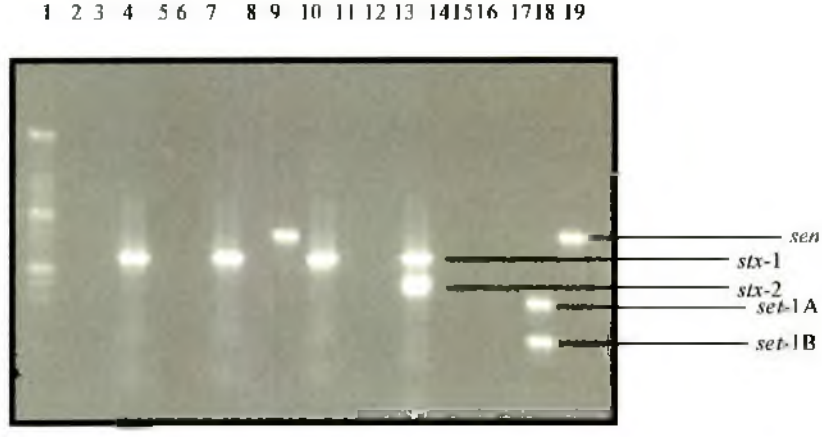


Figure 3.1: Detection of *set (set1A & set1B), sen, and stx (stx1 & stx2) gene* by PCR. Lane1: Marker 100bp ladder, Lane 2,3,4: KO-220 (140 less *S. dysenteriae* 1), Lane: 5,6,7: KO-234 (140 less *S. dysenteriae* 1), Lane 8,9,10: K-573 (140 containing *S. dysenteriae* 1), Lane-11,12,13: VTEC-3, Lane14,15,16: MC 1061, Lane: 17,18,19: YSH-6000 *S flexneri* 2a.

3.2 Estimation of Protein Concentration in Culture Supernatant by Bio-Rad Protein Assay

Culture supernatants were prepared as the source of Shiga toxin 1 from *S. dysenteriae* 1. The BIO-RAD protein assay revealed that the total protein concentration was increased remarkably during concentration procedure. Comparison to a standard curve provides a relative measurement of protein concentration. The total protein concentration of the culture supernatants was raised from 0.251± 0.04 mg/ml to 0.563± 0.06 mg/ml after concentration process.

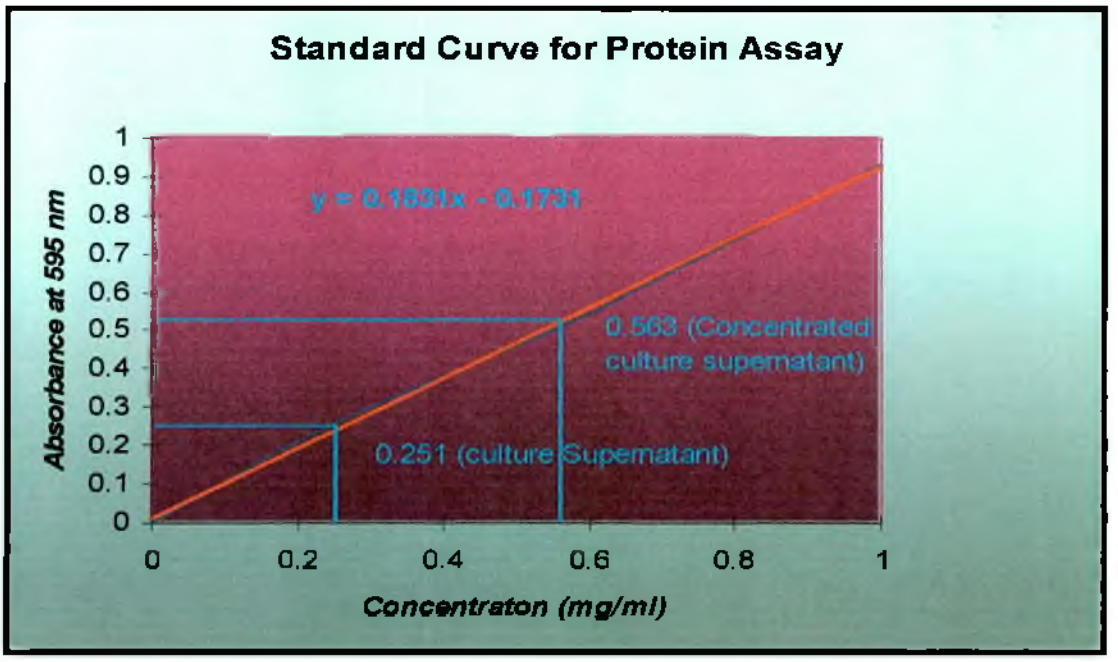


Figure 3.2: Standard Curve for BIO-RAD Protein Assay.

3.3 Shiga Toxin 1 Induced Fluid Accumulation in Rabbit Ileal Loop

Culture supernatant was studied for their ability to induce secretion of fluids in rabbit ileal loops. It was found that *S. dysenteriae* 1 and VTEC-3 (a verotoxin producing *E coli*) induces fluid accumulation in rabbit ileal loops (Table 3.1). By PCR it was found that *S. dysenteriae* 1 contains *stx*1 gene and VTEC-3 contains both *stx*1 and *stx*2 gene (Fig. 3.1). Culture supernatant from control strain, MC-1061 (absence of toxin genes) did not induce fluid accumulation in rabbit ileal loop (Table 3.1).

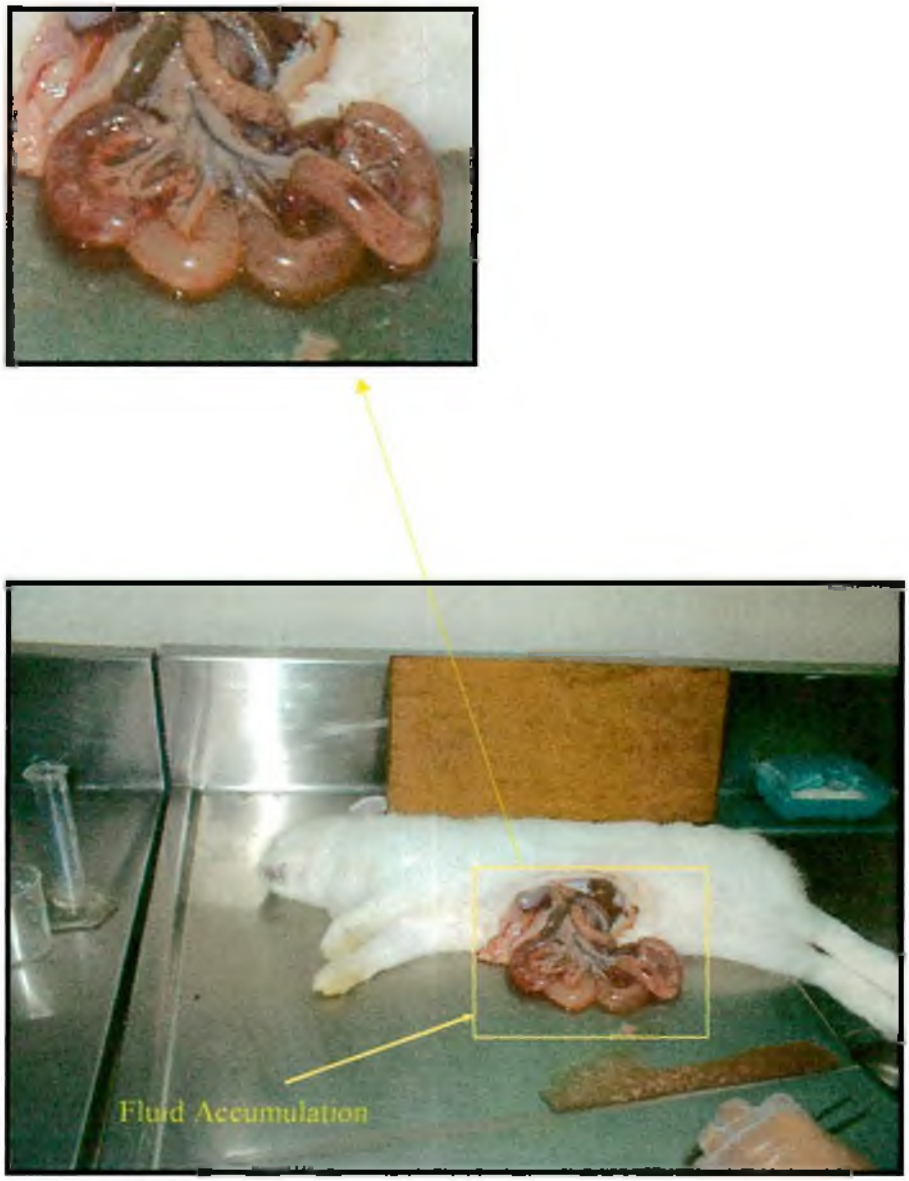


Figure 3.3: Photograph of STX1 induced fluid accumulation in Rabbit Ileal Loop

Table 3.1: Shiga Toxin 1 induced fluid accumulation in Rabbit Ileal Loops

Strains	Fluid accumulation in Rabbit Ileal Loop
*S. dysenteriae*1 (STX1)	0.70-0.78 ml/cm
VTEC-3 (positive control)	0.98-1.5 ml/cm
MC-1061 (negative control)	0.02 ml/cm

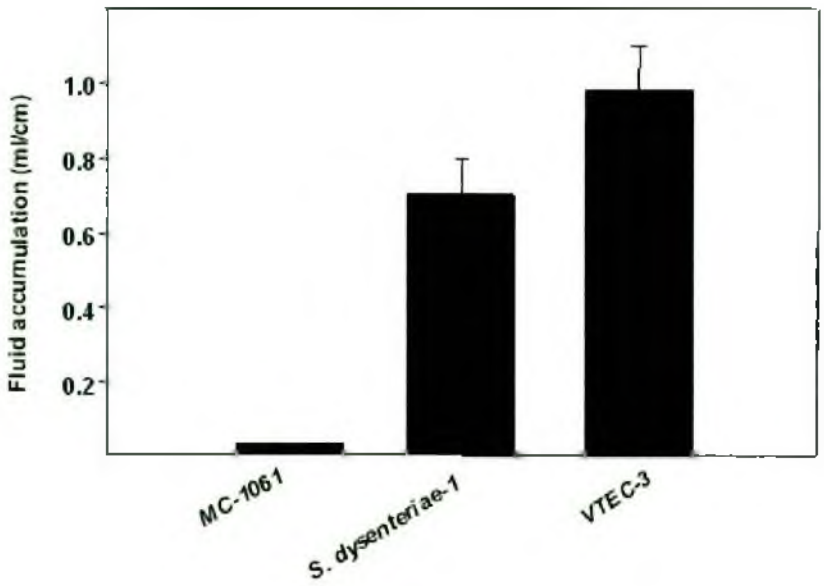


Figure 3.4: Shiga Toxin-dependent fluid accumulation in Rabbit Ileal Loops

3.4 Shiga Toxin 1 Exhibit Cytotoxic Activity in HeLa Cell

Bacterial culture supernatants were prepared from MC-1061, *S dysenteriae* 1, and VTEC-3. Different concentrations of bacterial culture supernatants were added to the tissue culture wells containing 200 µl of Dublecco's Modified Eagle's Medium (DMEM) and the 18 hr old HeLa cells. Cytotoxic activity of the STX gene containing bacterial strains were observed by inverted microscopy (Fig. 3.5) and later determined by nuclear condensation (DAPI staining) assay. It was found that treatment of HeLa cells with culture supernatants from STX gene containing bacteria induced nuclear condensation in a dose (Fig. 3.7) dependant manner. Culture supernatant from a STX lacking bacteria did not show any significant cytotoxic activity (Fig. 3.5c). Number of apoptotic nuclei (condensed chromatin) was determined from random microscopic fields (Fig. 3.5).

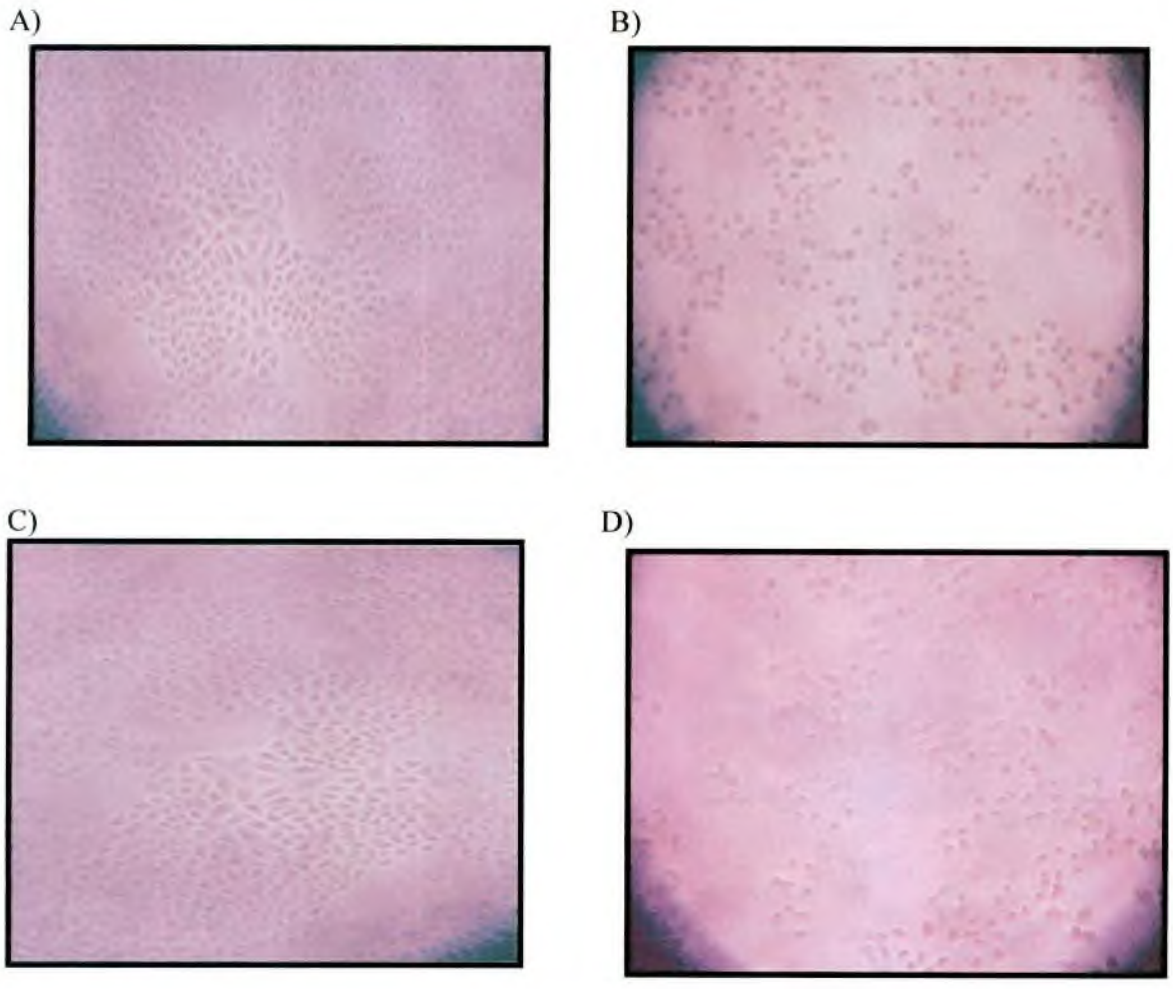
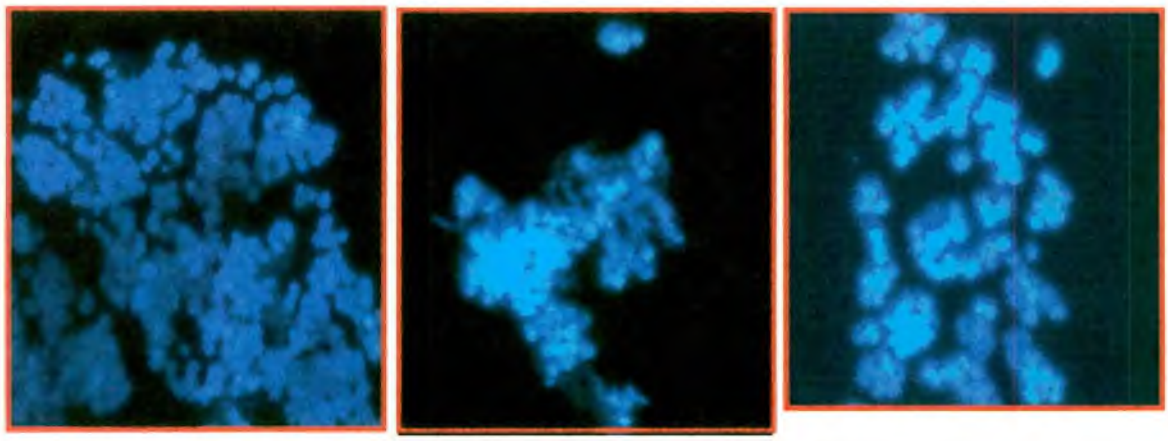


Figure 3.5: Effect of bacterial culture supernatant on HeLa cells. HeLa cells were seeded on culture plates and incubated overnight. Cells were treated with buffer (A), culture supernatants from VTEC-3 (B), MC-1061 (C) and *S. dysenteriae* 1 (D) for 24 hrs. HeLa cell were fixed and images were captured.

3.5 Chromatin Condensation Assay by DAPI Staining

Cytotoxic activity of purified STX1 was determined by nuclear condensation assay. Number of apoptotic nuclei (condensed chromatin) was determined by counting in random microscopic fields (Fig. 3.6). It was found that treatment of HeLa cells with STX1 induced nuclear condensation in dose dependant manner (Fig. 3.7).



a) HeLa cells (untreated)

b) HeLa cell treated with Positive control (VTEC-3)

c) HeLa cell treated with STX1.

Figure 3.6: Induction of chromatin condensation by STX1 in HeLa cells. HeLa cells were treated with STX1 for 24 hours. HeLa cells were fixed and stained with DAPI as described in Materials and Methods. a) Untreated HeLa cells b) Culture supernatant of VTEC-3 treated HeLa cells c) STX1 1 treated HeLa cells.

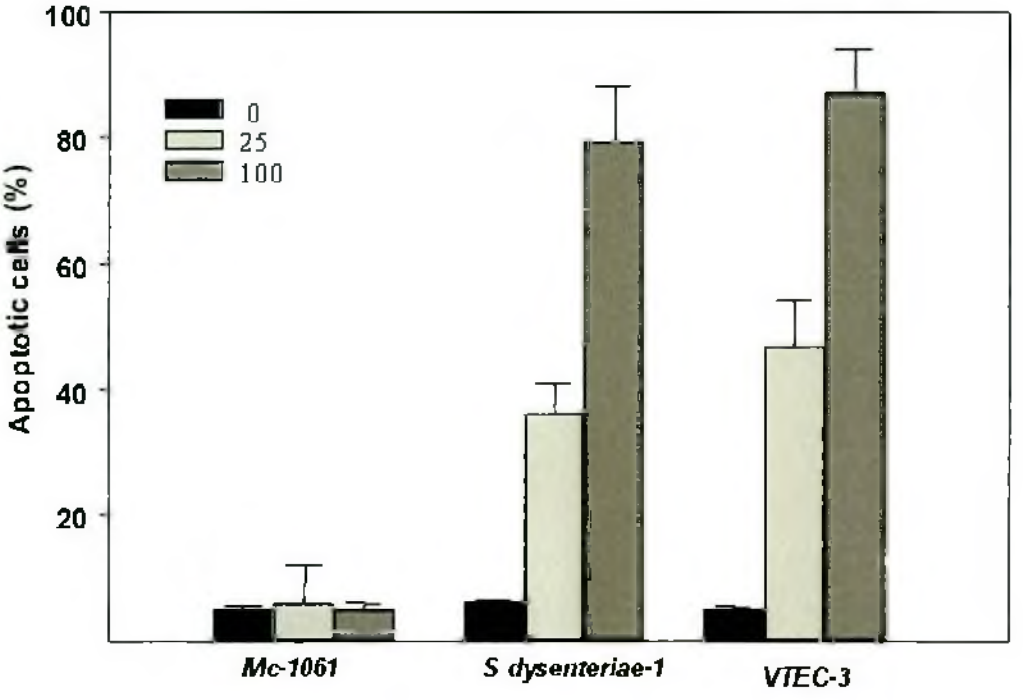


Figure3.7: Induction of HeLa cell death by STX1 in a Dose Dependant Manner.
STX-1 induces HeLa cell death. HeLa cells were treated with 0 (buffer), 25 or 100 ng per ml of culture supernatants prepared from MC1061, S dysenteriae-1, or VTEC-3. HeLa cells were fixed and stained with DAPI. Number of chromatin-condensed nuclei were counted from 10 random microscopic fields. Induction of HeLa cell death by STX1 in a Dose Dependant Manner.

3.6 Cytotoxicity of Shiga Toxin 1 with HeLa, MEF and Caco-2 Cell Line

A possible toxic effect of purified STX1 in inducing apoptosis in other mammalian cells were assayed. HeLa, mouse embryo fibroblasts (MEF), and Caco-2 (a human intestinal) cells were treated with purified STX1. After 24 hours, viability of cell was determined by MTT assay (Mosmann, 1983). It was found that STX1 inhibited the growth of all three mammalian cell lines with a stronger effect on Caco-2 cells (Fig. 3.8).

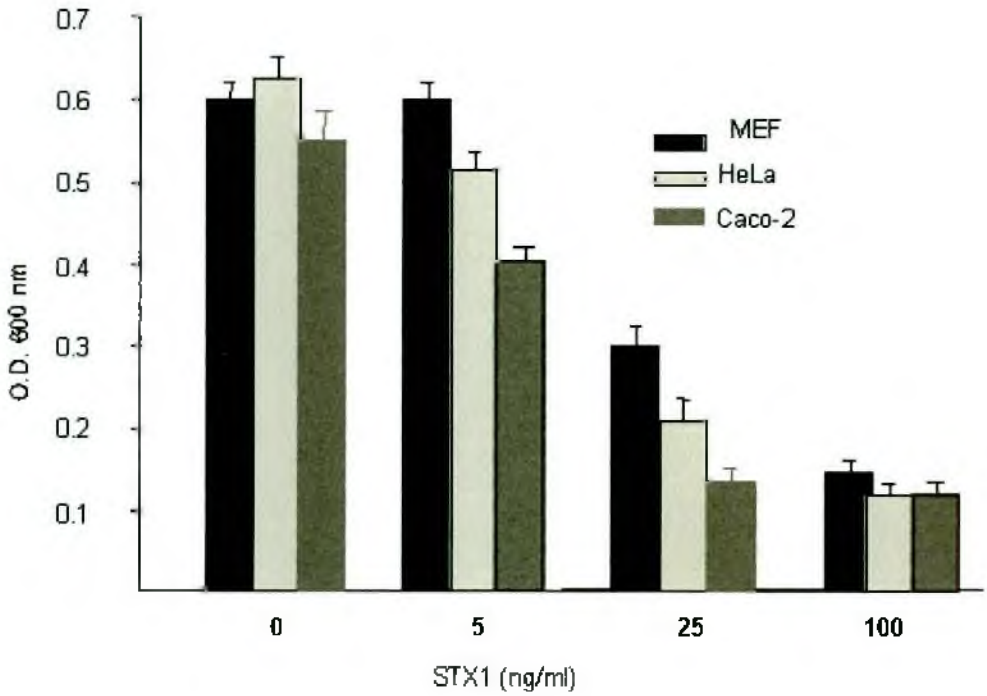


Figure 3.8: Induction of Mammalian cells (MEF, HeLa and Caco-2) death by STX1. Mammalian cells (MEF, HeLa and Caco-2) cells were cultured to semiconfluency. STX-1 (5, 25 or 100 ng/ml) or equal volume of culture medium (0) was added to cells. After 24 hours, viability of cell was determined by MTT assay (Mosmann, 1983) as described in "Materials and Method".

3.7 DNA Fragmentation Assay

Markers of apoptosis such as fragmentation of chromosomal DNA was studied. HeLa Cells were grown in six-well plate in an amount so that each of the well contains $3x10^6$ cells. After confluence growth of monolayer, the cells were then treated with STX1 or CHX. After 24 hours of treatment, the cells were lysed and DNA fragmentation analysis was performed. It was found that purified STX1 induced fragmentation of chromosomal DNA (Fig 3.9a) in HeLa cells. Cycloheximide (a potent inhibitor for protein synthesis) did not cause any significant fragmentation of chromosomal DNA (Fig 3.9) in HeLa cells.

C: culture medium
CHX: cycloheximide

Figure 3.9: STX-1 induces chromosomal DNA fragmentation in HeLa cells. HeLa cells were treated with C: culture medium, CHX: cycloheximide and STX1 (25 and 100ng/ml). Fragmented DNA was isolated from HeLa cells. DNA fragments were separated by agarose gel electrophoresis (1.5%) and DNA visualized by Ethidium bromide staining.

3.8 Induction of Cytochrome C Release from the Mitochondria by Shiga Toxin 1

Cytochrome C release from the mitochondria as one of the markers of apoptosis was studied. HeLa Cells were grown in six-well plate in an amount so that each of the well contains $3x10^6$ cells. After confluence growth of monolayer, the cells were then treated with STX1 (25 ng/ml). After 24 hours of treatment, the cells were harvested. Total cellular proteins were extracted, resolved by SDS-PAGE and western blotting was performed using anti-cytochrome c. It was also found that STX1 induced cytochrome C release from the mitochondria (Fig. 3.10). Equal loading of protein extract was confirmed by blotting the membrane with antibodies against topoisomerase I.

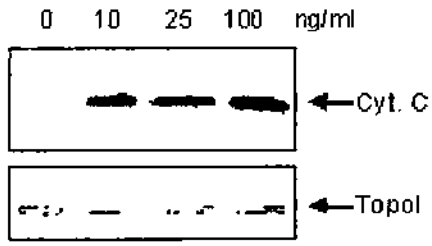
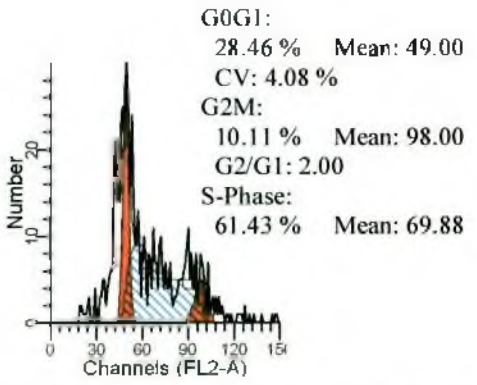


Figure 3.10: Treatment of HeLa cells with STX-1 induced cytochrome c release from the mictochondria. HeLa cells were cultured to semiconfluency. STX-1 (10, 25 or 100 ng/ml) or equal volume of culture medium (0) was added to cells. After 4 hours of treatment, cells were collected. Nuclear fraction was prepared previously (Nur et al., 2004). Proteins present in nuclear fraction were dissolved in SDS Laemmli sample buffer. Western blotting was performed using cytochrome c. Equal loading of protein extract was confirmed by blotting the membrane with actin antibody (Topol).

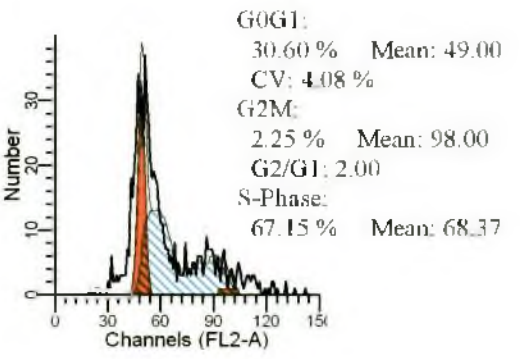
3.9 Shiga Toxin 1 Induced Cell Cycle Arrest

3.9.1 Effect of STX on Cell Cycle.

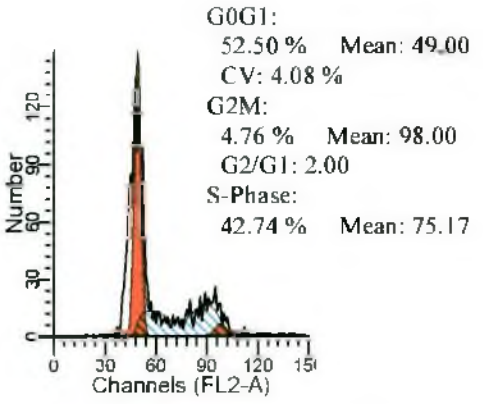
The cell cycle phase in which apoptosis has been triggered can be analyzed. Percentage of cell population in different inter phases (G1, S and G2) of cell cycle at different time intervals (2hr, 4hr, 6 hr, 8 hr and 24 hr) were measured by FACS analysis. It was found that untreated HeLa cells progressed through normal cell cycle (Figure 3.11).



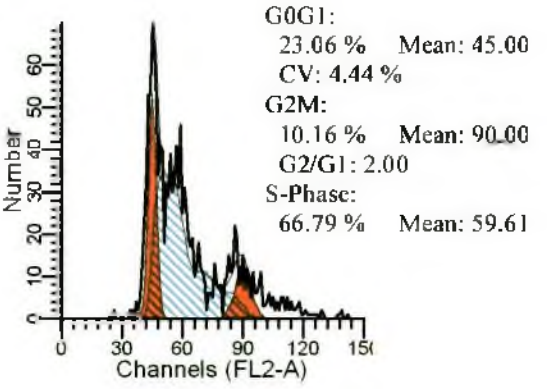
a) Distribution of cells in G1, S and G2 after 2hr



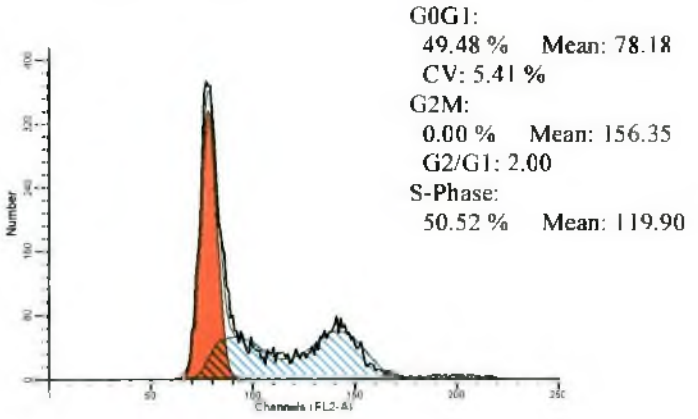
b) Distribution of cells in G1, S and G2 after 4hr



c) Distribution of cells in G1, S and G2 after 6hr



d) Distribution of cells in G1, S and G2 after 8hr



e) Distribution of cells in G1, S and G2 after 24hr

Figure 3.11: Distribution of Cell population at different inter phases (G1, S and G2) of Untreated HeLa Cell populations after 2hr, 4hr, 6hr, 8hr and 24hr analyzed by Modfit LT Software.

Table 3.2: Percentage of cell population in different inter phases (G1, S and G2) of cell cycle of untreated HeLa cell line at different time interval analyzed by Cell quest (BD) followed by Modfit LT software.

Time intervals	Percent distribution of cells		
	G0G1	G2M	S-Phase
2hr	28.46	10.11	61.43
4hr	30.60	2.25	67.15
6hr	52.50	4.76	42.74
8hr	23.06	10.16	66.79
24hr	49.49	0.0	50.52

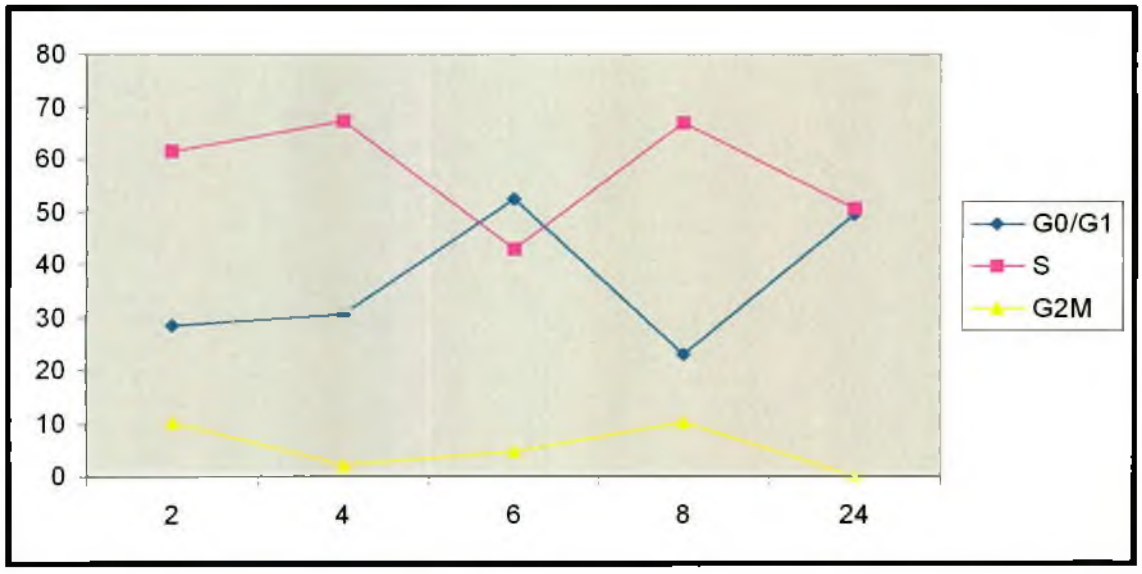
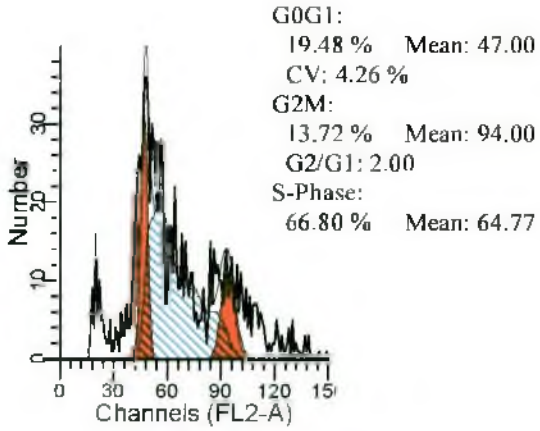


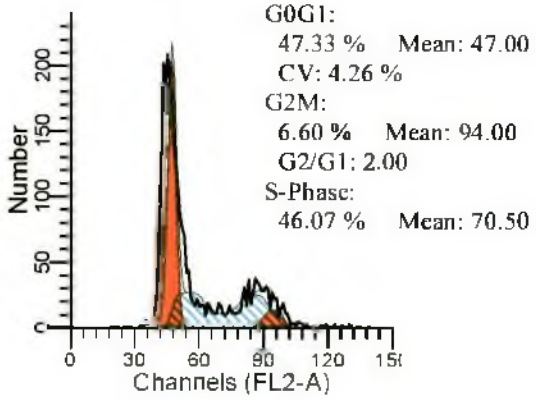
Figure 3.12: Graphical presentation of cell population in G0G1, S and G2M phase at 2, 4, 6, 8 and 24 hour of untreated HeLa cell line.

3.9.2 Determination of the Percentages of MC-1061 Treated HeLa Cell Population at Different Cell Cycle Phases

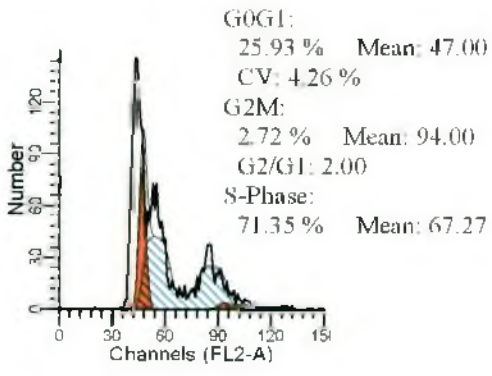
Percentage of cell population in different inter phases (G1, S and G2) of cell cycle of MC-1061 treated HeLa cell line at different time intervals (2hr, 4hr, 6 hr, 8 hr and 24 hr) were measured by FACS analysis. It was found that MC-1061 treated HeLa cells progressed through normal cell cycle like the untreated cells (Figure 3.13).



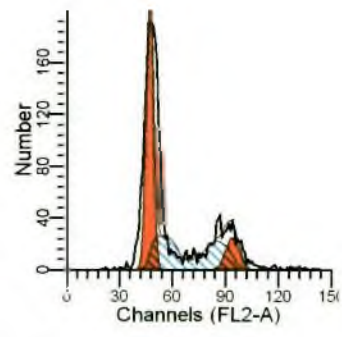
a) Distribution of cells in G1, S and G2 after 2hr



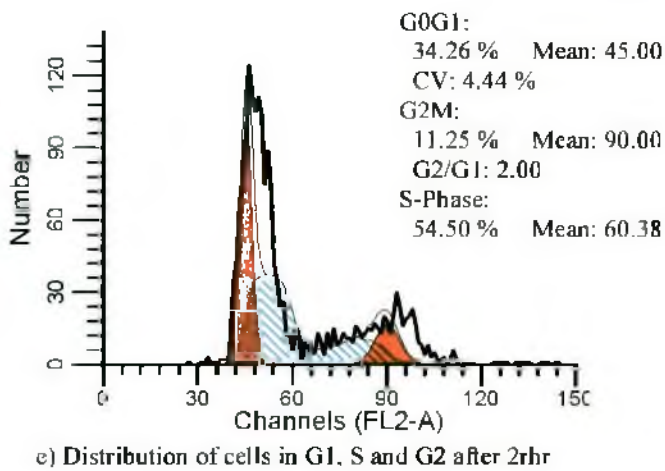
b) Distribution of cells in G1, S and G2 after 4hr



c) Distribution of cells in G1, S and G2 after 6hr



d) Distribution of cells after in G1, S and G2 after 8hr



e) Distribution of cells in G1, S and G2 after 2rhr

Figure 3.13: Distribution of Cell population at different inter phases(G1, S and G2) of culture supernatant of MC-1061(Negative control) treated HeLa Cell populations after 2hr, 4hr, 6hr, 8hr and 24hr analyzed by Modfit LT Software.

Table 3.3: Percentage of cell population in different phases (G1, S and G2) of cell cycle of MC-1061(negative control) treated HeLa cell line at different time interval analyzed by Cell quest (BD) followed by Modfit LT software.

Time intervals	Percent distribution of cells		
	G0G1	G2M	S-Phase
2hr	19.48	13.72	66.80
4hr	47.33	6.60	46.07
6hr	25.93	2.72	71.37
8hr	50.82	11.01	38.17
24hr	34.26	11.25	54.50

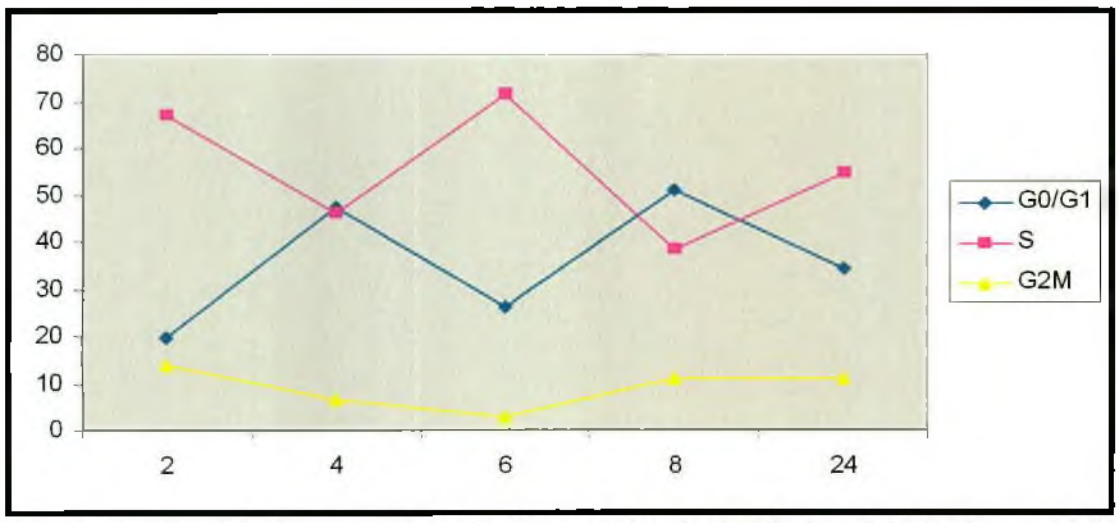
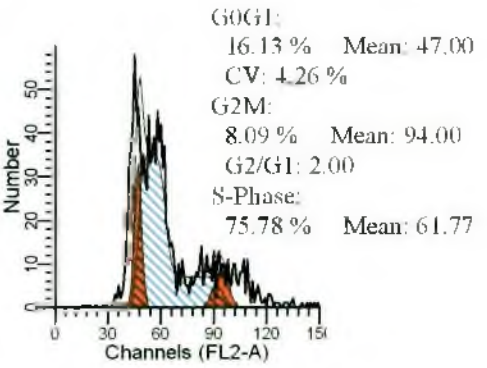


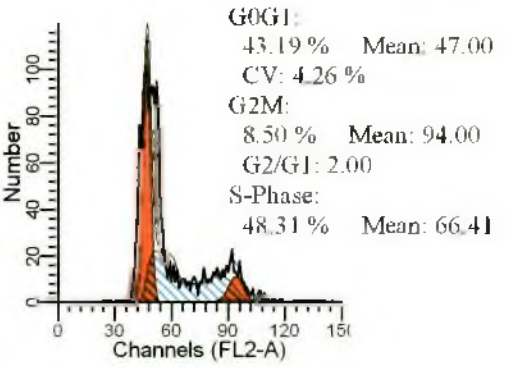
Figure 3.14: Graphical presentation of cell population in G0G1, S and G2M phase at 2,4, 6,8 and 24 hour of MC-1061 treated HeLa cell line.

3.9.2 Determination of the Percentages of VTEC-3 Treated HeLa Cell Population at Different Cell Cycle Phases

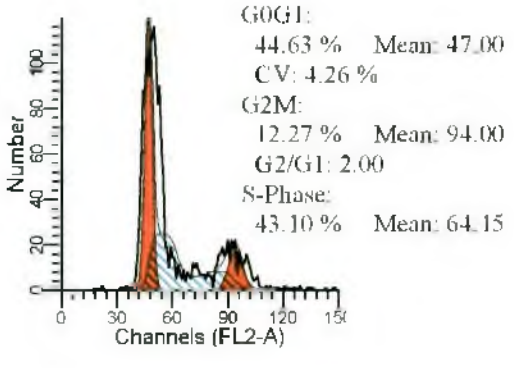
Percentage of cell population in different inter phases (G1, S and G2) of cell cycle of VTEC-3 treated HeLa cell line at different time intervals (2hr, 4hr, 6 hr, 8 hr and 24 hr) were measured by FACS analysis. The data from the flow cytometric (FACS) analysis indicated that VTEC-3 treated HeLa cells were arrested at G0/G1 phase and the cell percentage in that phase increased gradually from 16.13 to 59.77 (Figure 3.15).



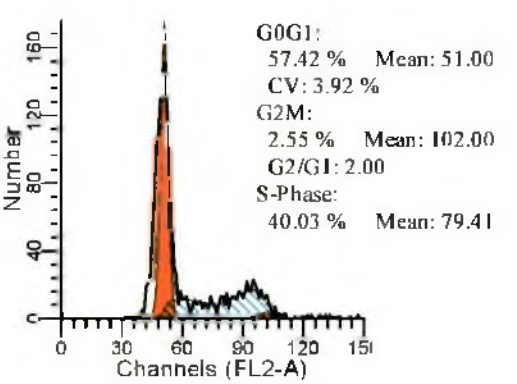
a) Distribution of cells in G1, S and G2 after 2hr



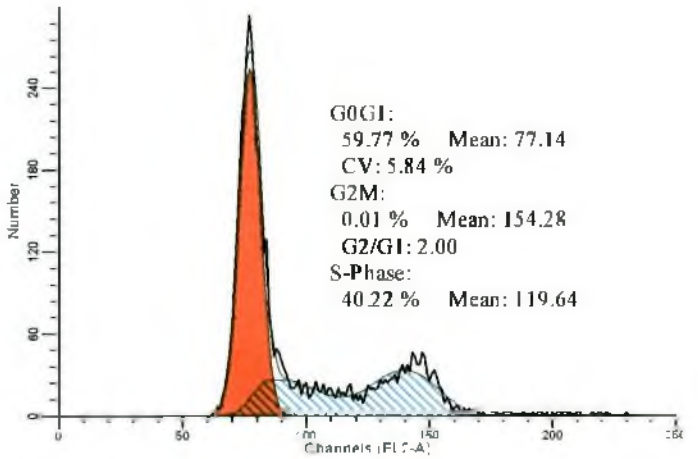
b) Distribution of cells in G1, S and G2 after 4hr



c) Distribution of cells in G1, S and G2 after 6hr



d) Distribution of cells in G1, S and G2 after 8hr



e) Distribution of cells in G1, S and G2 after 24hr

Figure 3.15: Distribution of HeLa Cell populations at different inter phases (G1, S and G2) after 2hr, 4hr, 6hr, 8hr and 24hr analyzed by Modfit LT Software. HeLa cells were treated with culture supernatant of VTEC-3 (Positive control).

Table 3.4: Percentage of cell population in different phases (G1, S and G2) of cell cycle of VTEC-3(Positive control) treated HeLa cell line at different time interval analyzed by Cell quest (BD) followed by Modfit LT software.

Time intervals	Percent distribution of cells		
	G0G1	G2M	S-Phase
2hr	16.13	8.09	75.78
4hr	43.19	8.50	48.31
6hr	44.63	12.27	43.10
8hr	57.42	2.25	40.03
24hr	59.77	0.01	40.22

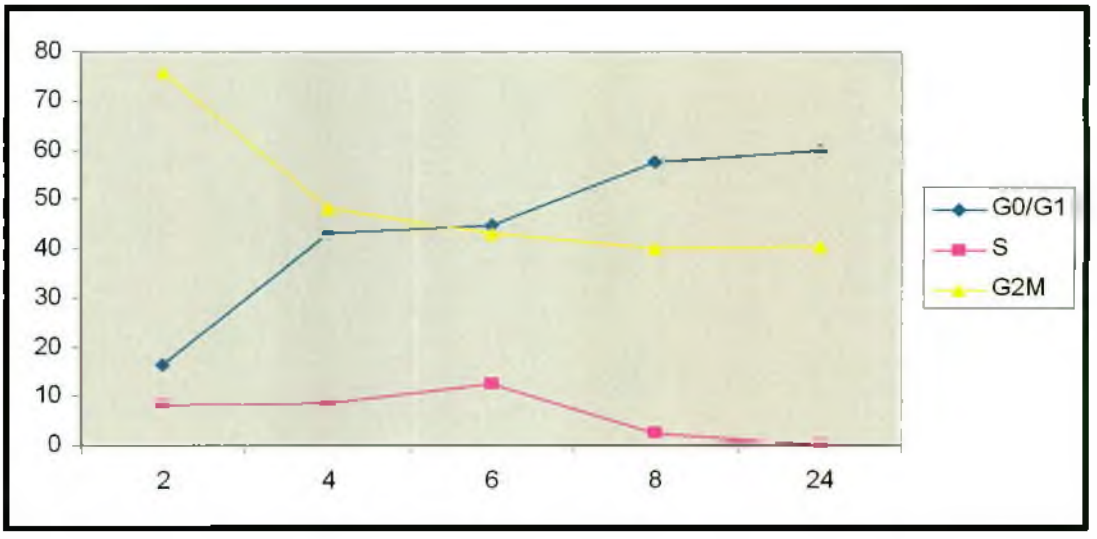
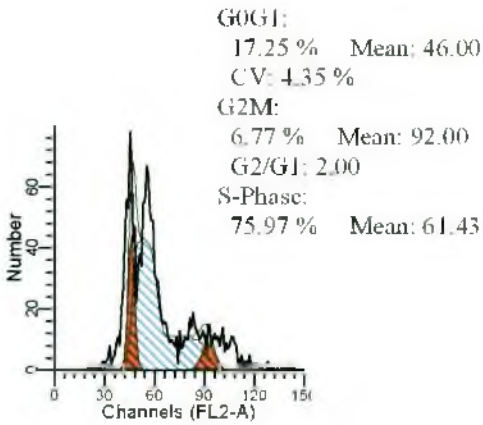


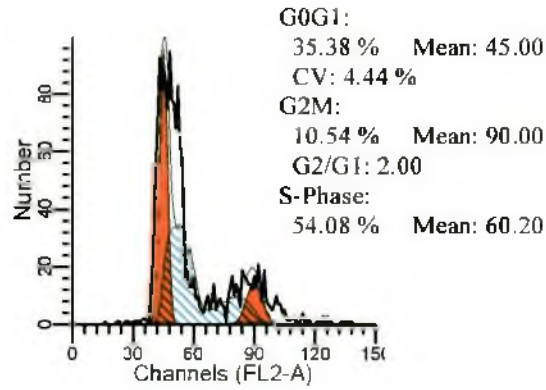
Figure 3.16: Graphical presentation of cell population in G0G1, S and G2M phase at 2,4, 6.8 and 24 hour of VTEC-3 treated HeLa cell line.

3.9.4 Determination of the Percentages of Shiga Toxin 1 Treated HeLa Cell Population at Different Cell Cycle Phases

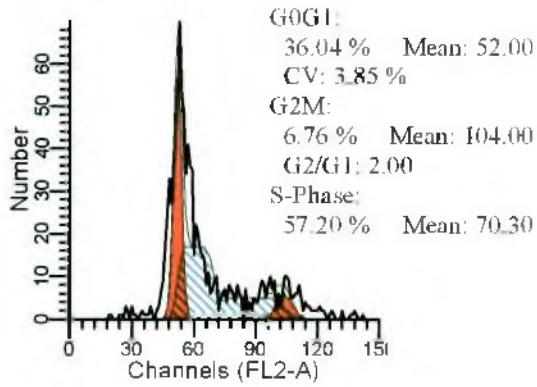
Percentage of cell population in different inter phases (G1, S and G2) of cell cycle of STX-1 treated HeLa cell line at different time intervals (2hr, 4hr, 6hr, 8hr and 24hr) were measured by FACS analysis. The data from the flow cytometric (FACS) analysis indicated that STX-1 treated HeLa cell were arrested at G0/G1 phase and the percentage of cells in that phase increased gradually from 17.25 to 51.52 (Figure 3.17).



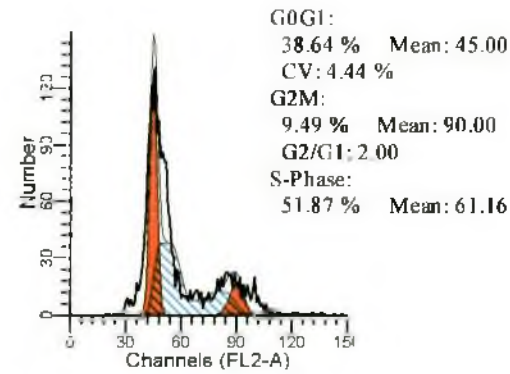
a) Distribution of cells in G1,S, G2 after 2hr



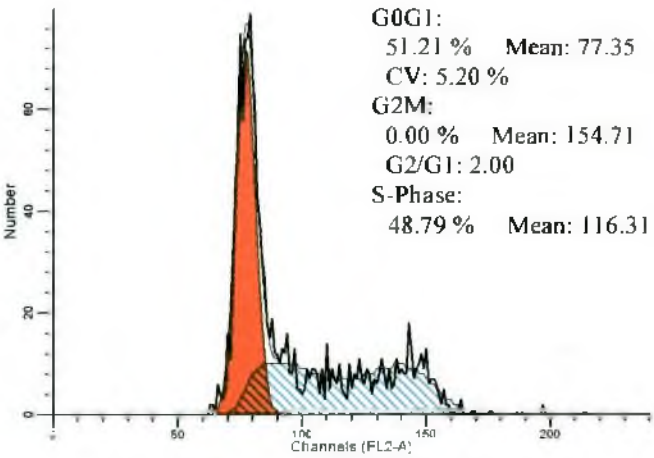
b) Distribution of cells inG1,S, G2 after 4hr



c) Distribution of cells in G1,S, G2 after 6hr



d) Distribution of cells inG1,S, G2 after 8hr



e) Distribution of cells in G1, S and G2 after 24hr

Figure 3.17: Distribution of HeLa Cell populations at different inter phases (G1, S and G2) after 2hr, 4hr, 6hr, 8hr and 24hr analyzed by Modfit LT Software. HeLa cells were treated with STX 1.

Table3.5: Percentage of cell population in different phases (G1, S and G2) of cell cycle of STX1 treated HeLa cell line at different time interval analyzed by Cell quest (BD) followed by Modfit LT software.

Time intervals	Percent distribution of cells		
	G0G1	G2M	S-Phase
2hr	17.25	6.77	75.97
4hr	35.38	10.54	54.08
6hr	36.04	6.76	57.20
8hr	38.64	9.49	51.87
24hr	51.52	0.00	48.79

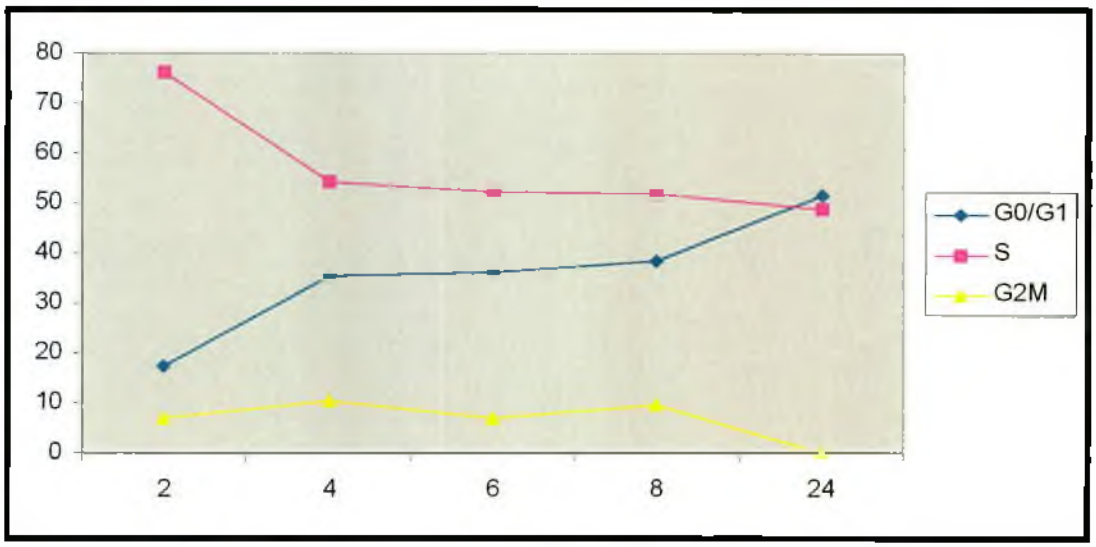


Figure 3.18: Graphical presentation of cell population in G0G1, S and G2M phase at 2, 4, 6.8 and 24 hour of STX1 treated HeLa cell line.

3.10 Activation of DNA Damage Signaling Pathway by STX1.

Markers of apoptosis such as chromatin condensation, fragmentation of chromosomal DNA, and nuclear translocation of cytochrome c was studied. It was found that STX1 induced chromatin condensation (Fig. 3.6) and fragmentation of chromosomal DNA (Fig. 3.9) in HeLa cells. It was also found that STX1 induced cytochrome c release from the mitochondria (Fig. 3.10). STX1 activates DNA damage signaling pathway was studied. STX1 treated cells were studied for activation of PARP cleavage and phosphorylation of H2AX. As shown in Fig. 3.19, STX1 induced phosphorylation of H2AX and activated PARP cleavage within 4 hours in HeLa cells. These results indicate that STX1-dependent activation of DNA damage signaling may have an important role in inducing apoptosis in mammalian cells. Equal loading of protein extract was confirmed by blotting the membrane with antibodies against actin (total cell extract).

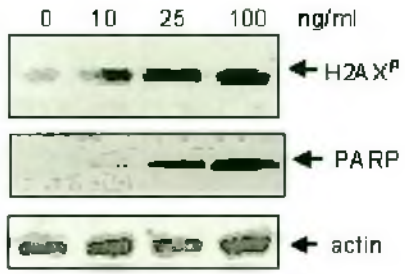


Figure 3.19. Induction of H2AX phosphorylation and PARP cleavage by STX-1 in HeLa cells. HeLa cells were treated with STX1 as described above. Cells were lysed in SDS-Laemmli sample buffer to prepare total cellular protein extract. Proteins present in total cell extract were separated by SDS-PAGE (12%) and transferred onto nylon membrane. Western blotting was performed using phospho-H2AX and PARP antibodies using ECL kit according to the protocol provided by suppliers. Equal loading of protein extract was confirmed by blotting the membrane for actin.

3.11 Shiga Toxin 1 Induced Up-regulation of p53 in HeLa Cells

STX1 induced up-regulation of p53 in HeLa cells was studied. Treatment of HeLa cells with STX1 was found to up-regulate the expression of p53 (Fig 3.20) in HeLa cells. Treatment of HeLa cells with a caspase 3 inhibitor (Z-VED) significantly reduced STX1-induced up-regulation of p53 suggesting an involvement of p53 in transducing STX-1-induced apoptosis in mammalian cells. Equal loading of protein extract was confirmed by blotting the membrane with antibodies against actin (total cell extract).

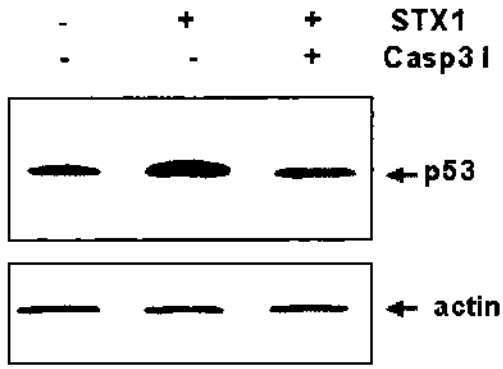


Figure 3.20: Caspase-3-dependent activation of p53 by STX-1 in HeLa cells. HeLa cells were cultured in DMEM containing 10% calf serum to semiconfluency. STX-1 (25 ng/ml) or BSA (25 ng/ml) was added to the cell medium. After 6 hours of treatment, cells were collected. Cells were lysed in Laemmli SDS sample buffer to prepare total cellular protein extract. Proteins were resolved by SDS-PAGE (10%) and transferred onto nylon membrane. Western blotting was performed using antibodies against p53. Equal loading was confirmed by blotting the membrane with actin.

3.12 Shiga Toxin 1 Induced Phosphorylation of ATM in HeLa Cells

STX1 induced phosphorylation of ATM in HeLa Cells was studied. Treatment of HeLa cells with STX1 was found to induce phosphorylation of ATM (Fig. 3.21) in HeLa cells. Treatment of HeLa cells with a caspase 3 inhibitor (Z-VED) significantly reduced STX1-induced phosphorylation of ATM suggesting an involvement of ATM in transducing STX-1-induced apoptosis in mammalian ceils. Equal loading of protein extract was confirmed by blotting the membrane with antibodies against ATM.

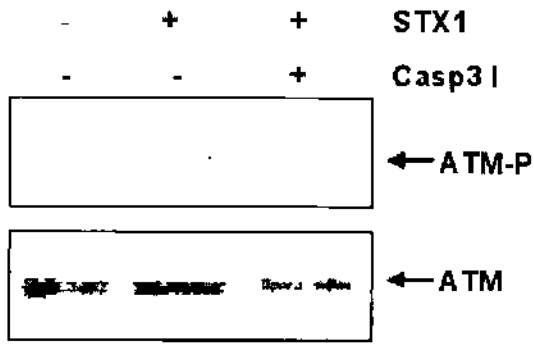


Figure 3.21: Caspases-3-dependent activation of ATM by STX-1 in HeLa cells. HeLa cells were cultured in DMEM containing 10% calf serum to semiconfluency. STX-1 (25 ng/ml) or BSA (25 ng/ml) was added to the cell medium. After 6 hours of treatment, cells were collected. Cells were lysed in Laemmli SDS sample buffer to prepare total cellular protein extract. Proteins were resolved by SDS-PAGE (6%) and transferred onto nylon membrane. Western blotting was performed using antibodies phospho-ATM. Equal loading was confirmed by blotting the membrane with ATM.

3.13 Involvement of p53/ATM-dependent DNA Damage Signaling Pathway in STX1-induced Mammalian Cell Death.

To further confirm the involvement of p53/ATM in STX1-induced mammalian cell death, mammalian cells deficient in p53 or ATM were treated. It was found that STX1 treatment induced chromatin condensation in about 65% of wild type ($p53^{+/+}$) cells while a significant reduction (~28%) in chromatin condensation was found in p53-deficient ($p53^{-/-}$) cells (Fig. 3.22). Similarly, STX1 induced chromatin condensation in wild type ATM ($ATM^{+/+}$) cells (~70%) and a significant reduction (~30%) in chromatin condensation was found in ATM deficient ($ATM^{-/-}$) cells (Fig. 3.22). These results indicate that p53 and ATM are involved in transducing STX1 signals for induction of apoptosis in HeLa cells.

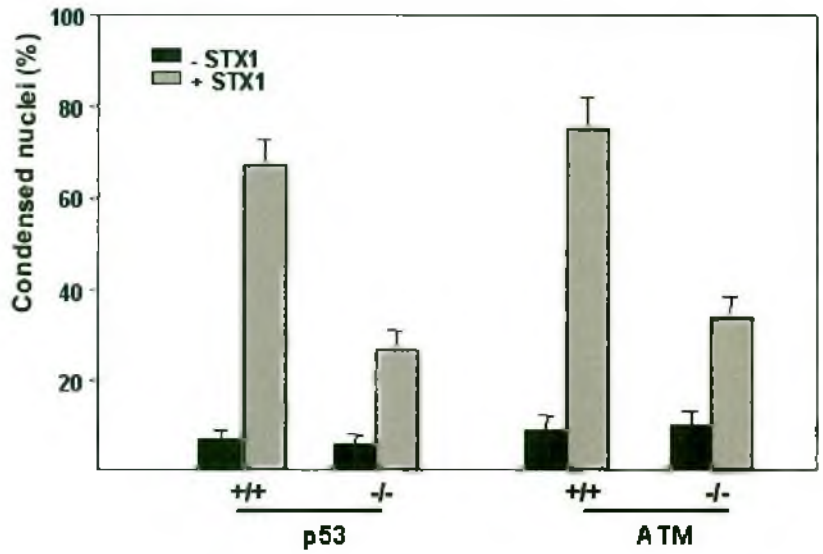


Figure 3.22: Requirement of p53 and ATM in STX-induced apoptosis. p53 (HCT116 ($p53^{+/+}$ and HCT-116 p53) and ATM {YZ5 ($ATM^{+/+}$) and FT169A (ATM deficient)} cells were cultured and treated with 25 ng/ml STX1 (+STX1) or BSA (-STX) as described above. After 24 hours incubation, cells were fixed. Nuclei were stained with propidium iodide (5 µg/ml). Chromatin condensation was viewed in several random fields under a fluorescent microscope (Zeiss, Axioplan2).

Chapter 4

DISCUSSION

4.0 DISCUSSION

Shigellosis has been shown to be associated with development of hemolytic uremic syndrome (HUS) and/or CNS complications (Paton and Paton, 1998; Proulx *et al.*, 2001). Toxins produced by *Shigella* strain are believed to be responsible for these complications. Shiga toxin (STX) has been shown to induce mammalian cell death by different groups suggesting that STX-induced apoptosis plays a critical role in acute renal failure, seizures, paralysis, coma, and death. However, molecular mechanism of STX-induced mammalian cell death has remained to be understood. In this study I have attempted to explore the molecular insight into STX1-induced mammalian cells death. As a first step, I have demonstrated that culture supernatant from *Shigella dysenteriae* 1 (producing STX1) induces mammalian cell death in different cell lines such as MEF, Caco-2, and HeLa. In addition to STX, *Shigella dysenteriae* 1 culture supernatant may contain other toxins. I have screened bacterial for the presence of some bacterial toxin genes.

The representative strains of each *Shigella* serotype was analyzed for the presence of toxin genes such as *set*, *sen*, *stx*-1 and *stx*-2 by PCR. *Shigella dysenteriae* 1 harbored the *stx*-1 gene. A few *Shigella dysenteriae* 1 strains were negative for *sen* gene. Both *set*1A and *set*1B genes were absent in all *Shigella dysenteriae*1 strains. Therefore, *Shigella dysenteriae* 1 strains bearing STX gene were negative for *set* and *sen* genes) suggesting cytotoxic activity of these culture supernatant is most likely due to STX1.

I have also determined the ability of these shigella cell culture supernatant to accumulate fluid in rabbit ileal loop. STX producing bacteria was found to induce fluid accumulation in rabbit ileal loop. *Shigella* strains have previously been shown to induce accumulation of fluid and mammalian cell death (Philpott *et al.*, 2000; Sandvig, 2001). A relationship between Shiga toxin producing bacteria and incidence of HUS has recently been reported (Bennish *et al.*, 2006). The results were consistent with others (Nakagawa *et al.*, 1999; Iwata *et al.*, 2001; Fujii *et al.*, 2003).

To confirm the effect of STX1 on mammalian cell death, I used purified STX-1 for cytotoxicity assay. Mammalian cells (MEF, HeLa and Caco-2) were cultured to semiconfluency and STX-1 (5, 25 or 100 ng/ml) was added to each cell line. After 24 hours, viability of cell was determined by MTT assay. It was also found that purified Shiga toxin1 exhibited cytotoxic activity in different mammalian cells such as HeLa, MEF (mouse embryo fibroblast) and Caco-2 (a human intestinal primary fibroblast cell line) cells. I did not find any difference in pattern of killing mammalian cells by *S. dysenteriae* 1 culture supernatant and purified STX-1 suggesting that cytotoxic activity of *S. dysenteriae* 1 culture supernatant is due to STX-1.

Apoptosis, or programmed cell death, is a genetically regulated process, which is characterized by plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation and formation of apoptotic bodies (Whyllie *et al.*, 1997). To understand the molecular mechanism of STX-induced mammalian cell death, I studied whether the cytotoxicity induced by STX1 was due to activation of apoptosis.

During apoptosis cell rounds up along with chromatin condensation, leading to the formation of crescent shaped masses aggregating at the membrane (Kerr *et al.*, 1971). These initial changes are followed by the cytoplasmic and nuclear compaction and ultimately by fragmentation of the cells. The nuclear fragments formed in this process are still surrounded by the nuclear membranes (Kerr *et al.*, 1971; Oberhamer *et al.*, 1993). Thus chromatin condensation was done for the further confirmation of apoptosis. During the chromatin condensation assay one of the major problem sought was the aggregation of the cells. After staining with DAPI the apoptotic cells were shown to exhibit marked fluorescence due to the binding of DNA among the condensed chromatin compared with the control cell line. The control HeLa cell line was less florescent where culture supernatant of *S. dysenteriae* 1 /purified STX1 treated cells showed marked florescence. More 150 cells were counted per field and the average percentage of chromatin condensation was measured. The experiment was repeated at least three times showing similar results.

DNA fragmentation is one of the critical markers, which is used to identify apoptotic cells (Fraziska *et al.*, 1994). Thus DNA fragmentation of the HeLa cell line was observed after treating with Culture supernatant of *Shigella dysenteriae* 1 / purified STX1. The chromosomal DNA was extracted by a previously described method which (Tayeb *et al.*, 1999). The agarose gel electrophoresis revealed the pattern of DNA in the affected cells. The DNA was found readily fragmented in case of HeLa cell line treated with culture supernatant of *Shigella dysenteriae* 1 /purified STX1. The DNA fragmenting pattern caused by STX1 was almost similar to the positive control exhibiting a long smear of the chromosomal DNA molecules. The control cell line was also extracted for the DNA content and run as well, which showed no fragmented DNA in its lane. Again, no fragmented DNA was found in case of HeLa cells being treated with cycloheximide (a potent inhibitor for protein synthesis). A clear laddering of the DNA was found when resolved in the Agarose Gel electrophoresis. The extraction procedure is set in such a way so that only the fragmented DNA could be purified from the cellular bodies. Members of the ribosome inactivating proteins (RIPs) that includes STX and ricin has been shown to deadenylate ribosomal RNA (Barbieri *et al.*,1 994; Barbieri *et al.*, 1997; Barbieri *et al.*, 1998; Barbieri *et al.*, 2000; Nicolas *et al.*, 2000) suggesting that inhibition of protein synthesis may play a key role in RIP-induced cell death. However, protein synthesis inhibitory role of STX in inducing apoptosis in mammalian cells has remained controversial (Sakamoto *et al.*, 2003). In fact, I did not find any significant apoptosis in HeLa cells treated with cycloheximide (a potent inhibitor for protein synthesis) while STX1 induced apoptosis under similar condition. Interesting, cycloheximide has been reported to block STX-induced cell lysis (Sandvig and van Deurs, 1992). STX has also been found to induce expression of proapoptotic proteins (Jones *et al.*, 2000). These results suggest that STX might activate additional signaling pathway(s) leading to apoptosis. Recently, RIPs have been shown to induce DNA and RNA fragmentation in vitro and in vivo (Barbieri *et al.*, 1997; Brigotti *et al.*, 2002). It has also been shown that small pieces of DNA induce apoptosis in mammalian cells (Schiavone *et al.*, 2000; Tidd *et al.*, 2000; Milyavsky *et al.*, 2001; Nur *et al.*, 2003). Although the DNA fragmentation proved that the cellular death is probably mediated by apoptosis, it was unable to depict the degree of DNA fragmentation in this study.

Checkpoints are layers of control that act to delay CDK activation when defects in the division program occur. As the CDKs functioning at different points in the cell cycle are regulated by different means, the various checkpoints differ in the biochemical mechanisms by which they elicit their effect. However, all checkpoints share a common hierarchy of a sensor, signal transducers and effectors that interact with CDKs. Cells whose DNA is damaged by irradiation with UV light or chemical modification become arrested in G1 and G2 until the damage is repaired. Arrest in G1 prevents copying of damaged bases, which would fix mutation in the genome. Arrest in G2 allows DNA double stranded breaks to be repaired before mitosis. If the DNA damage is too extensive to repair, the cell commits suicide via apoptosis.

I studied whether STX affects progression of cell cycle in HeLa cells FACS analysis. HeLa cells at different time interval (2hr, 4hr, 6hr, 8hr and 24hr) of treatment were measured by FACS analysis. The data from the flow cytometric (FACS) analysis indicated that untreated HeLa cells and MC-1061 treated HeLa cells were progress through normal cell cycle while culture supernatants of VTEC-3 and STX-1 treated HeLa cell were arrested at G0/G1 phase and the percentage increase gradually from 16.13 to 59.77 and 17.25 to 51.52 respectively indicated that STX1 induced G0/G1 cell cycle arrest and apoptosis. Mammalian cells respond to DNA-damaging agents by activating cell cycle checkpoints. These control mechanisms determine a temporary arrest at a specific stage of the cell cycle to allow the cell to correct possible defects (Hartwell and Weinert, 1989; Hartwell and Kastan, 1994). At least two checkpoints monitor DNA damage: one at the G_1/S transition and the other at the G_2/M transition. The G_1 checkpoint prevents replication of damaged DNA, whereas the G_2/M transition is inhibited by damaged and/or incompletely replicated DNA (Hartwell and Weinert, 1989; Hartwell and Kastan, 1994). Several findings have demonstrated that the product of the p53 tumor suppressor gene is responsible for the G_1 checkpoint (Kastan *et al.*, 1991; Kuerbitz *et al* , 1992), In response to genotoxic stress, the levels of p53 protein increase and this increase determines a transient arrest of cell cycle progression in the G_1 phase (Kastan *et al* , 1991; Kuerbitz *et al* , 1992), or triggers apoptosis (Clarke *et al* , 1993; Lowe *et al* , 1993). The arrest in G_1 is thought to give the cells time to repair critical damage before DNA replication occurs, thereby avoiding the propagation of genetic

lesions to progeny cells. The cell cycle can resume once the damage has been repaired or, if the damage is too extensive, the cell will undergo apoptosis.

I did not find any significant apoptosis in HeLa cells treated with cycloheximide while STX1 induced apoptosis under similar condition. Interestingly, cycloheximide has been reported to block STX-induced cell lysis (Sandvig and van Deurs, 1992). STX has also been found to induce expression of proapoptotic proteins (Jones *et al.*, 2000). These results suggest that STX 1 might activate additional signaling pathway(s) leading to apoptosis. Recently, RIPs have been shown to induce DNA and RNA fragmentation in vitro and in vivo (Barbieri *et al.*, 1997; Brigotti *et al.*, 2002). It has also been shown that small pieces of DNA induce apoptosis in mammalian cells (Schiavone *et al.*, 2000; Tidd *et al.*, 2000; Milyavsky *et al.*, 2001; Nur *et al.*, 2003). RIP-dependent degradation of cellular DNA/RNA may activate DNA damage signaling leading to cell death (Saxena *et al.*, 1989; Brigotti *et al.*, 2002). In fact, it has recently demonstrated that ssDNA activates ATM/p53-dependent DNA damage signaling pathway (Saxena *et al.*, 1989; Huang *et al.*, 1996; Schiavone *et al.*, 2000; Nur *et al.*, 2003). A role of ATM in DNA repair and apoptosis has recently been demonstrated (Shiloh, 2003). ATM is a ubiquitously expressed protein and its level in cells has not been found to alter in response to radiation (Brown *et al.*, 1997). ATM is primarily activated through post-translational modification by ionizing radiation and other DNA damaging agents that give rise to DNA double-strand breaks (Banin *et al.*, 1998; Canman *et al.*, 1998; Khanna *et al.*, 1998). Once activated, ATM phosphorylates multiple substrates, which are primarily involved in DNA damage signaling (Shiloh and Kastan, 2001). For example, IR radiation activates ATM to phosphorylate H2AX at Serine-139 (Burma *et al.*, 2001). ATM has also been shown to regulate cell cycle checkpoints. In the case of G1/S checkpoint, activated ATM phosphorylates p53 at Serine-15 (Banin *et al.*, 1998; Canman *et al.*, 1998; Khanna *et al.*,1 998). These reports suggest that degradation of cellular DNA/RNA by STX 1 may cause activation of ATM-dependent DNA damage signaling pathways causing mammalian cell death. These results indicate that STX-induced DNA/RNA fragmentation may activate a unique apoptotic signaling pathway(s) in mammalian cells.

The PI3KK family of proteins has recently been shown to act as sensor for DNA damage in cells. These proteins form complex with ssDNA and RNA in the cell and activate DNA damage signaling pathway (Cortez *et al.*, 2001; Nur-E-Kamal *et al.*, 2003; Namiki and Zou, 2006). It was demonstrated that ssDNA activate ATM/p53-mediated DNA damage signaling pathway to induce apoptosis in mammalian cells (Nur-E-Kamal *et al.*, 2003). In this study, a critical role of ATM/p53 in inducing apoptosis by STX1 in mammalian cells was found. Since RIPs have been demonstrated to induce fragmentation of DNA and RNA (Barbieri *et al.*, 1997; Brigotti *et al.*, 2002), it remains to investigate whether fragmented DNA or RNA or both are involved in STX-induced cell death in this experiments. The STX1 could potentially induce fragmentation of RNA/DNA producing small pieces of RNA and DNA. These DNA/RNA oligos activate the nuclear kinase ATM as evidenced by phosphorylation of the ATM target protein H2AX in the nucleus. Activation of ATM then phosphorylates downstream target including p53 leading to induction of apoptosis. In my studies I found that purified STX1 was found to activate ATM/p53-dependent DNA damage signaling pathway and induce apoptosis in mammalian cells. Interestingly, a significant reduction of apoptosis was found in cells deficient of either ATM or p53. This is supported previous observation that STX1-induced cell death is significantly reduced in ATM or p53 knockout cells. It is possible that DNA/RNA oligos produced by STX1 may mimic a DNA damage signal in the nucleus to activate ATM/p53. Activated ATM/p53 signals are transduced to the mitochondria causing release of cytochrome C that leads to activation of caspase pathway and apoptotic cell death of mammalian cells. Consistent with this hypothesis pancreatic RNase (HP-RNase) has been shown to be cytotoxic (Piccoli *et al.*, 1999). It has also been shown that ribonucleases from frog oocytes and bovine tissues have been shown to exhibit antitumor activity (Schein, 1997; Hu *et al.*, 2001; Leland *et al.*, 2001). While it has been interpreted that degradation of RNAs by RNases causes cell death by blocking protein synthesis, it is conceivable that degraded RNA may signal cell death. It is, therefore, possible that STX-induce degradation of RNA causes inhibition of protein synthesis as well as produce DNA damage signal(s). More studies are required to determine whether inhibition of proteins synthesis and/or degraded RNA responsible for STX-induced mammalian cell death.

Chapter 5

REFERENCES

5. REFERENCES

Acheson D.W.K., and Keusch G.T. (1995) Shigella and enteroinvasive Escherichia coli. In: Infections of the Gastrointestinal Tract (Blaser, M.J., Smith, P.D., Ravdin, J.I., Greenberg, H.B. and Guerrant, R.L., Eds.), pp. 763-784. Raven Press, New York.

Agarwal M. L., Agarwal A., Taylor W. R., and Stark G. R.. (1995) p53 Controls Both the G_2/M and the G_1 Cell Cycle Checkpoints and Mediates Reversible Growth Arrest in Human Fibroblasts; Proc Natl Acad Sci USA. **92:** 8493–8497.

Arrowsmith C.H. (1999) Structure and function in the p53 family. Cell Death Differ. **6:** 1169-1173.

Barbieri L., Gorini P., Valbonesi P., Castiglioni P., Stirpe F. (1994) Unexpected activity of saporins. Nature **372:** 624.

Barbieri L., Valbonesi P., Bonora E., Gorini P., Bolognesi A., Stirpe F. (1997) Polynucleotide:adenosine glycosidase activity of ribosome-inactivating proteins: effect on DNA, RNA and poly(A). Nucleic Acids Res **25:** 518-522.

Barbieri L., Valbonesi P., Brigotti M., Montanaro L., Stirpe F., Sperti S. (1998) Shiga-like toxin I is a polynucleotide:adenosine glycosidase. Mol Microbiol **29:** 661-662.

Barbieri L., Valbonesi P., Righi F., Zuccheri G., Monti F., Gorini P., Samori B., Stirpe F. (2000) Polynucleotide:Adenosine glycosidase is the sole activity of ribosome-inactivating proteins on DNA. J Biochem (Tokyo) **128**: 883-889.

Bellacosa A. (2001) Functional interactions and signaling properties of mammalian DNA mismatch repair proteins. Cell Death Differ. **8:** 1076-1092.

Banin S., Moyal L., Shieh S., Taya Y., Anderson C.W., Chessa L., Smorodinsky N.I., Prives C., Reiss Y., Shiloh Y., Ziv Y. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science **281:** 1674-1677.

Benchimol S. (2001) p53-dependent pathways of apoptosis . Cell Death Differ. **8:** 1049-1051

Bennish M.L., Harris J.R., Wojtyniak B.J., Struelens M. (1990) Death in shigellosis: incidence and risk factors in hospitalized patients. J Infect Dis **161:** 500-506.

Bennish M.L., Wojtyniak B.J. (1991) Mortality due to shigellosis: community and hospital data. Rev Infect Dis 13 Suppl **4:** 245-251.

Bennish M. L., Salam M. A., Hossain M. A., Myaux J., Khan E. H., Chakraborty J., Henry F., and Ronsmans C. (1992) Antimicrobial resistance of *Shigella* isolates in Bangladesh, 1983-1990: increasing frequency of strains multiply resistant to ampicillin, trimethoprim-sulfamethoxazole, and nalidixic acid. Clin. Infect. Dis. **14:** 1055-1060.

Bennish M..L, Khan W.A., Begum M., Bridges E.A., Ahmed S., Saha D., Salam M.A., Acheson D., Ryan E.T. (2006) Low risk of hemolytic uremic syndrome after early effective antimicrobial therapy for Shigella dysenteriae type 1 infection in Bangladesh. Clin Infect Dis **42:** 356-362.

Blanco J., Blanco M., Gonzalez E.A., Alonso M.P. and Garabal J.I. (1990) Comparative evaluation of three tests for the detection of *Escherichia coli* cytotoxic necrotizing factors(CNF1 and CNF2) using filtrates of cultures treated with mytomycin C. FEMS Microbial. Lett. **69:** 311-316

Bonnet R., Souweine B., Gauthier G., Rich C., Livrelli V., Sirot J., Joly B., and Forestier C. (1998) Non-O157:H7 Stx2-Producing *Escherichia coli* Strains Associated with Sporadic Cases of Hemolytic-Uremic Syndrome in Adults **J. Clin.** Microbiol. **36:** 1777-1780.

Brigotti M., Accorsi P., Carnicelli D., Rizzi S., Gonzalez V. A., Montanaro L., Sperti S. (2001) Shiga toxin 1: damage to DNA in vitro. Toxicon **39:** 341-348.

Brigotti M., Alfieri R., Sestili P., Bonelli M., Petronini P.G., Guidarelli A., Barbieri L., Stirpe F., Sperti S. (2002) Damage to nuclear DNA induced by Shiga toxin 1 and ricin in human endothelial cells. Faseb J **16:** 365-372.

Brown K.D., Ziv Y., Sadanandan S.N., Chessa L., Collins F.S., Shiloh Y., Tagle D.A. (1997) The ataxia-telangiectasia gene product, a constitutively expressed nuclear protein that is not up-regulated following genome damage. Proc Natl Acad Sci U S A **94:** 1840-1845.

Burma S., Chen B.P., Murphy M., Kurimasa A., Chen D.J. (2001) ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem **276:** 42462-42467.

Burrows W. and Musteikis G.M. (1966) Cholera infection and toxin in the rabbit ileal loop. J Infect Dis **116:** 183-190.

Canman C.E., Lim D.S., Cimprich K.A., Taya Y., Tamai K, Sakaguchi K., Appella E., Kastan M.B., Siliciano J.D. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Scicnce **281:** 1677-1679.

Chen Y., Smith, M. R., Thirumalai, K., and Zychlinsky, A. (1996) A bacterial invasin induces macrophage apoptosis by binding directly to ICE. *EMBO J.* **15:** 3853–3860

Ching J.C.Y., Jones N.L., Ceponis P.J.M., Karmali M.A. and Sherman P.M. (2002) Escherichia coli Shiga-like toxins induce apoptosis and cleavage of poly(ADP-ribose) polymerase via in vitro activation of caspases. Infect. Immun. **70:** 4669-4677.

Clarke A. R., Purdie C. A., Harrison D. J., Morris R. G., Bird C. C., Hooper M. L., Wyllie A. H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways Nature (London). **362:** 849–852.

Cortez D., Guntuku S., Qin J., Eiledge S.J. (2001) ATR and ATRIP: partners in checkpoint signaling. Science **294:** 1713-1716.

Cohen, G M, Sun, X M Snowden, , R T, D Dinsdale, and Skillcter D N.(1992). Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem J. **286:** 331–334.

Crissman H.A. and Tobey R.A. (1974) Cell-cycle analysis in 20 minutes. Scicnce 184: 1297–1298.

Darzynkiewicz Z., Traganos F., Sharpless T.K., Melamed M.R. (1977) Cell cycle-related changes in nuclear chromatin of stimulated lymphocytes as measured by flow cytometry. Cancer Res. **37:** 4635–4640.

Darzynkiewicz Z., Bruno S., Del Bino G., Gorczyca W., Hotz M.A., Lassota P., Traganos F., (1992) Features of apoptotic cells measured by flow cytometry. Cytometry **13:** 795– 808.

Darzynkiewicz Z., Li X., Gong J., (1994) Assays of cell viability: discrimination of cells dying by apoptosis. In: Darzynkiewicz, Z., Robinson, J.P., Crissman, H.A. (Eds.), Flow Cytometry, 2nd edn. Methods Cell Biol., vol. 41. Academic Press, San Diego,pp. 16– 38.

Darzynkiewicz Z., Juan G., Li X., Gorczyca W., Murakami T., Traganos F., (1997) Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry. **27:** 1–20.

Degli Esposti, M. and Dive, C. (2003) Mitochondrial membrane permeabilisation by Bax/Bak. Biochem. Biophys. Res. Commun. **304:** 455-461.

De S.N. and Chatterjee. (1953) An experimental study of the mechanism of action of *vibrio cholerae* on the intestinal mucus membrane. J. Path. Bact. **66:** 559-562.

Dupoint H. L., Levine M. M., Hornick R. B., and Formal S. B. (1989) Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis. **159:** 1126-1128.

El-Deiry W.S. (2001). Insights into cancer therapeutic design based on p53 and TRAIL receptor signaling. Cell Death Differ. **8:** 1066-1075.

El-Gendy A., El-Ghorab N. E. M., Lane R. A., Elyazeed N. I. A., Carlin M. M., Mitry B. A., Kay S. J. Savarino, and Peruski L. F. Jr. (1999) Identification of *Shigella flexneri* subserotype 1c in Rural Egypt. J. Clin. Microbiol. **37:** 873-874.

Endo Y. and Tsurugi K. (1987) RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J Biol Chem **262:** 8128-8130.

Endo Y., Tsurugi K., Lambert J.M (1988a) The site of action of six different ribosome-inactivating proteins from plants on eukaryotic ribosomes: the RNA N-glycosidase activity of the proteins. Biochem Biophys Res Commun **150:** 1032-1036.

Endo Y., Tsurugi K., Yutsudo T., Takeda Y., Ogasawara T., Igarashi K. (1988b) Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur J Biochem **171:** 45-50.

***Enterobacteriaeae* Subcommittee Reports.** (1954) Int Bull Bacteriol Nomcnel Taxon. **4:** 1-94.

Epstein J.H. and Cleaver J.E. (1992) 3-aminobenzamide can act as a cocarcinogen for ultraviolet lightinduced carcinogenesis in mouse skin. Cancer Res **52:** 4053-4054.

Ewing W. H. (1949). The Relationship of *SHIGELLA* dispar to certain coliform bacteria. J. Bacteriol. **57:** 497

Ewing W. H. (1950). Shigella grouping antiserums. J. Lab. Clin. Med. **36:** 471.

Erwert R.D., Winn R.K., Harlan J.M. and Bannerman D.D. (2002) Shiga-like toxin inhibition of FLICE-like inhibitory protein expression sensitizes endothelial cells to bacterial lipopolysaccharide- induced apoptosis. J. Biol. Chem. **277:** 40567-40574.

Erwert R.D., Eiting K.T., Tupper J.C., Winn R.K., Harlan J.M. and Bannerman, D.D. (2003) Shiga toxin induces decreased expression of the anti-apoptotic protein Mcl-1 concomitant with the onset of endothelial apoptosis. Microb. Pathog. **35:** 87-93.

Ewing W. H. (1986) Edwards and Ewing's identification of *Enterobacteriaceae* Elsevier, Newyork, Amsterdam, Oxford. Fourth edition. pp137-172.

Fan S., El-Deiry W. S., Bae I., Freeman J., Jondle D., Bhatia K., Fornace A. J. Jr., Magrath I., Khon K. W., (1994) *p53* Gene Mutations Are Associated with Decreased Sensitivity of Human Lymphoma Cells to DNA Damaging Agents O'Connor P M. Cancer Res. **54:** 5824–5830.

Fischer H., Koenig U., Eckhart L. and Tschachler E. (2002) Human caspase 12has acquired deleterious mutations. Biochem. Biophys. Res. Commun. **293:** 722-726.

Flatt P. M., Tang L. J., Caroline D. S., Suzanne T., S., and Jennifer A. P. (2000) p53 Regulation of G_2 Checkpoint Is Retinoblastoma Protein Dependent Mol. Cell. Biol. **20:** 4210-4223.

Fasano A., Levine M. M., Nataro J. P., Noriega F. (1991) Isolated DNA molecule encoding ShET2 of Shigella flexneri 2a . Infect. Immun., **58:** 3717-3723

Fasano A., Noriega F., Mancvel D. R., Chanasongeram S., Rossell R., Guandalini S., and Levine M. M. (1995) *Shigella* enterotoxin 1: an enterotoxin of *Shigella flexneri* 2a active in rabbit small intestine in vivo and in vitro. J. Clin. Invest. **95:** 2853-2861.

Fasano A., F. Noriega F., Liao M. and Levine M. M. (1997) Effect of *Shigella* enterotoxin 1 (ShET1) on rabbit intestine in vitro and in vivo. Gut. **40:** 505-511.

Fontaine A., Arondel J., and Sansonetti P. J. (1988) Role of Shiga toxin in the pathogenesis of bacillary dysenteriae 1. Infect. Immun. **56:** 34099-34109.

Frantzen, E. (1950). Acta Pathol. Microbiol. Scand. **26:** 879.

Franziska A., Oberhammer, Hochegger K., Gertraud F., TiefenbacherR, and Pavelka M. (1994) Chromatin Condensation during Apoptosis Is Accompanied by Degradation of Lamin A +B, without Enhanced Activation of cdc2 Kinase

Frankel G., Giron J.A., Valmassoi J., Schoolnik G.K. (1989) Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. Mol Microbiol **3:** 1729-1734.

Fraser M.E., Chernaia M.M., Kozlov Y.V. and James M.N.G. (1994) Crystal structure of the holotoxin from Shigella dysenteriae at 2.5 AW resolution. Nat. Struct. Biol. **1:** 59-64.

Fujii J., Matsui T., Heatherley D.P., Schlegel K.H., Lobo P.I., Yutsudo T., Ciraolo G.M., Morris R.E. and Obrig T.G. (2003) mRapid apoptosis induced by Shiga toxin in HeLa cells. Infect. Immun. **71:** 2724-2735.

Gannon V.P. and Gyles C.L. (1990) Characteristics of the Shiga-like toxin produced by Escherichia coli associated with porcine edema disease. Vet Microbiol **24:** 89-100.

Gao L. Y. and Abu Kwaik Y. (2000) Hijacking of apoptotic pathways by bacterial pathogens. Microb Infect. **2:** 1705–1719.

Ghayur T., Banerjee S., Hugunin M., Butler D., Herzog L., Carter A., Quintal, L., Sekut L., Talanian R., Paskind M., Wong W., Kamen R., Tracey D., and Allen H. (1997) Nature **386:** 619–623

Gong J., Traganos F., Darzynkiewicz Z., (1994) A selective procedure for DNA extraction from apoptotic cells suitable for gel electrophoresis and flow cytometry. Anal. Biochem. **218:** 314–319.

Guillouf C., Rosselli F., Krishnaraju K., Moustacchi E., Hoffman B., Liebermann D. A. (1995) Oncogene.**10:** 2263–2270.

Gu Y., Kuida K., Tsutsui H., Ku G., Hsiao K., Fleming M. A., Hayashi N., Higashimo K., Okamura H., Nakanishi K., Kurimoto M., Tanimoto T., Flavell R. A., Sato V., Harding M. W., Livingston D. J., and Su M. S.-S. (1997) Science 275: 206–209

Gu Y., Turck C. W., Morgan D. O. (1993) Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. Nature (London). **366:** 707–710.

Hale T.L. and Formal S.B. (1980) Cytotoxicity of Shigella dysenteriae 1 for cultured mammalian cells. Am J Clin Nutr **33:** 2485-2490

Hale T.L. (1991) Genetic basis of virulence in Shigella species. Microbiol Rev **55:** 206-224.

Harrington E.A., Jacqueline L., Bruce, Harlow E.d., and Dyson N. (1998) pRB plays an essential role in cell cycle arrest induced by DNA damage. Proc. Natl. Acad. USA **95:** 11945- 11950.

Hartwell L. H. and Weinert T. A. (1989) Checkpoints: Controls That Ensure the Order of Cell Cycle Events. Science. **246**: 629–634.

Hartwell L. H. and Kastan M. B. (1994) Cell Cycle control and cancer . Science. **266:** 1821–1828.

Harper J. W., Adami G. R., Wei N., Keyomarsi K., Elledge S. J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases .Cell. **75:** 805–816.

Henderson B., Poole S. and Wilson M. (1996) Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60:** 316-341.

Hilbi H., Moss J. E., Hersh D. and 7 other authors. (1998) Shigellainduced apoptosis is dependent on caspase-1 which binds to IpaB. J Biol Chem **273:** 32895–32900.

Hoey D.E.E., Sharp L., Currie C., Lingwoo, C.A., Gally D.L. and Smith D.G.E. (2003) Verotoxin 1 binding to intestinal crypt epithelial cells results in localization to lysosomes and abrogation of toxicity. Cell. Microbiol. **5:** 85-97.

Hossain M. A., M. Rahman Q. S. Ahmed M. A. Malek R. B. Sack, and. Albert M. J. (1998) Increasing frequency of mecillinam-resistant *Shigella* isolates in urban Dhaka and rural Matlab, Bangladesh: a 6-year observation. J. Antimicrob. Chemother. **42:** 99-102

Hu C.C., Tang C.H., Wang J.J. (2001) Caspase activation in response to cytotoxic Rana catesbeiana ribonuclease in MCF-7 cells. FEBS Lett **503**: 65-68.

Huang L.C., Clarkin K.C., Wahl G.M. (1996) Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. Proc Natl Acad Sci U S A **93:** 4827-4832.

Hughes A.K., Stricklett P.K., Schmid D., Kohan D.E. (2000) Cytotoxic effect of Shiga toxin-1 on human glomerular epithelial cells. Kidney Int **57:** 2350-2359

Hurley B.P., Jacewicz M., Thorpe C.M., Lincicome L.L., King A.J., Keusch, G.T. and Acheson D.W.K. (1999) Shiga toxins 1 and 2translocate dilerently across polarized intestinal epithelial cells. Infect. Immun. **67:** 6670-6677.

Ikawa S., Nakagawara A. and Ikawa Y. (1999) p53 family genes: structural comparison, expression and mutation . Cell Death Differ. **6:** 1154-1161.

Irmler M., Thome M., Hahne M., Schneider P., Hofmann K., Steiner V., Bodmer J.-L., Schro ter, M., Burns K., Mattmann C., Rimoldi D., French L.E. and Tschopp J. (1997) Inhibition of death receptor signals by cellular FLIP. Nature. **388:** 190-195.

Irwin M., Marin M.C., Andrew C., Phillips R. S., Seelan D. I., Smith L. W., Elsa R.. Flores K. Y., Tsai J. T., Karen H. V. and William G. K. Jr. (2000) Role for the p53 homologue p73 in E2F-1-induced apoptosis Nature. **407:** 645 -648.

Islam D., Veress B., Bardhan P. K., Lindberg A. A., and Christensson B. (1997) In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis Infect. Immunol. **65:** 739–749

Ito H., Terai A., Kurazono H., Takeda Y., Nishibuchi M. (1990) Cloning and nucleotide sequencing of Vero toxin 2 variant genes from Escherichia coli O91:H21 isolated from a patient with the hemolytic uremic syndrome. Microb Pathog **8:** 47-60.

Iwata M., Hotta H., Higuchi K., Nishiuchi Y., Fujiwara Y., Arakawa T., Yano I., Kobayashi K. (2001) Induction of thymic apoptosis by Shiga toxin from Escherichia coli O157:H7 in vivo and in vitro. Osaka City Med J **47:** 11-22.

Jones N.L., Islur A., Haq R., Mascarenhas M., Karmali M.A., Perdue M.H., Zanke B.W., Sherman P.M. (2000) Escherichia coli Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. Am J Physiol Gastrointest Liver Physiol **278:** G811-819.

Kanduc D., Mittelman A., Serpico R., Sinigaglia E., Sinha A.A., Natale C., Santacroce R., di Corcia M.G., Lucchese A., Dini L., Pani P., Santacroce S., Simone S., Bucci R. and Farber E. (2002) Cell death: apoptosis versus necrosis. Int. J. Oncol. **21:** 165-170.

Kaper J.B. (1998) Enterohemorrhagic Escherichia coli. Curr. Opin. Microbiol. **1:** 103-108.

Karmali M.A., Petric M., Lim C., Fleming P.C., Steele B.T. (1983) Escherichia coli cytotoxin, haemolytic-uraemic syndrome, and haemorrhagic colitis. Lancet **2:** 1299-1300.

Karpman D., Hakansson A., Perez M.-T.R., Isaksson C., Carlemalm, E., Caprioli A. and Svanborg C. (1998) Apoptosis of renal cortical cells in the hemolytic-uremic syndrome: in vivo and in vitro studies. Infect. Immun. **66:** 636-644.

Kastan M. B., Onyekwere O, Sidransky D., Vogelstein B., Craig R. (1991) Cancer Res. **51:** 6304–6311.

Kauffmann, F. (1949) On the serology of the Klebsiella group. Acta. Pathol. Microbiol. Scand. **26:** 879.

Kerr J. F. R. (1971) Shrinkage necrosis: a distinct mode of cellular death. J. *Pathol.* **105:**13-20.

Keusch G.T., Jacewicz M., Donohue-Rolfe A. (1986) Pathogenesis of shigella diarrhea: evidence for an N-linked glycoprotein shigella toxin receptor and receptor modulation by beta-galactosidase. J Infect Dis **153:** 238-248.

Keusch, G.T. and Bennish M. L. (1998) Shigellosis. Evans *et. al.*, editors. Bacterial Infections of Humans: Epidemiology and control, 3rd edition. New York, Plenum Publishing Crop, pp. 631-656.

Keusch G. T. (1998) Shigellosis. In: Gorbach *et. al.*, editors. Infectious diseases. 2nd edition. Philadelphia, WB Saunders Company. pp 694-698.

Khanna KK., Keating K.E., Kozlov S., Scott S., Gatei M., Hobson K., Taya Y., Gabrielli B., Chan D., Lees-Miller S.P., Lavin M.F. (1998) ATM associates with and phosphorylates p53: mapping the region of interaction. Nat Genet **20:** 398-400.

Khanna K.K., Lavin M.F., Jackson S.P. and Mulhern T.D. (2001) ATM, a central controller of cellular responses to DNA damage Cell Death Differ **8:** 1052-1065.

Kim J.S., He L. and Lemasters J.J. (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem. Biophys. Res. Commun. **304:** 463-470.

Knudsen K,E,, DBooth D., Naderi S., Sever-Chroneos Fribourg A.F., Hunton I.C., Feramisco JR,Jean Wang J.R.J., and Erik S. and Knudsen E.S .(2000) RB-Dependent S-Phase Response to DNA Damage. Mol. Cell. Biol. **20:** 7751-7763.

Knutton S., Baldwin T.J., Haigh R.D., Williams P.H., Manjarrez- Hernandez A. and Aitken A. (1989) Actin accumulation at sites of bacterial adhesion to tissue culture cells : basis of a new diagnostic test for enteropathogenic and enterohemorrhagic Escherichia coli. Infect. Immun. **57:** 1290-1298.

Kodama T., Nagayama K., Yamada K., Ohba Y., Akeda Y. and Honda T. (1999) Induction of apoptosis in human renal proximal tubular cells by Escherichia coli verocytotoxin 1 in vitro. Med. Microbiol. Immunol. **188:** 73-78.

Kopecko, D. J., J. Holcombe, and Formal S. B. (1979) Molecular characterization of plasmids from virulent and spontaneously occurring avirulent colonial variants of *Shigella flexneri.* Infect. Immun. **24:** 580-582.

Korsmeyer S.J., Sallan S.E., Lander E.S., Golub T.R. (1999) MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia Cold Spring Harb. Symp. Quant. Biol. **64:** 343-350

Kuerbitz S. J., Plunkett B. S., Walsh W. V., Kastan M. B. (1992) Proc Natl Acad Sci USA. **89:** 7491–7495.

Lane D. P. (1992) p53, guardian of the genome Nature (London).**358:** 15–16.

Leland P.A., Staniszewski K.E., Kim B.M., Raines R.T. (2001) Endowing human pancreatic ribonuclease with toxicity for cancer cells. J Biol Chem **276:** 43095-43102.

Levrero M., Laurenzi V.D., Costanzo A., Gong J., G Melino G. and Wang J. Y. J. (1999) Structure, function and regulation of p63 and p73. Cell Death Differ. **6:** 1146-1153.

Li C-Y, Nagasawa H., Dahlberg W. K., Little J. B. (1995) Diminished capacity for p53 in mediating a radiation-induced G1 arrest in established human tumor cell lines. Oncogene. **11:** 1885–1892.

Lindahl T., Satoh M.S., Poirier G.G. and Klungland A. (1995) Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *Trends Biochem. Sci.* **20:** 405–411.

Lindberg A. A. and Pa´ I. T. (1993) Strategies for development of potential candidate Shigella vaccines. Vaccine **11:** 168–179

Lingwood C.A. (1996). Role of verotoxin receptors in pathogenesis. Trends Microbiol. **4:** 147-153.

Lin Z., Kurazono H., Yamasaki S., Takeda Y. (1993) Detection of various variant verotoxin genes in Escherichia coli by polymerase chain reaction. Microbiol Immunol **37:** 543-548.

Lissy N.A., Davis P.K., Irwin M., Kaelin W.G., Dowdy S.F.. (2000) A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. Nature **407:** 642- 645.

Little J. B., Nagasawa H., Keng P. C., Yu Y., Li C-Y. (1995) Absence of radiation-induced G1 arrest in two closely related human lymphoblast cell lines that differ in p53 status. J Biol Chem. **270:** 11033–11036.

Liu J., Akahoshi T., Sasahana T., Kitasato H., Namai R., Sasaki T., Inoue M. and Kondo H. (1999) Inhibition of neutrophil apoptosis by verotoxin 2de rived from Escherichia coli O157:H7. Infect. Immun. **67:** 6203-6205.

Livingstone L., White A., Sprouse J., Livanos E., Jacks T., Tlsty T. (1992) Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell. **70:** 923–935.

Lohrum M.A.E. and Vousden K.H. (1999). Regulation and activation of p53 and its family members Cell Death Differ. **6:** 1162- 1168.

Lowe S. W., Schmitt E. M., Smith S W., Osborne B. A., Jacks T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes Nature (London). **362:** 847–849.

Lowe S. W., Ruley H. E., Jacks T., Housman D. E. (1993) p53-dependent apoptosis modulates the cytoxicity of anticancer agents. Cell. **74:** 957–967.

Lu X. and Lane D.P. (1993) Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell **75:** 765-778.

Makino S., Sasakawa C., Kamata K., Kurata T., and Yoshikawa M. (1986) A virulence determinant required for continuous reinfection of adjacent cells on large plasmid in *Shigella flexneri* 2a. Cell. **46:** 551-555.

Malanga M., Pleschke J.M., Kleczkowska H.E. and Althaus F.R. (1998) Poly(ADP-ribose) binds to specific domains of p53 and alters its DNA binding functions. J Biol Chem **273:** 11839-11843.

Mamun K. Z., S. Tabassum M. A. H., and Shears P.. (1997) Antimicrobial susceptibility of *Shigella* from a rural community in Bangladesh. Ann. Trop. Med. Parasitol. **91:** 643-647.

Maurelli A. T., B. Baudry H., d'Hauteville T. L. H., and Sansonetti P. J. (1985) Cloning of plasmid DNA sequences involved in invasion of Hela cells by *Shigella flexneri.* Infect. Immun. **49:** 164-171

McDonough M.A. and Butterton J.R. (1999) Spontaneous tandem amplification and deletion of the Shiga toxin operon in Shigella dysenteriae 1. Mol. Microbiol. **34:** 1058-1069.

McIlwrath A. J., Vasey P. A., Ross G. M., Brown R. (1994) Cell cycle arrests and radiosensitivity of human tumor cell lines: dependence on wild-type p53 for radiosensitivity.Cancer Res. **54:** 3718–3722.

Mead P.S., Slutsker L., Dietz V., McCaig L.F., Bresee J.S., Shapiro C., Griffin P.M. and Tauxe R.V. (1999) Food related illness and death in the United States. Emerg. Infect. Dis. **5:** 607-625.

Melton-Celsa A.R. and O'Brien A.D. (1998) Structure, biology and relative toxicity of Shiga toxin family members for cells and animals. In: Escherichia coli O157:H7 and Other Shiga Toxin-Producing E. coli Strains (Kaper, J.B. and O'Brien, A.D., Eds.), pp. 121-128. ASM Press, Washington, DC.

Me´nard R., Dehio C., and Sansonetti P. J. (1996) Bacterial entry into epithelial cells: the paradigm of. Shigella Trends Microbiol. **4:** 220–226

Mills J. A., Buysse J. M. and Oaks E.V. (1988) *Shigella flexneri* invasion plasmid antigens B and C: epitope location and characterization with monoclonal antibodies. Infect. Immun. **56:** 2933-2942.

Milyavsky M., Mimran A., Senderovich S., Zurer I., Erez N., Shats I., Goldfinger N., Cohen I., Rotter V. (2001) Activation of p53 protein by telomeric (TTAGGG)n repeats. Nucleic Acids Res **29:** 5207-5215.

Miura M., Zhu H., Rotello R., Hartwieg E. A., and Yuan J. (1993) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis . Cell **75:** 653–660

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods **65:** 55-63.

Moxley R.A. and Francis D.H. (1998) Overview of animal models. In: Escherichia coli O157:H7 and Other Shiga Toxin-Producing E. coli Strains (Kaper, J.B. and O'Brien, A.D., Eds.), pp. 249-260. ASM Press, Washington, DC.

Nagasawa H., Li C-Y, Maki C. G., Imrich A. C., Little J. B. (1995) Loss of Normal G1 Checkpoint Control Is an Early Step in Carcinogenesis, Independent of p53 Status 1 Cancer Res..**55:** 1842–1846.

Nagata S. (2000) Apoptotic DNA fragmentation. Exp. Cell Res. **256:** 12-18.

Naheed A., Kalluri P., Talukder K.A., Faruque A.S., Khatun F., Nair G.B., Mintu E.D., Breiman R.F. (2004) Fluoroquinolone-resistant Shigella dysenteriae type 1 in northeastern Bangladesh. Lancet Infec Dis. **4(10):** 607-8.

Nakagawa I., Nakata M., Kawabata S. and Hamada S. (1999) Regulated expression of the Shiga toxin B gene induces apoptosis in mammalian fibroblastic cells. Mol. Microbiol. **33:** 1190-1199.

Nakagawa T., Zhu H., Morishima N., Li E., Xu J., Yankner B.A. and Yuan J. (2000) Caspase 12 mediates endoplasmic reticulum-specific apoptosis and cytotoxicity by amyloid-L. Nature **403:** 98-103.

Namiki Y., Zou L. (2006) ATRIP associates with replication protein A-coated ssDNA through multiple interactions. Proc Natl Acad Sci U S A **103:** 580-585.

Nataro J. P., J. A. Serwatana A. Fasano D. R. Maneval L. D. Guers F. Noriega F. Dubovsky M. M. Levine and Morris J. G. (1995) Identification and cloning of a novel plasmid-encoded enterotoxin of enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. Infect. Immun. **63:** 4721-4728.

Nicolas E., Beggs J.M., Taraschi T.F. (2000) Gelonin is an unusual DNA glycosylase that removes adenine from single-stranded DNA, normal base pairs and mismatches. J Biol Chem **275:** 31399-31406.

Nordstrom W. and Abram J (2000) Guardian ancestry: Fly p53 and damage-inducible apoptosisCell Death Differ. **7:** 1035- 1038.

Noriega F. R., Liao F. M., Formal S. B., Fasano A., and Levine M. M. (1995) Prevalence of *Shigella* enterotoxin 1 among *Shigella* clinical isolates of diverse serotypes. J. Infect. Dis. **172:**1408-1410.

Nur-E-Kamal A., Varga M., Maruta H. (1993) The GTPase-activating NF1 fragment of 91 amino acids reverses v-Ha-Ras-induced malignant phenotype. J Biol Chem **268:** 22331-22337.

Nur-E-Kamal A., Li T.K., Zhang A., Qi H., Hars E.S., Liu L.F. (2003) Single-stranded DNA induces ataxia telangiectasia mutant (ATM)/p53-dependent DNA damage and apoptotic signals. J Biol Chem **278:** 12475-12481.

Nur-E-Kamal A., Gross S.R., Pan Z., Balklava Z., Ma J., Liu L.F. (2004) Nuclear translocation of cytochrome c during apoptosis. J Biol Chem **279:** 24911-24914.

Oberhammer F., Fritseh G., Schrnied M., Pavelka M., Print.z D., Purchio T., Lassmann H., and Schnlte-Hermann R.. (1993a) Condensation of the chromatin at the nuclear membrane of an apoptotic nucleus is not associated with activation of an endonuclease. J. Cell Sci. **104**: 317-326.

Oberhammer F., Wilson J. W., Dive C., Morris I. D., Hickman J. A., Wakeling A. E., Walker P. R., and Sikorska M.. (1993b) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kilobase fragments prior to or in the absence of internucleosomal fragmentation. EMBO (Fur. Mol. Biol. Organ.) J. **12:** 3679-3684.

O'Brien A.D., Tesh V.L., Donohue-Rolfe A., Jackson M.P., Olsnes S., Sandvig K., Lindberg A.A. and Keusch G.T. (1992) clarification. Shiga toxins: biochemistry, genetics, mode of action and role in pathogenesis. Curr. Top. Microbiol. Immunol. **180:** 65-94.

Obrig T.G., Moran T.P., Brown J.E. (1987) The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. Biochem J **244:** 287-294

O'Connor P. M., Jackman J., Jondle D., Bhatia K., Magrath L, Kohn K. W. (1993) Role of the p53 tumor suppressor gene in cell cycle arrest and radiosensitivity of Burkitt's lymphoma cell lines. Cancer Res. **53:** 4776–4780.

O'Loughlin E.V. and Robins-Browne R.M. (2001) Effect of Shiga toxin and Shiga-like toxins on eukaryotic cells. Microb. Infect. **3:** 493-507.

Ormerod M.G., (1998) The study of apoptotic cells by flow cytometry. Leukemia **12:** 1013–1025.

Ormerod M.G. (2000) Flow cytometry in the study of apoptosis. In: Ormerod, M.G. (Ed.), Flow Cytometry: A Practical Approach, 3rd edn. IRL Press at Oxford University Press, Oxford, pp. 235– 247.

Ormerod, M.G., (2000 a). Analysis of DNA—general methods. In: Ormerod, M.G. (Ed.), Flow Cytometry: A Practical Approach, 3rd edn. IRL Press at Oxford University Press, Oxford, pp. 83–97.

Ormerod M.G., (2001) Using flow cytometry to follow the apoptotic cascade. Redox Report . **6:** 275–287.

Ormerod M.G. (2002) Investigating the relationship between the cell cycle and apoptosis using flow cytometry. J. Immunol Methods, **265:** 73–80.

Parsot C., and Sansonetti P. J. (1996) Invasion and the pathogenesis of *Shigella* infections. Curr. Top. Microbiol. Immuno. **209:** 25-42.

Paton A.W., Ratcliff R.M., Doyle R.M., Seymour-Murray J., Davos D., Lanser J.A., Paton J.C. (1996) Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing Escherichia coli. J Clin Microbiol **34:**1622-1627.

Paton A.W., Voss E., Manning P.A., Paton J.C. (1997) Shiga toxin-producing Escherichia coli isolates from cases of human disease show enhanced adherence to intestinal epithelial (Henle 407) cells. Infect Immun **65:** 3799-3805.

Paton J.C. and Paton A.W. (1998) Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. Clin. Microbiol. Rev. **11:** 450-479.

Pellegata N. S., Cajot J-F, Stanbridge E. J. (1995) The basic carboxy-terminal domain of human p53 is dispensable for both transcriptional regulation and inhibition of tumor cell growth. Oncogene. **11:** 337–349.

Piccoli R., Di Gaetano S., De Lorenzo C., Grauso M., Monaco C., Spalletti-Cernia D., Laccetti P., Cinatl J., Matousek J., D'Alessio G. (1999) A dimeric mutant of human pancreatic ribonuclease with selective cytotoxicity toward malignant cells. Proc Natl Acad Sci U S A **96:** 7768-7773.

Philpott D.J., Ackerley C.A., Kiliaan A.J., Karmali M.A., Perdue M.H. and Sherman P.M. (1997) Translocation of verotoxin 1 across T84 monolayers: mechanisms of bacterial toxin penetration of epithelium. Am. J. Physiol. **273:** G1349-G1358.

Philpott D.J., Edgeworth J.D., Sansonetti P.J. (2000) The pathogenesis of Shigella flexneri infection: lessons from in vitro and in vivo studies. Philos Trans R Soc Lond B Biol Sci **355:** 575-586.

Pijpers A.H.J.M., van Setten P.A., van den Heuvel L.P.W.J., Assmann K.J.M., Dijkman H.B.P.M., Pennings A.H.M., Monnens L.A.H. and van Hinsbergh V.W.M. (2001) Verocytotoxin-induced apoptosis of human microvascular endothelial cells. J. Am. Soc. Nephrol. **12:** 767-778.

Pozniak C.D., Radinovic S, Yang A., McKeon F., Kaplan D.R., Miller F.D. (2000) An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. Science. **289:** 304- 306.

Proulx F., Seidman E.G. and Karpman D. (2001) Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome. Pediatr. Res. **50:** 163-171.

Riley L.W., Remis R.S., Helgerson S.D., McGee H.B., Wells J.G., Davis B.R., Hebert R.J., Olcott E.S., Johnson L.M., Hargrett N.T., Blake P.A., Cohen M.L. (1983) Hemorrhagic colitis associated with a rare Escherichia coli serotype. N Engl J Med **308:** 681-685

Purnell M.R. and Whish W.J.D. (1980) Novel inhibitors of poly(ADP-ribose) synthetase. Biochem J **185:** 775-777.

Rahaman M. M., Khan M. M., Aziz K. M. S., Islam M. S., and Kibriya A. K. M. G. (1975) An outbreak of dysentery caused by *Shigella dysenteriae* type 1 on a coral island in the Bay of Bengal. J. Infect. Dis. **132:**15-19.

Robinson J.P., Carter W.O., Narayanan P.K. (1994) Oxidative Product Formation Analysis by Flow Cytometry. In Methods in Cell Biology: Flow Cytometry; Darzynkiewicz, Z., Robinson, J.P., Crissman, H.A., Eds.; Academic Press: New York, pp. 437–447.

Rowe B., and Gross R. J. (1981) The genus *Shigella*, the prokaryotes, volume 2 eds Starr MP, Stolph *et. al.*, Springer-verlag, Berlin, Heidelberg and Newyork, pp.1248-1259.

Ruscetti T., Lehnert B.E., Halbrooks J., Trong H.L., Hoekstra M.F., Chen D.J. and Peterson S.R. (1998) Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase. J Biol Chem **273:** 14461-14467.

Sakamoto C., Suzuki K., Hato F., Akahori M., Hasegawa T., Hino M., Kitagawa S. (2003) Antiapoptotic effect of granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and cyclic AMP on human neutrophils: protein

synthesis-dependent and protein synthesis-independent mechanisms and the role of the Janus kinase-STAT pathway. Int J Hematol **77:** 60-70.

Sansonetti P. J., Kopecko D. J., and Formal S. B. (1982) Involvement of a plasmid in the invasive ability of *Shigella flexneri.* Infect. Immun. **35:** 852-860.

Sansonetti P. J., Hale T. L., Dammin G. J., Kapfer C., Collins H., and Formal S. B. (1983) Alterations in the pathogenecity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri.* Infect. Immun. **39:**1392-1402.

Sansonetti P. J., and Phalipon A. (1996) Shigellosis: From molecular pathogenesis of infection to protective immunity and vaccine development. Res. Immuno. **147:** 595-602.

Sansonetti P.J. (1998) Molecular and cellular mechanisms of invasion of the intestinal barrier by enteric pathogens. The paradigm of Shigella. Folia Microbiol (Praha) **43:** 239-246

Sandvig K. (2001) Shiga toxins. Toxicon **39:** 1629-1635.

Sandvig K. and van Deurs B. (2002) Transport of protein toxins into cells: pathways used by ricin, cholera toxin and Shiga toxin. FEBS Lett. **529:** 49-53.

Sandvig K., van Deurs B. (1992) Toxin-induced cell lysis: protection by 3-methyladenine and cycloheximide. Exp Cell Res **200:** 253-262.

Sanyal S.C., Saraswathi B., Sharma P. (1980) Enteropathogenicity of Plesiomonas shigelloides. J Med Microbiol; **13:** 401-409.

Sasakawa C., Kamata K., Sakai T., Murayama S. Y., Makino S., and Yoshikawa M.. (1986) Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and Congo red binding activity in *Shigella flexneri.* Infect. Immun. **51:** 470-475.

Sasakawa C., Kamata K., Sakai T., Makino S., Yamada M., Okada N., and Yoshikawa V. (1988) Virulence-associated genetic regions comprising 31 kilobase of the 230-kilobase plasmid in *Shigella flexneri* 2a. J. Bacteriol. **170:** 2480-2484.

Saxena S.K., O'Brien A.D., Ackerman E.J. (1989) Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA N-glycosidases of 28 S RNA when microinjected into Xenopus oocytes. J Biol Chem **264:** 596-601.

Schein C.H. (1997) From housekeeper to microsurgeon: the diagnostic and therapeutic potential of ribonucleases. Nat Biotechnol **15:** 529-536.

Schiavone N., Papucci L., Luciani P., Lapucci A., Donnini M., Capaccioli S. (2000) Induction of apoptosis and mitosis inhibition by degraded DNA lipotransfection mimicking genotoxic drug effects. Biochem Biophys Res Commun **270:** 406-414.

Schmidt, H. (2001) Shiga toxin-coverting bacteriophages. Res. Microbiol. **152:** 687-695.

Schmitt C.K., Meysick K.C. and O'Brien A.D. (1999) Bacterial toxins: friends or foes? Emerg. Infect. Dis. **5:** 224-234.

Schmitz K. E. F. (1917) Eine neuer types aus der grouppe der ruhrbazillen als erreger einer grosseren epidemic. Z. Hyg. Ifekt. Kr. **84:** 449-516.

Shahid N. S., Rahaman M. M., Haider K., Banu H., and Rahman N.. (1985) Changing pattern of resistant Shiga bacillus (*Shigella dysenteriae* type 1) and *Shigella flexneri* in Bangladesh. J. Infect. Dis. **152:**1114-1119.

Shiloh Y., Kastan M.B. (2001) ATM: genome stability, neuronal development, and cancer cross paths. Adv Cancer Res **83:** 209-254.

Shiloh Y. (2003) ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer **3:** 155-168.

Sereny B. (1957. Experimental keratoconjunctivitis *Shigella*. Acta. Microbiol. Acad. Sci. Hung. **2:** 293-296.

Shears P. (1996) *Shigella* infections.. Ann. Trop. Med. Parasitol. **90:**105-114.

Shiga K. (1898). Dysentery bacillus. Zentralbl. Bakteriol. **23:** 913-918.

Siegler R.L. (1992) Central nervous system involvement in the hemolytic uremic syndrome. In: Hemolytic Uremic Syndrome and Thrombotic Thrombocytopenic Purpura (Kaplan, B.S., Trompeter, R.S. and Moake, J.L., Eds.), pp. 113-129. Marcel Dekker, New York.

Singh S. J., S.C. Sanayal. (1978) Enterotoxicity of the so called NAG vibrios. Ann. Soc. Belg Med. Trop. **58:** 133-140

Slebos R. J. C., Lee M. H., Plunkett B. S., Kessis T. D., Williams B. O., Jacks T., Hedrick L., Kastan M. B. (1994) Cho K. Proc Natl Acad Sci USA **91:** 5320–5324. **Slichenmyer W J, Nelson W G, Slebos R J, Kastan M B.** (1993). Cancer Res. **53:** 4164–4168.

Smulson M.E., Simbulan-Rosenthal C.M., Boulares A.H., Yakovlev A., Stoica B., Iyer S., Luo R., Haddad B., Wang Z.Q., Pang T., Jung M., Dritschilo A. and Rosenthal D.S. (2000) Roles of Poly(ADP-ribosyl)ation and PARP in apoptosis, DNA repair, genomic stability and functions of p53 and E2F-1. Advan Enzyme Regul **40:** 183-215.

Smith W.E., Kane A.V., Campbell S.T., Acheson D.W.K., Cochran B.H. and Thorpe C.M. (2003) Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. Infect. Immun. **71:** 497-504.

Stennicke H.R. and Salvesen G.S. (2000) Caspases - controlling intracellular signals by protease zymogen activation. Biochim. Biophys. Acta **1477:** 299-306.

Stoll B. J., Glass R. I., Huq M. I., Khan M. U., Banu H., and Holt J. (1982) Epidemoiologic and clinical features of patients infected with *Shigella* who attended a diarrhoeal disease hospital in Bangladesh. J. Infect. Dis. **146:** 177-183.

Stewart N., Hicks G. G., Paraskevas F., Mowat M. (1995) **Evidence for a second cell cycle block at G2/M by p53.** Oncogene. **10**:109–115.

Suzuki A., Doi H., Matsuzawa F., Aikawa S., Takiguchi K., Kawano H., Hayashida M. and Ohno S. (2000) Bcl-2 antiapoptotic protein mediates verotoxin II-induced cell death: possible association between Bcl-2and tissue failure by E. coli O157:H7. Genes Dev. **14:** 1734-1740.

Talukder K. A. Personal communication

Talukder K. A., Dutta D. K., and Albert M. J. (1999) Evaluation of pulsed field gel electrophoresis for typing of *Shigella dysenteriae* type 1. J. Med. Microbiol. **48:** 781-784.

Talukder K. A., Dutta D. K., Safa A., Ansaruzzaman M., Hassan F., Alam K., Islam K. M. N., Carlin N. I. A., Nair G. B., and Sack D. A. (2001) Altering trends in the dominance of *Shigella flexneri* serotypes and emergence of serologically atypical *S. flexneri* strains in Dhaka, Bangladesh. J. Clin. Microbiol. **39:** 3757-3759.

Talukder K. A., Dutta D.K., Islam M. A., Safa A., Hassan F., Faruque A. S. G., MHossain. A., Alam K., Nair G. B., andSack D. A. (2002) Epidemiology and molecular characterization of *Shigella flexneri* in Bangladesh. 10th Annual scientific conference (ASCON) pp.114.

Talukder K. A., Islam Z., Islam M. A., Dutta D. K., Safa A., Ansaruzzaman M., Faruque A.S.G., Shahed S. N., Nair G. B., and Sack D. A.. (2003) Phenotypic and genotypic characterization of provisional serotype *Shigella flexneri* 1c and clonal relationships with 1a and 1b strains isolated in Bangladesh. In press. J. Clin. Microbiol. **41:** 2490-2497

Talukder K.A., Khajanchi B.K., Islam M.A., Dutta D.K., Islam Z., Safa A., Khan G.Y., Alam K., Hossain M.A., Malla S., Niyogi S.K., Rahman M., Watanabe H., Nair G.B., Sack D.A. (2004) Genetic relatedness of ciprofloxacin-resistant Shigella dysenteriae type 1 strains isolated in south Asia.J Antimicrob Chemother. **54:** 730-734.

Tayeb K. and William T.B. (1999) Mebarone, a catalytic inhibitor of DNA topoisomerase II, induces apoptosis in CEM cells through activation of ICE/ CED-3-like protease. Mol pharmacology, **55:** 548-556.

Tidd D.M., Spiller D.G., Broughton C.M., Norbury L.C., Clark R.E., Giles R.V. (2000) Oligodeoxynucleotide 5mers containing a 5'-CpG induce apoptosis through a mitochondrial mechanism in T lymphocytic leukaemia cells. Nucleic Acids Res **28:** 2242-2250.

Thornberry N. A., Rano T. A., Peterson E. P. and 9 other authors. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. J Biol Chem **272:** 17907–17911.

Thorpe C.M., Hurley B.P., Lincicome L.L., Jacewicz M.S., Keusch G.T. and Acheson D.W.K. (1999) Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. Infect. Immun. **67:** 5985-5993.

te Loo D.M.W.M., Monnens L.A.H., van der Velden T.J.A.M., Vermeer M.A., Preyers F., Demacker P.N.M., van den Heuvel L.P.W.J. and van Hinsbergh V.W.M. (2000) Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. Blood **95:** 3396-3402.

te Loo D.M.W.M., van Hinsbergh V.W.M., van den Heuvel L.P.W.J. and Monnens L.A.H. (2001) Detection of verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with hemolytic uremic syndrome. J. Am. Soc. Nephrol. **12:** 800-806.

Tong W, Hande M. P., Lansdorp P M., and Wang Z. (2001) DNA Strand Break-Sensing Molecule Poly(ADP-Ribose) Polymerase Cooperates with p53 in Telomere Function, Chromosome Stability, and Tumor Suppression Mol. and Cell. Biol, **21:** 4046-4054.

Towbin H., Stachlin T., Gordon J. (1979) **Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications.** Proc Natl Acad Sci USA . **76:** 4350–4354.

Vargas M., Gascon J., de Anta M. T. J., and Vila J. (1999) Prevalence of *Shigella* enterotoxin 1 and 2 among *Shigella* strains isolated from patients with Traveler's diarrhoea. J. Clin. Microbiol. **37:** 3608-3611.

Van Setten P.A., van Hinsbergh V.W., Van den Heuvel L.P., van der Velden T.J., van de Kar N.C., Krebbers R.J., Karmali M.A., Monnens L.A. (1997) Verocytotoxin inhibits mitogenesis and protein synthesis in purified human glomerular mesangial cells without affecting cell viability: evidence for two distinct mechanisms. J Am Soc Nephrol **8:** 1877-1888.

Venkatesan M. M., Buysse J. M., Vandendries E. V., and Kopecko D. J. (1988) Development and testing of invasion associated DNA probes for the detection of *Shigella* Spp. and enteroinvasive *Escherichia coli*. J. Clin. Microbiol. **26:** 261-266.

Venkatesan M. M., Buysse J. M., and Kopecko D. J.. (1989) Use of *Shigella flexneri ipaC* and *ipaH* gene sequences for the general identification of *Shigella* spp. and enteroinvasive *Escherichia coli*. J. Clin. Microbiol. **27:** 2687-2691.

Victora C. G., Huttly S. R. A., Fuchs S. C., Barros F. C., Garenne M., Leroy O., Fontaine O., Beau J. P., Fauveau V., Chowdhury H. R., Yunus M., Chakraborty J., Sarder A. M., Kapoor S. K., Bhan M. K., Nath L. M., and Martines J. C. (1993) International differences in clinical patterns of diarrhoeal deaths: a comparison of children from Brazil, Senegal, Bangladesh and India. J. Diarrhoeal Dis. Res. **11:** 25-29.

Whitacre C.M., Hashimoto H., Tsai M.L., Chatterjee S., Berger S.J., Berger N.A. (1995) Involvement of NAD-poly(ADP-ribose) metabolism in p53 regulation and its consequences. Cancer Res **55:** 3697–3701

Wylie A.H. (1997) Apoptosis: an overview. Br Med Bull **53:** 451-456

World Health Organization. (1987) Programme for control of diarrhoeal disease. In: Manual for laboratory investigation of acute enteric infections. World Health Organization, Geneva. **CDD/93.3, Rev.1**, pp.9-20.

World Health Organization. (1999). Genetic protocol to estimate the burden of *Shigella* diarrhoea and dysenteric mortality. **WHO/V and B/99**

Xiong Y., Hannon G. J., Zhang H., Casso D., Kobayashi R., Beach D. (1993) Nature (London). **366:** 701–704.

Yang A., Walker N., Bronson R., Kaghad M., Oosterwegel M., Bonnin J., Vagner C., Bonnet H., Dikkes P., Sharpe A., McKeon F., Caput D. (2000) p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature **404:** 99-103

Yin Y., Tainsky M., Bischoff F. Z., Strong L. C., Wahl G. M. (1992) Wild-type p53 restores cell cycle control and inhibits gene amplification in celis with mutant p53 alleles. Cell. **70:** 937–948.

Zimmermann K.C., Bonzon C. and Green D.R. (2001) The machinery of programmed cell death. Pharmacol. Ther. **92:** 57-70.

Zychlinsky A., Prevost M. C., and Sansonetti P. J. (1992) Shigella flexneri induces apoptosis in infected macrophages. Nature **358:** 167–168

Zychlinsky A., Thirumalai K., Arondel J., Cantey J. R., Aliprantis A., and Sansonetti P. J. (1996) In vivo apoptosis in Shigella flexneri infections. Infect. Immunol. **64:** 5357–5365

Zychlinsky A., and Sansonetti P. J. (1997) Apoptosis as a proinflammatory event: what can we learn from bacteria-induced cell death? Trends Microbiol. **5:** 201–204

APPENDIX-1

Media Composition

The composition of the media used in this thesis work are given below:

Unless otherwise mentioned all media were autoclaved at 120^0 C for 15 minutes at 15 lbs pressure.

1. MacConkey Agar (DIFCO)

Ingredients	Amounts (g/l)
Peptone	17.0
Protease peptone	3.0
Lactose	10.0
Bile salts no. 3	1.5
Sodium Chloride	5.0
Agar	13.5
Neutral red	0.03
Crystal Violet	0.001
Distilled Water	1l
Final pH = 7.1 ± 0.2	

2. Trypticase soy borth

Ingredients	Amounts (g/l)
Pancreatic digest of casein	15.0
Papaic digest of soyabean meal	5.0
Sodium Chloride	5.0
Distilled Water	1l
Final pH = 7.3 ± 0.2	

3. Dulbecco's Modified Eagles Medium (DMEM)*

Ingredients	Amounts (g/l)
Glucose	High
L-glutamine	Supplemented
Pyridoxine HCl	Supplemented
Na-pyruvate	110 mg/L
$NaHCO_3$	3.7 g/L
Distilled water	1L
pH	7.2 ± 0.2

Note: Should be store at 2-8° C with Dark and dry condition

APPENDIX-II

Preparation of the stock solutions used in this thesis work are given below:
(All the working solutions used in the work were prepared from the stock solutions).

Tris-HCl

121.1 g tris-base was dissolved in 800 ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl, and the final volume was made up to 1l with distilled water. The solution was sterilized by autoclaving and stored at room temperature (RT).

3 M NaCl

175.3 g of NaCl was dissolved in distilled water to a final volume of 1l. The solution was autoclaved and stored at RT.

Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 2 g of KH_2PO_4 in 800 ml of distilled water. pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1l by distilled water. The solution was sterilized by autoclaving for 20 minutes and stored at RT.

RNase A stock solution (10 mg/ml)

RNase A stock solution was prepared by dissolving 100 mg of RNase A in 10 ml of distilled water and stored in -20° C until use.

TE buffer (pH 8.0)

10 mM tris-Cl (pH 8.0), 1 mM EDTA was prepared by diluting concentrated stocks of 1M tris-Cl (pH 8.0) and 0.5 M EDTA. The buffer was stored at 4^{0} C.

TBE buffer (GIBCO-BRL)

The total content of a bag having the formula of 100 mM tris, 90 mM Boric acid, 1.0 mM EDTA was mixed with 0.99 l of distilled water to make the 1x concentrated TBE buffer. The buffer was stored at RT.

Ethidium bromide solution

Ethidium bromide was dissolved in distilled water at a concentration of 10 mg/ml and stored at 4^0 C in the dark.

SDS Laemmli Buffer

0.5 Tris HCL(pH)	1.0 ml
Glycerol	0.4 ml
10% Sucrose	0.4 ml
10% SDS	1.6 ml
2-marcapto ethanol	0.4 ml
5% bromo phenol blue	80 µi
Distilled Water	To make 8 ml sample buffer

Gel loading buffer

6% concentrated loading buffer consisted of 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (type 400; Pharmacia), 0.5 µg/ml Rnase in water. it was stored at 4^0 C in 1 ml aliquot.

Separating Gel:

Separating Gel	10%	12.5%
Distilled water	1.8 ml	1.325 ml
Lower Gel Buffer	1.25 ml	1.25 ml
Acryl amide Bis Acryl amide solution	1.9 ml	2.375 ml
10% SDS	50 ml	50 ml
10% APS	4.5 ml	4.5 ml
TEMED	20 ml	20 ml
	5.70 ml	5.70 ml

Acrylamide
30% X V1 = 5.70 X 10 %

V1= 5.70 X 10 / 30
= 1.9 ml

Acrylamide
30% X V1 = 5.70 X 12.5 %

V1= 5.75 X 12.5 / 30
= 2.375 ml

Spacer Gel/Stacking Gel: 5%

Spacer Gel/Stacking Gel	5%
Distilled water	1.4 ml
Upper Gel Buffer	650 ml
Acryl amide Bis Acryl amide solution	400 ml
10% SDS	50 ml
10% APS	2.5 ml
TEMED	10 ml

Upper Gel Buffer (TRIS CHLORIDE 0.5 M)

121.1/2 gm Tris base dissolved in 800 ml of DH_2O then adjust the P_H to 6.8

Lower Gel Buffer (TRIS CHLORIDE 1.5 M)

121.1*3/2 gm Tris base dissolved in 800 ml of DH_2O then adjust the P_H to 8.8

Running Buffer ph 8.3

Tris 3.0 gm, 14.4 gm Glycine, SDS 1 gm, 1000 ml DH_2O

10% SDS

10 gm SDS Dissolved in 100 ml DH_2O

30% Acryl amide and Bis Acryl amide

29.5 gm Acryl amide and .5 gm Bis Acryl amide Dissolved in 100 ml DH_2O.

10% APS

10 gm APS dissolved in 100 ml DH_2O.

0.1% BMB (Tracking dye)
0.1% BMB, DH_2O 100 ml.

Commassie Stain

Commassie 0.25 gm, Methanol 45 ml, Acetic Acid 10 ml, Distilled Water 45 ml.

Destain

Methanol 45 ml, Acetic Acid 10 ml, Distilled Water 45 ml

TEMED

Stock present

Sample Loading Buffer
0.5 M Tris Chloride 1ml, 10% SDS 1 ml, B- markepto ethanol 0.1 ml, glycerol 1 ml, Water 1.9 ml.