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Salmonella enterica serovar Typhimurium promotes apoptosis in activated neutrophils

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SALMONELLA ENTERICA SEROVAR TYPHIMURIUM PROMOTES APOPTOSIS IN ACTIVATED NEUTROPHILS.

BY

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ABSTRACT

SALMONELLA ENTERICA SEROVAR TYPHIMURIUM PROMOTES APOPTOSIS IN ACTIVATED NEUTROPHILS.

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Apoptosis is an extremely important and highly regulated function in neutrophils. As the most abundant, but shortest-lived white blood cells, they must initiate this process in the absence of pathogens and selectively release their potent cytotoxic components when pathogens are present. Many pathogens are able to modulate this process in neutrophils, either for the host's benefit or for their own. Previous work has shown that transmigration across a T84 intestinal epithelial crypt cell monolayer increases the ability of neutrophils to bind and kill *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium).

The goal of this work was to see how *S.* Typhimurium affects the cell death rate of human PMN. *S.* Typhimurium had little to no effect on inactivated neutrophils. When neutrophils were activated, either chemically through phorbol 12-myristate 13-acetate (PMA) or physiologically through transmigration, however, their normal apoptosis rate was delayed ($p<0.0001$). When *S.* Typhimurium was present at a ratio of ten bacteria to one neutrophil, apoptosis

was significantly increased in these activated neutrophils more prominently when anaerobically grown (p=0.0027) compared to aerobically grown (p=.012).

Caspase-3 (p=0 .0129), but not -8 or -9, was associated with bacterial induced apoptosis in these studies. We conclude that *S.* Typhimurium modulates the rate of apoptosis in neutrophils, but only once these host cells have become activated. Our results provide further insights into the early events involved in *Salmonella* infections.

CHAPTER I

INTRODUCTION

Salmonella-associated Diseases

Salmonella species are Gram-negative, facultative anaerobic bacteria transmitted through the fecal-oral route that invade the enteric tract in humans and cause a wide variety of diseases. The type of disease seen in humans depends upon the serotype with which the patient is infected. This can range from mild gastroenteritis and similar food-borne illnesses (salmonellosis), caused by *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) and others, to sometimes fatal systemic illnesses like typhoid fever caused by *Salmonella enterica* serotype Typhi (*S.* Typhi). There are approximately 2000 serotypes that cause human disease but the three main serotypes seen in the United States are *S.* Typhimurium, *Salmonella enterica* serotype Enteritidis (*S.* Enteritidis) and *Salmonella enterica* serotype Newport (*S.* Newport) (CDC, 2005). Almost every case of disease in humans and domesticated animals belong to one of the serotypes of *enterica* (Fierer, 2001).

It is estimated that there are approximately 1.4 million cases of salmonellosis in the U.S. per year, but not all cases are actually reported and/or confirmed by culture. The actual current number of co-culture confirmed cases of salmonellosis per year is approximately 30,000 with greater than 500 of these

cases becoming fatal (CDC, 2005). This is estimated to cost approximately $3 billion annually in the U.S. alone (USDA, 2003). Cases of salmonellosis caused by either *S.* Typhimurium and *S.* Enteritidis have decreased 24% and 22% respectively since 1996, but cultures collected recently have shown increasing resistances to many antibiotics (CDC, 2005). This resistance is especially seen in fluoroquinolones, which is thought to be due to their use in livestock, since the prevalence of fluoroquinolone-resistant *Salmonella* increased rapidly after its use was sanctioned in food animals (WHO, 2005) Such clinical challenges make it more important to understand all the mechanisms behind *Salmonella* infections.

Infection from *Salmonella* species in humans normally occurs from ingestion of contaminated water and of food of animal origin including, but is not limited to meat, poultry, eggs and milk. Cases have been reported from vegetables infected from manure used as fertilizer as well. Transmission can also occur via human-to-human and animal-to-human contact (WHO, 2005). Symptoms of infection by *Salmonella* usually manifest within 5-7 days after the initial contact and include headaches, fever, abdominal cramps and diarrhea (CDC, 2005). Normally, approximately 10^5 to 10^{10} organisms are needed to overcome stomach pH and competition with normal microbiota to establish an infection (Darwin, 1999). Once colonization in the lower intestine has occurred, the bacterium begins to invade the mucosal layer, where it encounters host defenses. Diarrhea is one such defense against infection, as the production of fluid is a response generated in order to flush the intestines of the invading organisms. Healthy adults can usually resolve the infection without intervention,

but salmonellosis can be a severe problem in immunocompromised patients, infants, and the elderly where it can spread from the intestinal mucosa to the bloodstream and cause a systemic infection (CDC, 2005).

Salmonella Interaction with the Intestinal Epithelium

Since it is an intracellular pathogen, *Salmonella* must first gain entry into host cells. The bacterium achieves this through specific virulence factors affecting both the surface of the host cell and its intracellular components. About 4% of the *S.* Typhimurium genome encodes for virulence factors (Bowe et al., 1998). Once the bacterium comes into close contact with the host cells, it initiates a novel program, which basically allows a normally non-phagocytic cell to phagocytose the bacterium, helping bypass host defenses. Depending on the host, *Salmonella* targets different cells in the epithelium for invasion. For instance in the case of the mouse *Salmonella* will target M cells (Jones, 1994), while in calves it will target both M cells and enterocytes, but prefers neither (Santos, 2002). *Salmonella* is able to do this through one of two type III secretion systems encoded in its genome, specifically at Salmonella pathogenicity island-1 (SPI-1), which contains genes involved in intracellular invasion (Galán, 1999). There are at least 19 proteins secreted by this system that act in conjunction to deliver the bacterial proteins into the host cell (Galán and Collmer, 1999). Four of these proteins (SpaO, InvJ, PrgI and PrgJ) are part of what is termed the "needle complex", a rigid complex spanning both the inner and outer membranes of the bacterial membrane, which is structurally similar to

flagellar complexes (Collazo et al., 1995). Three other proteins are needed for protein translocation across the host cell membrane, specifically SipB, SipC, and SipD (Collazo and Galán, 1997). All other proteins injected into the host are known as effector proteins and carry out various functions inside the host cell (Galán, 2001).

Salmonella uses these proteins to up- and down-regulate host cell signals to enter host epithelial cells in the intestines in a similar manner to that of phagocytosis. It is able to do this by rearranging the actin filaments in the cytoskeleton of the host cells, which creates what are termed "membrane ruffles" that engulf the bacterium. The first bacterial effector proteins involved are SopE1 and SopE2, which act as guanine nucleotide exchange factors for Rho GTPases, specifically Cdc42 and Rac1, two proteins in the host cells that regulate the actin cytoskeleton (Hardt et al., 1998). A mutant strain of *Salmonella* lacking SopE1 and SopE2 can still initiate cytoskeletal rearrangements. It achieves this through an alternative effector protein, SopB, which, unlike SopE and SopE2, does not act as an exchange factor for Rho GTPases (Galyov, 1997). Instead, the mode of action for SopB is through the manipulation of phosphoinositide metabolism, which indirectly stimulates Cdc42, but not Rac1 (Zhou et al., 2001).

Other important effector proteins involved in the initial cytoskeletal rearrangements are SipA and SipC, which bind actin and alter the dynamics of the actin in the host cell (Finlay, 2000). SipA is required for the bacterium to enter the host cell efficiently. It acts by inhibiting depolymerization of F-actin filaments causing them to undergo conformational changes, which triggers them

to straighten and lose their natural shape (Zhou et al. 1999). The exact method by which the bacterium achieves this is unknown. SipC has two functions; the first is the translocation of the bacterial proteins across the host membrane. SipC also works to modulate actin inside the host cell, but does so in a slightly different method than SipA. Specifically it has been shown that SipC is able to nucleate and bundle actin *in vitro* (Hayward, 1999), although a mutant lacking in all three activators of Rho GTPases (SopE, SopE2, SopB) cannot initiate cytoskeletal arrangement despite the fact SipC is fully able to translocate across the host membrane (Zhou et al., 2001). This indicates that SipC alone cannot mediate bacterial entrance into the host cell, despite its actin modulating properties.

The final step in *Salmonella* invasion is the reversal of all the previous steps to return the cell to its normal conformation. The effector protein responsible for this is SptP, and mutants lacking this protein are able to initiate the cytoskeletal rearrangements but unable to return the host cell back to its normal configuration (Fu, 1999). While SopE1 and SopE2 act as exchange factors for Rho GTPases, SptP has GTPase-activating protein activity for Rac1 and Cdc42, but not for other related GTPases like Rho (Fu, 1999). In essence, when *Salmonella* releases SptP it is inactivating what SopE1 and SopE2 originally activated and returning the host cell to its original state, except that the bacterium is now inside the cell and can more easily evade host immune responses. Once inside the host cell, *Salmonella* downregulates SPI-1, and upregulates SPI-2, which is responsible for intracellular survival.

Innate Host Immune Response in the Intestinal Lumen

The innate immune system is extremely complex and this review will focus on only some of the major contributors involved in the initial association of *Salmonella* and the intestinal epithelium. The first line of defense against most pathogens is the epithelial gut lining, which acts as a barrier to small molecules and ions as well as invading pathogens (Köhler, 2002). Since *Salmonella* has developed strategies to bypass this barrier, as described above, other host defenses play a more significant role in providing protection against the invading bacteria. One of the first defense mechanisms comes from specialized enterocytes called goblet cells, which secrete large amounts of mucus. This mucus serves to aggregate adherent bacteria in the gut and reduce the force of the luminal stream (Neish, 2002). Another common host response involves flushing out the invading pathogens through fluid release. Typically, when epithelial cells receive certain signals, e.g., a spike in cAMP concentration, the enterocytes will release a chloride-rich fluid that causes the diarrhetic symptoms seen with *Salmonella* infection (Neish 2002). Another possible source of fluid release is the direct tissue damage caused by the influx of polymorphonuclear leukocytes (PMN or neutrophils) to the site of infection (Zhang, S., 2003). Epithelial cells also release various factors to communicate with and alter the hosts immune cells to better fight infection (Canny, 2006).

Neutrophil Response

Neutrophils play an important role in the innate host immune response. They are short-lived, typically living about one to two days *in vivo* or about twelve hours *in vitro,* but are very potent killers during that time. They are also the most abundant leukocyte in the body, comprising up to 80% of the population of white blood cells. Typically, they are the first immune cells to arrive at the site of infection and attempt to kill as many pathogens as possible through phagocytosis and the release of reactive oxygen species (ROS) and cytotoxic granules. They also play a key role in inflammation and contribute widely to inflammatory diseases (Kobayashi, 2003). At the onset of infection, large numbers of PMN will migrate to the site of infection to destroy invading pathogens. A problem arises when the host cells themselves are also exposed to these cytotoxic components, and this may lead to the destruction of host tissue along with the pathogen, should the neutrophil undergo these defense processes (Weiss, 1989).

The first step in the neutrophil response is the migration of large quantities of neutrophils from circulation to the site of infection. To do this, the PMN must travel across the endothelium, through the lamina propria and across the epithelial layer to get into the intestinal lumen, and they do this in response to specific chemotactic signals released by epithelial cells, other phagocytes, and products of the pathogenic organisms themselves (McCormick, 1998). The most common and well-known chemoattractants are microbe-derived *N*-formyl peptides (Omann, 1987), which cause neutrophils to polarize and orient their

leading edge in the direction of the highest concentration of the chemoattractant and begin migrating towards it. *Salmonella*, however, does not use this method of migration (McCormick 1993). Instead, *Salmonella* induces the production of a substance called pathogen-elicited epithelial chemoattractant (PEEC) by epithelial cells, which serves to stimulate neutrophil transmigration in a manner independent of the *N*-formyl peptide receptor pathway (McCormick, 1998). The PEEC pathway is similar to other chemoattractants in most areas except that with PEEC there is no upregulation of superoxide production or degranulation of the PMN (McCormick, 1998). Transmigrated neutrophils also have altered surface properties after crossing the intestinal epithelial barrier. There is an upregulation of the CD47 receptor, but this has no effect on the number of bacteria internalized by the neutrophil, suggesting it plays a role in helping the PMN transmigrate, but does not effect phagocytosis (Hofman, 2000). Complement receptor 3 (CR3, CD11b/CD18) on the other hand, is also upregulated upon transmigration (Hofman, 2000) and has previously been described as playing an important role in phagocytosis of complement opsonized (Detmers, 1987) and unopsonized (Gresham, 1991) bacteria, and in the apoptosis rate of neutrophils (Walzog, 1997).

After neutrophils have reached the site of infection, they must recognize and destroy the pathogenic organism. Various molecules on the pathogen cell surface, such as peptidoglycan on Gram-positive bacteria and lipopolysaccharide (LPS) on Gram-negative bacteria are recognized by receptors on the neutrophil (Kobayashi, 2003). Many receptors on the neutrophil cell surface play a role in

the recognition of foreign particles. As stated earlier, CD11b/CD18 plays a role in both complement-opsonized and unopsonized bacterial phagocytosis. Other important players are peptidoglycan recognition protein (Liu, 2000) and various Toll-like receptors (Armant, 2002). After recognition, the neutrophil will bind tightly with the pathogen and engulf it into a compact compartment called the phagosome. Granules inside the neutrophil will then bind to the phagosome and release their cytotoxic components into it within minutes of phagocytosis (Hampton, 1998).

Neutrophils have developed multiple mechanisms to kill invading pathogens. As stated above, some are detrimental not only to the invading pathogen, but also to the host itself. Probably the most effective killing mechanism by neutrophils is what is termed the oxidative burst, which is the production of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals, singlet oxygen, hypochlorous acid (HOCl), chloramines, nitric acid, and peroxynitrate (Hampton, 1998). These are produced through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which takes an electron from NADPH on the inside of the phagocyte and transfers it to an oxygen molecule on the outside of the cell to produce the aforementioned ROS (El-Benna, 2005). While superoxide is important in the neutrophil host response (Baboir, 1973) it is unclear whether it plays a direct role in pathogen destruction or is simply a precursor to hydrogen peroxide (Hampton, 1998). The same is true with hydrogen peroxide, which is only bactericidal at higher concentrations (Hampton, 1998) and is unlikely to play

a direct role in microbial killing. These molecules work in conjunction with other bactericidal molecules, mainly myeloperoxidase (MPO), to achieve their killing ability.

Myeloperoxidase is a heme peroxidase that uses hydrogen peroxide to oxidize aromatic compounds to produce substrate radicals and is responsible for the majority of the oxygen consumption during the oxidative burst (Hampton, 1998). MPO is found in more than one third of all granules in a neutrophil, which are termed primary granules because they are the first to appear during PMN maturation (Lehrer, 1990). MPO also oxidizes chloride ions and produces HOCl, which is the most potent oxidant in the neutrophil repertoire (Klebanoff, 1968). The myeloperoxidase/HOCl/H_2O_2 system is very important in the bactericidal effects of the neutrophil as seen with patients who suffer from chronic granulomatous disease (CGD). These patients have defects in the NADPH oxidase complex; their phagocytes cannot initiate an oxidative burst and are therefore much more susceptible to infections (Lekstrom-Heimes, 2000). The radicals produced can have varying targets on the bacterium including iron-sulfur proteins, membrane transport proteins, adenosine triphosphate (ATP)-generating systems, and the origin of replication site for DNA synthesis (Hampton 1998).

Along with the oxygen-dependent mechanisms discussed above, neutrophils also employ some oxygen-independent methods of microbial killing. These include antimicrobial polypeptides that reside in both the primary and secondary granules of the neutrophil (Lehrer, 1990). Primary granules contain polypeptides such as defensin, cathepsin G, azurocidin, bactericidal/permeability

increasing factor (BPI), elastase, collagenase and [beta]-glucuronidase and constitute the majority of the antimicrobial polypeptides in the neutrophil (Lehrer, 1990). Secondary granules contain lysozyme, lactoferrin, vitamin B-12 binding protein, adhesion receptors, chemoattractant receptors, gelatinase, and cytochrome b (Lehrer, 1990). These proteins act in various ways to help the neutrophil fight invading pathogens in an oxygen-independent manner. Both oxygen-dependent and -independent mechanisms result in the neutrophil undergoing necrosis and being phagocytosed by macrophages (Haslett, 1999). Another more recent theory for oxygen-independent microbial killing is through the use of what has been termed neutrophil extracellular traps (NETs) (Brinkmann, 2004). NETs are made up of granule proteins and chromatin, are used by the neutrophils to bind both Gram-positive and -negative bacteria, and degrade virulence factors produced by these bacteria. Bacteria trapped by these NETs are prevented from spreading and are closer to the antimicrobial response generated by the neutrophil.

Macrophage Response

In contrast to neutrophils, macrophage are much longer-lived cells and are the mediators of the immune response through the release of various cytokines, chemokines and other inflammatory mediators. They mature from monocytes and enter many different tissues throughout the body. Typically, they are the first cells to encounter pathogens, and begin signaling for neutrophils and eventually other macrophage to migrate to the site of infection (Janeway, 2005). Also unlike

neutrophils, macrophages have a decreased ability to produce ROS and are not as potent killers as neutrophils in this regard (DeLeo, 2004), although the ROS produced are the same as neutrophils (Janeway, 2005). Although they differ from neutrophils in these specific instances, their general method of action is still similar. While neutrophils generally phagocytose only pathogens, macrophages indiscriminately phagocytose almost any foreign particle to break it down and the process of recognition and phagocytosis is nearly identical to that of neutrophils.

One of the main functional differences between neutrophils and macrophages is that macrophages can act as antigen-presenting cells. Though less potent than dendritic cells, macrophages can be stimulated to produce co-stimulatory and MHC class II molecules to activate naïve T-cells (Janeway, 2005).

Neutrophil Cell Death

As neutrophils are naturally short-lived they must have a method of death that does not release their cytotoxic components and damage host cells. This method is through programmed cell death or spontaneous apoptosis and occurs without the release of the cytotoxic components. When neutrophils undergo apoptosis they lose their ability to release their cytotoxic granules and are cleared by macrophages without inducing the macrophage to undergo an inflammatory response (Haslett, 1999). Normally, when a neutrophil phagocytoses a pathogen, it releases its granule contents to destroy the stimulus that originally activated it. The neutrophil undergoes spontaneous apoptosis to

halt further granule release, which will lead to cell disintegration (necrosis); the cellular debris will eventually be cleaned up by macrophage (Haslett, 1999). If apoptotic neutrophils are not cleared by macrophages and allowed to undergo necrosis, they will release their cytotoxic components and cause more damage to the host, so regulation of neutrophil cell death is very important (Maianski, 2004). Cell death can be upregulated or downregulated by different stimuli and while apoptotic and necrotic cells share some characteristics, they also have unique characteristics, which allow them to be distinguished from one another.

Apoptosis is a natural form of cell suicide. Typical characteristics of apoptosis are membrane blebbing, nuclear condensation, DNA fragmentation, and cysteinyl-aspartate specific protease (caspase) production. In the case of the neutrophil it allows the cell to kill itself without harming other host cells around it. Neutrophils can undergo two routes of apoptosis, one of which is stimulated by outside factors that stimulate death receptors such as tumor necrosis factor-α (TNF-α) and Fas-ligands, which has been termed the extrinsic pathway. This pathway operates by activating caspase-8, which in turn activates caspase-3, the protease that triggers the cascade leading to cell death (Maianski, 2004). Obviously, neutrophils have a method of programmed cell death that can be utilized without outside influence though, as they are so short-lived. This method of cell death is achieved through the mitochondria, which activate caspase-3, like the extrinsic pathway, but does so through different intermediates, namely various Bcl-2 proteins and other caspases such as caspase-9 (Maianski, 2004). This has been termed the intrinsic pathway.

Caspase-3 is known as an executioner caspase and works by cleaving various substrates in the cell that lead to most of the classic cellular symptoms of apoptosis. In fact, of all the known caspase substrates 42 of the 58 are cleaved by caspase-3 (Porter, 1999). The first typical characteristic of apoptosis is membrane blebbing, where the membrane loses its tight formation and begins to "bleb" outwards. This is caused by the cleavage of actin-binding protein α-fodrin by caspase-3 (Nath, 1996). Actin is very important in controlling the structural integrity of the cell as discussed in relation to *Salmonella* and its ability to modulate actin to cause membrane ruffles during its infection strategy. Caspase-3 also cleaves gelsolin to produce fragments, which have been shown to break actin filaments and induce anchorage-dependent cells to detach, undergo nuclear condensation and DNA fragmentation (Kothakota, 1997). Another common trait of apoptosis is DNA fragmentation, which is also mediated by caspase-3. In this case, the CAD endonuclease is responsible for the DNA fragmentation seen during apoptosis, but its function is normally inhibited by ICAD/DFF-45 (Sakahira, 1998). Caspase-3 works by cleaving ICAD/DFF-45 at the carboxy-terminal end and thereby activating the CAD endonuclease activity (Tang, 1998).

Apoptosis can be triggered by many external or internal signals. Some signals are created by host cells themselves, presumably to help mediate the fine balance between the life and death of neutrophils that keep their destructive properties in check. Upregulation of certain receptors on the neutrophil cell membrane have been shown to promote apoptosis. For instance, after

transmigration the CR3 receptor is upregulated and this receptor has been shown to work in conjunction with TNF-α to promote apoptosis (Walzog, 1997). Growth factors such as G-CSF and GM-CSF, and cytokines such as IL-8, IL-1β and IFN-γ have also been shown to increase the survival time of neutrophils by staving off normal apoptotic function (Maianski, 2004).

Modulation of Apoptosis by Pathogens

Along with various chemical signals, apoptosis can be modified by the ingestion of pathogens; this is called phagocytosis-induced cell death (PICD). PICD is a form of apoptosis that uses the extrinsic pathway and is dependent on the CD11b/CD18 receptor and ROS production (Zhang, B., 2003). Apoptosis in neutrophils and macrophages have different characteristics and consequences in relation to the immune response. As reviewed earlier, macrophages have a much longer lifespan compared to neutrophils and have a much more active role in shaping the immune response. Also, the cytotoxic capabilities of macrophages are less than those of neutrophils; because of this, macrophages are much more susceptible to bacterial invasion (DeLeo, 2004), including *Salmonella* (see below). Conversely, neutrophils are extremely cytotoxic and very short-lived. They are very inhospitable to bacterial survival and growth. In this case, it is beneficial for bacteria to stop PICD and therefore delay neutrophil turnover. There have only been two bacterial pathogens shown to be able to not only survive, but replicate inside the neutrophil: *Anaplasma phagocytophilum* and *Chlamydia pneumoniae* (Deleo, 2004). Many bacteria, however, have been

shown to modulate neutrophil apoptosis through various molecules. Typical virulence factors that have been shown to modulate neutrophil apoptosis are: lipopolysaccharide (LPS) (Hofman, 2001), lipoteichoic acid (LTA) (Lotz, 2003), phenol-soluble modulin (Liles, 2001) and various enterotoxins (Moulding, 1999). In each case the bacteria modulate the normal cell death processes to the advantage of the bacterium, although PICD can override any adjustments by virulence factors (Englelich, 2001).

Salmonella Survival in Macrophage

Perhaps *Salmonella's* greatest advantage when infecting a host is its ability not only to survive, but also to replicate inside a human macrophage. It is able to achieve this by being able to modulate the cell death rate of the macrophage and its ability to stop the oxidative burst from occurring inside the macrophage. Along with SPI-1, discussed in an earlier section, *Salmonella* also has a pathogenicity island with genes dedicated to intracellular survival: Salmonella pathogenicity island-2 (SPI-2), a collection of genes at centisome 30 of the *Salmonella* chromosome (Vazquez-Torres, 2000). It was first shown that SPI-2 was important for intracellular survival by comparing wild-type *Salmonella* to SPI-2 mutants, which showed that macrophages incubated with the wild-type bacteria could not localize the NADPH-oxidase complex with the phagosome, while macrophages with the SPI-2 mutants showed no difference from that of control macrophages (Vazquez-Torres, 2000). This was later attributed to the

ability of *Salmonella* to exclude or remove flavocytochrome b_{558} from the phagosomal membrane, which subsequently prevents the NADPH-oxidase complex from assembling effectively, stopping the oxidative burst (Gallois, 2000).

Other virulence factors released by *Salmonella* have also been shown to mediate the cell death rate of the human macrophage. The factor SipB discussed above as playing a role in translocating other virulence factors across the host membrane has also been shown to induce apoptosis in human macrophages (Hersh, 1999). It achieves this by associating with and subsequently activating the host protein caspase-1, which is necessary for cytotoxicity and cell death.

Most virulence factors produced by *Salmonella* delay macrophage apoptosis. The first factors that modulate macrophage cell death are termed pathogen-associated molecular patterns (PAMPs), which are conserved regions on the pathogen recognized by phagocytes. These can be structures such as LPS and flagellin (Hueffer, 2004). The second group of molecules is the virulence factors produced through the two type III secretion pathways (SPI-1 and SPI-2) discussed earlier (Hueffer, 2004). The factors work to regulate the natural cell death rate in a way such that *Salmonella* can survive and replicate inside the macrophage.

Salmonella – Neutrophil Interaction

Salmonella was first shown to have an effect on human neutrophils in 1972 in studies on *S.* Typhi, which showed that *Salmonella* species do not

induce an oxidative burst (Miller, 1972). The events may be similar to what is seen in the macrophage: *Salmonella* is able to disrupt the normal neutrophil oxidative burst by misdirecting the NADPH-oxidase complex formation in the phagosomal membrane (Rosen, 2004). Also, some strains of *S.* Typhiumurium have been shown to induce lysis in human PMN (Chiu, 1999). Previous work in our laboratory has shown that when neutrophils transmigrate across a cell culture intestinal epithelial layer, they have an increased ability to bind to and kill *S.* Typhimurium, and that there is no oxidative burst released by the neutrophil (Nadeau, 2002). The current work focuses on the same events, but examines the effect of these bacteria on the cell death rate of the neutrophil.

Relevance

Salmonellosis is still a problem across the world today and understanding the mechanisms involved in its infection strategy is extremely important. This is especially important when examining neutrophils as they comprise up to 80% of all white blood cells, are the first to arrive during an infection, and have a devastating ability to destroy not only pathogens, but host cells as well. This ability is tightly regulated by their natural death rate: as they are short-lived and constantly being replaced, they need to die without releasing their cytotoxic components. Since bacteria have already been shown to be able to modify the natural death rate of neutrophils and *Salmonella* has been shown to alter macrophage cell death already, it is important to study how *Salmonella* influences this process in the neutrophil.

CHAPTER II

MATERIALS AND METHODS

Reagents

Unless stated, all reagents used were obtained from Sigma-Aldrich (St. Louis, Missouri).

Bacterial Strains and Growth Conditions

S. Typhimurium 14028 (wild-type) was cultured as previously described (Lee, 1990). Bacteria were grown and isolated on nutrient agar (NA) plates. One colony of bacteria was removed and grown in 20 mL of LB broth (Miller) (EM Science, Darmstadt, Germany) at 37ºC under agitating conditions to stationary phase, approximately 8 hours. To upregulate type III secretion, and subsequently virulence, the bacterial culture was then grown anaerobically by inoculating 100 µL of the aerobic culture into 20 mL of LB broth at 37ºC overnight, approximately 18 hours. This typically resulted in $5x10^8$-$1x10^9$ CFU/mL; the exact CFU/ml were calculated through plate counts on NA plates.

Cell Culture

T84 intestinal epithelial crypt cells (American Type Culture Collection, Rockville, Maryland) (passages 50-80) were grown in a 1:1 mixture of Dulbecco's

Modified Eagles Medium (DMEM) nutrient mixture and Ham's F12 supplemented with $NaHCO_3$ (14mM), Hepes buffer (15mM), penicillin (100,000 units/L), streptomycin (100mg/L) and 5% fetal bovine serum. Cells were grown at 37ºC, in 5% CO_2 in 75-cm^2 tissue culture flasks (Corning Inc., Corning, New York). Medium was replaced twice weekly with 15 mL of fresh medium and subcultured weekly.

T84 cells were subcultured by removing spent medium and washing in Ca^{++} and Mg^{++} free buffered saline for approximately 1 minute. Four-to-five milliliters of 0.25% trypsin EDTA was then added on top of the entire monolayer and allowed to incubate at 37ºC, 5% CO_2 for approximately 30 minutes or until the cells detached. The flask was then tapped smartly against a hard surface to detach all cells. The trypsin was inhibited by adding 10mL of fresh medium into the flask and the cell solution was transferred into a 50mL conical centrifuge tube and centrifuged for 10 minutes at 250xg. After centrifugation the supernatant was discarded, the pellet was resuspended in 2mL of fresh media, and the viable cell concentration was determined using trypan blue exclusion dye. New 75-cm^2 flasks containing 15mL of fresh medium were then seeded with approximately 1-5x10^6 viable cells.

T84 Growth on Transwell Inserts

T84 cells were grown on 6.5-mm diameter cell culture transwell inserts with 5.0-µm pore size polycarbonate membranes (Corning Inc., Corning, NY). The inserts were first removed from their 24-well plates and placed inverted in a

150 x 25-mm Petri dish (Becton Dickinson, Franklin Lakes, NJ). Approximately 100µL of 0.1mg/mL rat tail collagen was added to the tops of each of the inserts and allowed to incubate overnight under ultraviolet light to facilitate the cross-linking of the collagen. The collagen solution was then removed and the inserts were washed by dipping them in medium to remove the unattached collagen. Inserts were reinverted in the Petri dish and approximately $1x10^5$ to $1x10^6$ cells/mL were added on top of the inserts. The inserts were incubated at 37ºC, 5% CO_2 for 6-8 hours to allow cells to attach. After the incubation, the inserts were placed back into the wells of a 24-well plate with 1 mL of medium in the lower (apical) chamber and 100 µL of medium in the upper (basolateral) chamber. This allows for basolateral feeding similar to *in vivo* conditions. Medium in both chambers was replaced every 3-4 days. Monolayers typically became confluent after approximately 2 weeks; this was confirmed on a voltohmmeter using chopstick electrodes (World Precision Instruments, Sarasota, FL). Resistance ensures a confluent and polarized monolayer that mimics an *in vivo* state *in vitro*; this is typically associated with a reading of 400 Ω/cm^2. The inserts have an area $0.33cm^2$, so only inserts registering above 1200 Ω were used for transmigration.

Neutrophil Isolation

Human neutrophils were isolated from anticoagulated whole human blood using density centrifugation (English, 1974). Blood was collected from volunteers using Vacutainer brand Blood Collection Sets into Vacutainer tubes containing K_2

EDTA anticoagulant; filled tubes were inverted gently to ensure complete mixing of the blood and anticoagulant. In a 50-mL conical centrifuge tube the following were layered: 10mL of Histopaque 1119 on the bottom, 10 mL of Histopaque 1077 next and 20-25 mL of whole human blood on top. Each reagent was added very slowly to prevent any mixing between layers and ensure that a sharp definition between each layer could be seen. The tubes were then centrifuged at 700xg for 30 minutes at room temperature (RT). After centrifugation 6 distinct layers could be seen. The top plasma, mononuclear and histopaque 1077 layers were aspirated leaving the granulocyte layer on top and intact. This layer was then removed using a serological pipet and put into a new 50-mL centrifuge tube, mixed with an equal volume of balanced salt solution and centrifuged at 250xg for 10 minutes at RT. The supernatant was discarded and residual red blood cells were lysed through osmotic pressure. Specifically, the pellet was resuspended in 20 mL of cold 0.2% NaCl solution for approximately 30 seconds, then returned to isotonicity by the addition of 20 mL of 1.6% NaCl solution. The cell solution was then centrifuged at 250xg for 10 minutes at RT. This was repeated as many times as necessary until virtually all of the red blood cells were disrupted. Once the red blood cells were lysed, the neutrophils were resuspended in Hank's Balanced Salt Solution without divalent cations (HBSS-), and counted in 2% acetic acid in a hemocytometer. Neutrophils were then resuspended at the desired concentration in HBSS with divalent cations (HBSS+), and 10mM HEPES. This procedure creates a cell suspension in which 90% or more of the cells are morphologically neutrophils.

This study was approved by the University of New Hampshire's Institutional Review Board for the Protection of Human Subjects (approval #2609) and was conducted in accordance with all the guidelines set forth in that approval (See Appendix).

Bacterial/Neutrophil Association – Dose Response

To determine the ideal bacteria:neutrophil ratio, bacteria were added to neutrophils at 10-fold ratios from 1:1 to 1:1000. Neutrophils were isolated as described above and resuspended at a concentration of $1x10^5$ cells/mL. One milliliter of this solution was then added to 15 separate wells of a 24-well plate for each condition. An overnight bacterial culture (~$1x10^9$ CFU/mL), grown under the conditions noted above, was washed three times in HBSS+, diluted as appropriate, and subsequently plated for exact CFU determination. One-hundred microliters of each dilution, undiluted stock, or HBSS+ alone was then added to 1 ml of a neutrophil suspension. All samples were incubated for 12 hours.

Cell Death Enzyme-linked Immunosorbent Assay (ELISA)

Cell death was quantified using an ELISA kit detecting DNA fragments characteristic of apoptosis. This kit was obtained from Roche Diagnostics (product #1 774 425), Penzberg, Germany and the following protocol was adapted from the one provided by the manufacturer (Fig 1).

Sample preparation: In all assays neutrophils were resuspended at a concentration of $1x10^5$ cells/mL. For every sample tested a 100 µL aliquot

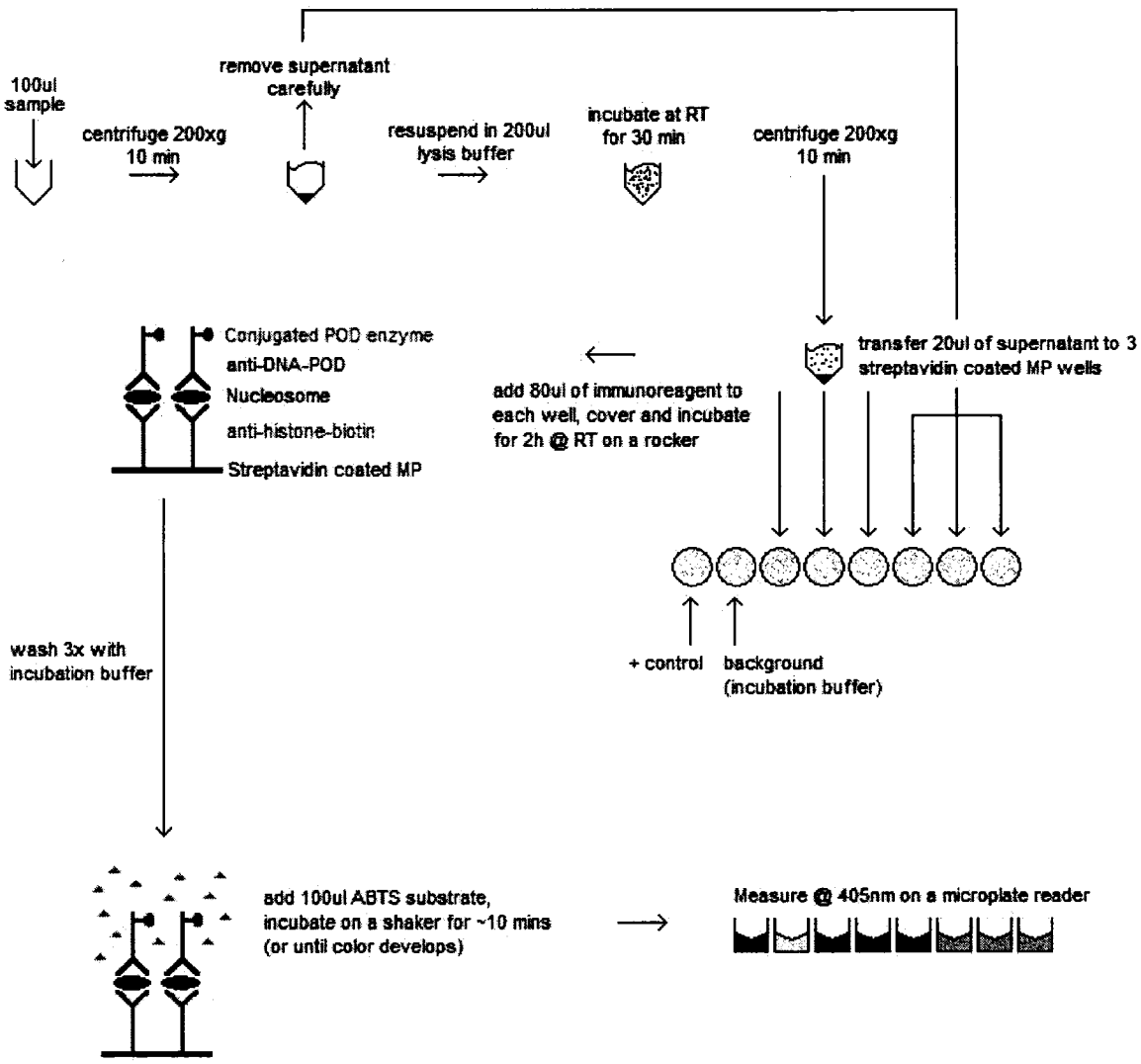


Figure 1: General cell death ELISA protocol. Adapted from Roche Diagnostic protocol 1 774 425.

(~1x10^4 cells) was added in triplicate to a 96-well v-bottom plate. The plate was then centrifuged at 200xg for 10 min at RT. The supernatant was removed from each well and saved for testing as the necrotic sample. The cell pellet was then resuspended in 200µL of lysis buffer and incubated at RT under gentle shaking conditions for 30 minutes.

ELISA: After the incubation the plate was centrifuged at 200xg for 10 min to remove cellular debris; this is the apoptotic sample. During the centrifugation the immunoreagent, containing both the anti-DNA peroxidase conjugated and anti-histone biotin conjugated antibodies, was prepared by adding 1/20^{th} of a volume of each antibody with 18/20^{th} of a volume of incubation buffer. In triplicate, 20 µL from both the apoptotic and necrotic samples was added to separate wells of the streptavidin coated microplate along with a background and positive control. The immunoreagent (80 µl) was then added to each well and the assay systems were incubated for 2 hours at RT, with gentle shaking. After incubation, each well was washed three times with 250 µL of incubation buffer, making sure to remove the buffer carefully after each wash. Freshly prepared 2,2"-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution (100 µl) was added to each well and the assays were incubated for approximately 10 minutes or until the color developed completely. The plate was then read on a Bio-Rad 3550 microplate reader at 405nm and a reference wavelength of 495 nm.

Phorbol 12-myristate 13-acetate optimization

Varying concentrations of phorbol 12-myristate 13-acetate (PMA) were added to replicate neutrophil samples of $1x10^5$ cells/ml from 100 ng/ml to 1 pg/ml. Aliquots (100 µL) of each sample were added in triplicate in a 96-well plate to the neutrophil samples; neutrophils with no added PMA were also included. After 8 h the samples were treated as described in the Sample Preparation section above and stored at 4ºC until the ELISA was performed.

Neutrophil activation with PMA and incubation with S. Typhimurium

S. Typhimurium was grown both aerobically and anaerobically for these studies. Neutrophils suspensions were prepared at a concentration of $1x10^5$ cells/mL and pretreated with PMA at a concentration of 100 pg/ml. Aliquots of *S.* Typhimurium, grown either anaerobically or aerobically, at a ratio of one neutrophil to 10 bacteria or 100 bacteria were then added. Ten different conditions were tested.

1) Neutrophils with no PMA or bacteria
2) Neutrophils with no PMA +1:10 anaerobic bacteria
3) Neutrophils with no PMA +1:100 anaerobic bacteria
4) Neutrophils with no PMA +1:10 aerobic bacteria
5) Neutrophils with no PMA +1:100 aerobic bacteria
6) Neutrophils with 100 pg/ml PMA, no bacteria
7) Neutrophils with 100 pg/ml PMA +1:10 anaerobic bacteria
8) Neutrophils with 100 pg/ml PMA +1:100 anaerobic bacteria
9) Neutrophils with 100 pg/ml PMA +1:10 aerobic bacteria
10) Neutrophils with 100 pg/ml PMA +1:100 aerobic bacteria

Seven 96-well plates were set up with triplicate wells and incubated at 5% CO_2, 37ºC. Every 4 h, up to 24 h, one plate was removed and the samples were

prepared as described above. These samples were stored at 4ºC until assessed by ELISA.

Neutrophil Transmigration

Once satisfactory T84 confluency was reached, inserts were washed in HBSS+ and placed in a low attachment 24-well plate (Corning Inc, Corning, New York) containing 1 mL of HBSS+ and 10 mM of HEPES in the lower chamber and 100 µL of HBSS+ in the upper chamber. The inserts were incubated for 1 h at 37ºC, 5% CO_2 to allow T84 cells to equilibrate in the HBSS+. Inserts were washed two more times with HBSS+. Three controls were set up along with the T84 cellular inserts: (a) acellular inserts (inserts without T84 cells), (b) fMLP (transmigration system with fMLP in the chamber), and (c) no transmigration (PMN that were not exposed to the transmigration system). The transmigration plates were set up as depicted in Figure 2. fMLP (10µL of 10^{-5}M) was then added to each of the transmigration, acellular control and fMLP control wells to create a chemotactic gradient to attract the neutrophils. No fMLP was added to the negative control. Neutrophils were isolated as described above and resuspended at a concentration of $5.0x10^7$ cells/mL and 20 µL of the neutrophil cell suspension was added to each of the insert upper chambers for the transmigration and acellular control and to the wells themselves for the fMLP and negative controls. Each plate was then incubated for 2 hours at 37ºC, 5% CO_2. At the end of the incubation inserts were removed with forceps and tapped gently

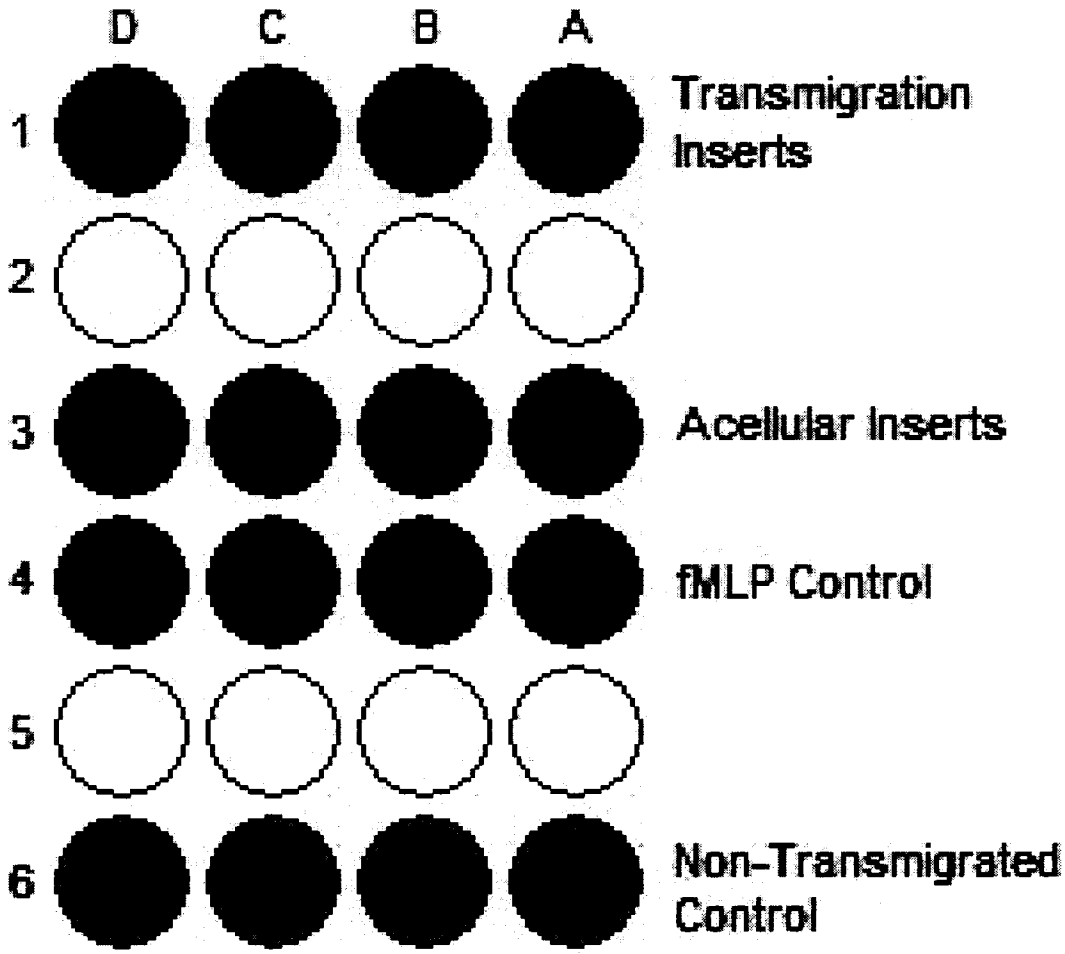


Figure 2. Design of transmigration assay. Neutrophils were extracted and transmigrated across T84 intestinal epithelial cells grown on special cell culture inserts and attracted across by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). Neutrophils were also exposed to three control conditions: 1) Migration across cell culture inserts with no T84 cells grown on them with fMLP to ensure crossing the permeable membrane on the insert was not causing activation, 2) Neutrophils just in the presence of the chemoattractant fMLP to show they were not being chemically activated and 3) Non-transmigrated neutrophils – negative control. The four wells for each condition were combined after a 2h incubation at 37C, 5% CO_2, each condition was then counted on a hemocytometer and resuspended at a concentration of $1x10^5$ cells/mL.

against the side of the well to remove all residual neutrophils. All wells were combined for each condition, and the PMN were counted and resuspended at a concentration of $1x10^5$ cells/mL. *Salmonella* was added at ratios of 10 and 100 bacteria to 1 neutrophil, for both transmigrated and non-transmigrated neutrophils. Seven identical 96-well plates were set up with 100µL of each condition in triplicate. At each time point samples were prepared from one 96-well plate; these samples were stored at 4ºC until assessed by ELISA.

Caspase-3, -8 and -9 colorimetric assay

The following protocol was adapted from Biovision protocol K106-25 (Mountain View, California, USA). Samples were set up at neutrophil concentrations of approximately $1\text{-}5x10^6$ cells/ml and incubated with PMA and varying ratios of neutrophils:*S.* Typhimurium as described above. Aliquots (100 µl) of each sample were placed in a sterile 96-well cell culture plate and samples were collected at time zero and 3 h and tested for caspase activity. Each plate was centrifuged at 250x*g* for 10 minutes and resuspended in 50 µL of chilled lysis buffer. All plates were kept on ice for 10 minutes and then centrifuged at 3,700x*g* for 2 minutes. The cytosolic extract (supernatant) was removed and put into new wells. The protein concentration of these wells was then determined using a microplate Bradford assay.

Briefly, 180 µl of Bradford's reagent was added to each well and a standard curve of bovine serum albumin (BSA) from 0 µg to 30 µg was established. Aliquots (20 µL) from each sample were tested. Each sample was

analyzed in triplicate. The cytosolic extract was diluted 1:10 and 1:100, and 20 μL aliquots of these dilutions were also analyzed. All wells were allowed to incubate for approximately 5 minutes and then read at 600 nm on a plate reader. The unknown cytosolic extract protein concentration was determined from the BSA standard curve.

Approximately 100-200 μg of protein was added to a new well in a 96-well plate and the total volume was increased to 50 μL with chilled lysis buffer. Aliquots (50 μL) of the reaction buffer with 10 mM of Dithiothreitol (DTT) were added to each well. Then 5 μL of the appropriate caspase substrate was added to it corresponding well: DEVD (N-Acetyl-Asp-Glu-Val-Asp) -pNA (p-nitroanilide) to caspase-3 wells, IETD (Z-Ile-Glu-Thr-Asp)-pNA to the caspase-8 wells and LEHD (N-Acetyl-Leu-Glu-His-Asp)-pNA to the caspase-9 wells. All plates were then incubated overnight at 37°C, and read on a plate reader at 405 nm.

Data Presentation

Two-way ANOVA with Bonferroni post test was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Due to the inherent variations involved with neutrophils, the resistances of the T84 monolayers on each insert, and color development in the ELISA, data were evaluated in each individual experiment and not between different experiments. The trends were observed in each experiment and these trends were repeatable throughout all experiments.

CHAPTER III

RESULTS

Normal human neutrophil apoptosis rate in vitro in HBSS+ peaks after twelve hours over the course of twenty-four hour period.

I initially determined the normal course of human neutrophil cell death once the cells were purified and removed from the body to ensure that the assay system selected produced results similar to previously published data. Neutrophils were purified from whole human blood and resuspended at a concentration of $1x10^5$ cells/mL in HBSS+. Over the course of a 24-h period neutrophil apoptosis rose sharply beginning at 15 min after purification and peaked at approximately 12 h, as expected (Fig. 3). The rate of apoptosis decreased slightly from 12 to 24 h. This was measured at both 405nm (Fig. 3A) and 490nm (Fig. 3B), as suggested by the manufacturer, but no obvious differences between the two conditions were seen.

The importance of bacterial dose upon normal human neutrophil cell death over twenty-four hours when exposured to *S.* Typhimurium.

To determine optimum bacterial dosage, purified human neutrophils were exposed to increasing ten-fold ratios of *S.* Typhimurium (from 1:1 to 1:1000). This was done to determine the optimal ratio of bacteria that did not disrupt the

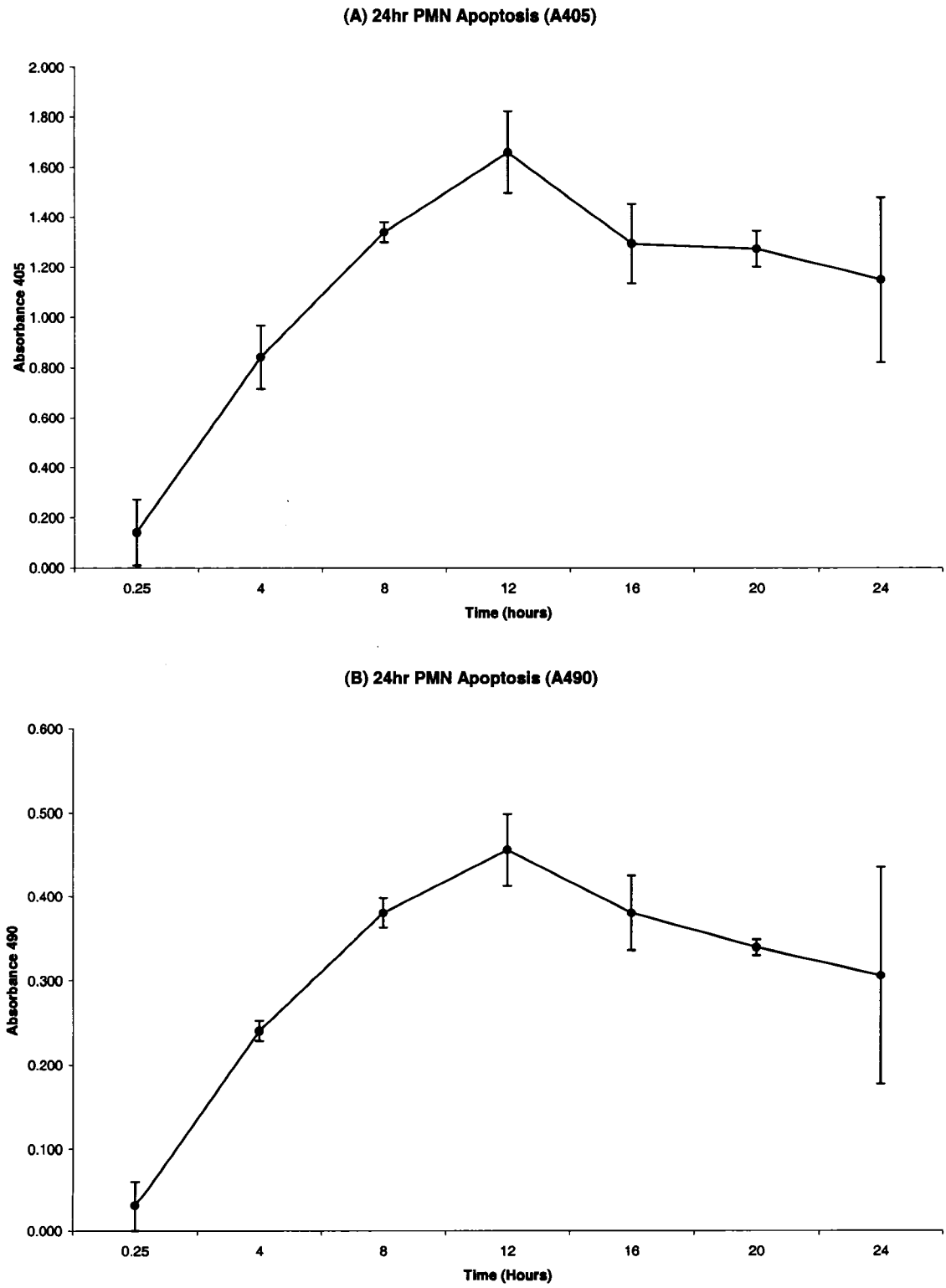


Figure 3: Initial assays to determine the normal human neutrophil apoptosis rate *in vitro*. Human neutrophil apoptosis increases until approximately twelve hours, where it peaks and levels off. There were no significant differences in measuring the system at 405nm (A) as compared to 490nm (B).

system used and to observe any dose response. The time point of 12 h was chosen as it is the point where cell death peaks and any change due to the bacteria can be more easily seen. As the bacterial dose increased, apoptosis decreased (Fig 4a), while necrosis remained constant until a ratio of 1:100, where the rate of necrosis rose sharply (Fig 4b). At a ratio of 1:1000 both the apoptotic and the necrotic rate fell sharply compared to the 1:1, 1:10 & 1:100 ratios. The optimal bacterial dose was determined to be 1 neutrophil to 10-100 bacteria, as these were the ratios in which the bacterial effect was clearly seen, without quenching all the color change in the ELISA system.

_S._ Typhimurium grown aerobically has no effect on apoptosis, while anaerobically grown _S._ Typhimurium has a slight pro-survival effect on inactivated human neutrophils.

As seen in Figure 5, when *S.* Typhimurium was grown anaerobically there is a slight pro-survival effect on the apoptosis rate seen over the course of twenty-four hours at either ratios: 1:10 (p=.024) and 1:100 (p=.002). Using the GraphPad statistical program, time accounted for most (82%) of the variation in each assay not bacterial treatment, which accounted for only 3.0% for the 1:10 ratio and 5.4% for the 1:100 ratio.

Figure 6 shows the effect of aerobically grown *S.* Typhimurium. There was no significant effect on neutrophil apoptosis at either the 1:10 PMN-to-10 aerobically grown *S.* Typhimurium (p=.498) or 1:100 (p=.690) ratios.

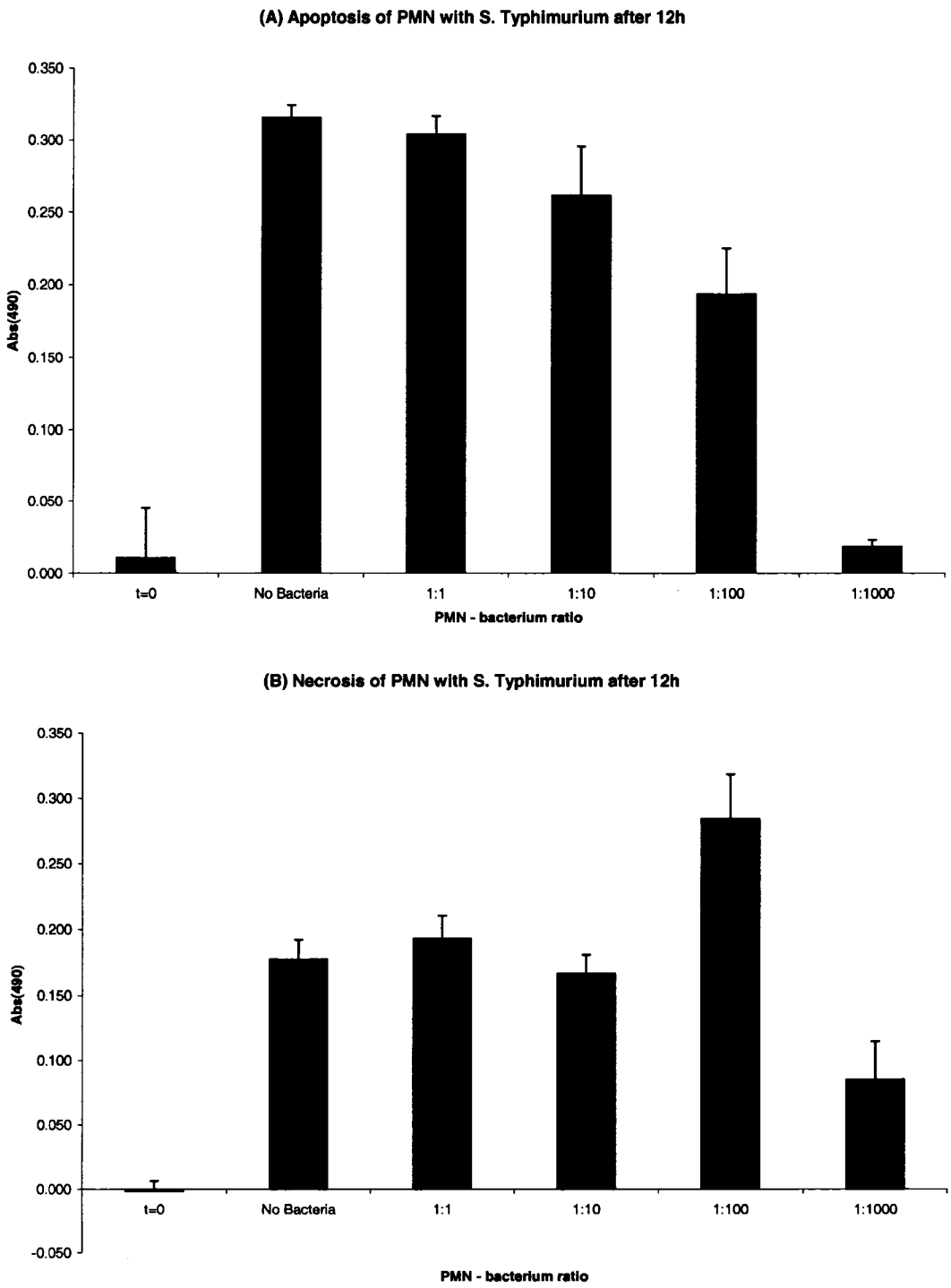
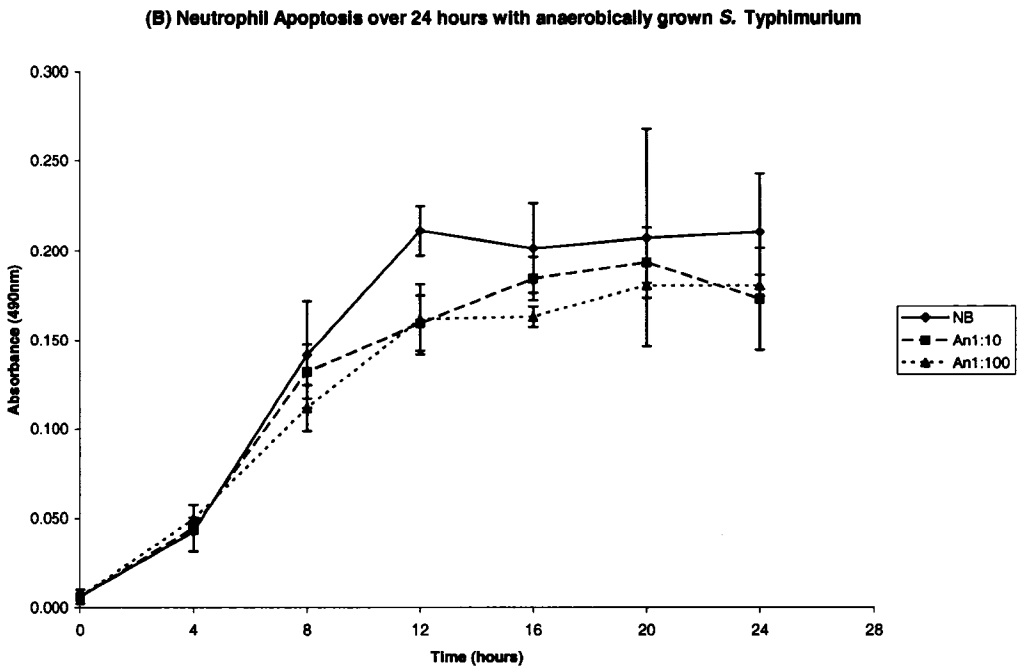


Figure 4: Bacterial dose assay. (A) Apoptosis slowly decreases as PMN: bacteria ratio increases up to 1:1000. (B) Necrosis remains relatively constant except for 1:100 where necrosis increases. Cell death levels at the time of the start of the assay are shown for initial values (t=0)



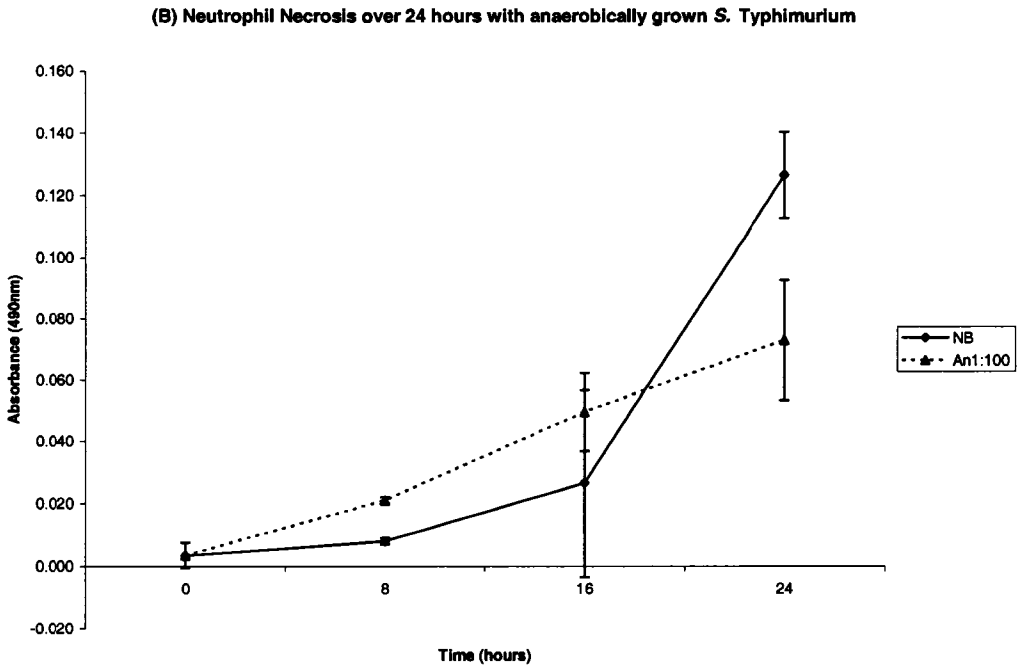


Figure 5: Analysis of the apoptosis (A) and necrosis (B) rates of human neutrophils in the presence anaerobically grown *S.* Typhimurium. (A) Anaerobically grown *S.* Typhimurium had a slight pro-survival effect on human neutrophils compared to the no bacteria control (NB) over 24 hours. (B) Necrosis remained higher for 16 hours but after 24 hours ended up much lower.

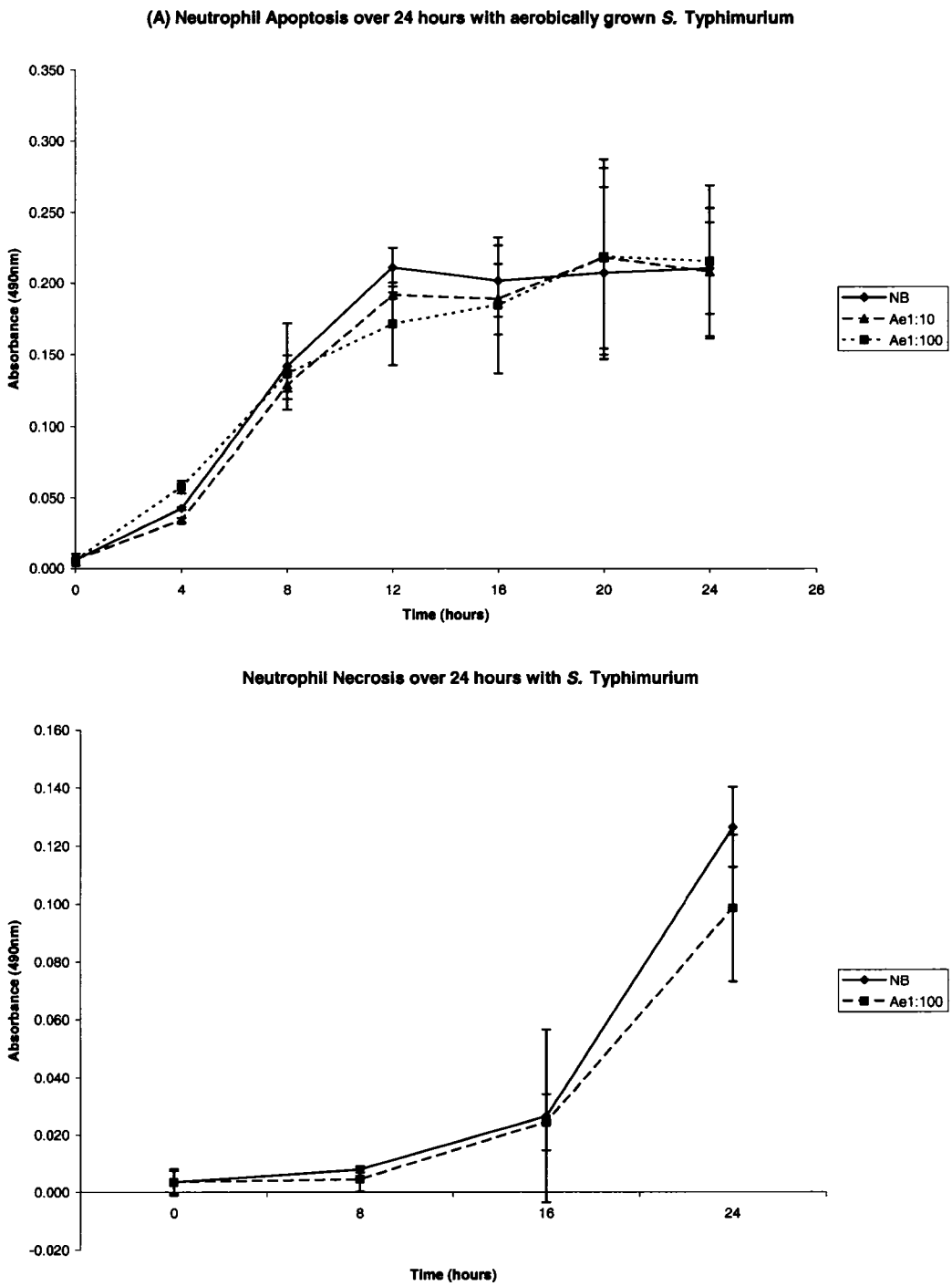


Figure 6: Analysis of the apoptosis (A) and necrosis (B) rates of human neutrophils in the presence aerobically grown *S.* Typhimurium. (A) Aerobically grown *S.* Typhimurium had no significant effect on the apoptosis rate of the neutrophils compared to the no bacteria control (NB). (B) There was no effect on the necrosis rate of the neutrophils.

not deviate significantly from the inactivated neutrophils. The anaerobically grown *S.* Typhimurium on the other hand, did have a slight effect on the rate of necrosis in neutrophils as it started higher than inactivated neutrophils and steadily increased and remained higher through sixteen hours; by twenty four hours, however, it ended up much lower.

Activation with phorbol 12-myristate 13-acetate has a pro-survival effect on the cell death rate of human neutrophils.

The next goal was to determine what effect, if any, phorbol 12-myristate 13-acetate (PMA) had on the cell death rate of human neutrophils. First, the optimal concentration of PMA needed to be determined so that it would not mask any effect seen when *S.* Typhimurium was added to the system. Figure 7 shows the effect that increasing concentrations of PMA from 1 pg/ml to 100ng/ml had on the neutrophils after 8 hours. As seen in this figure, PMA exerted a pro-survival effect on human neutrophils down to 0.1ng/ml. At lower concentrations of PMA apoptosis rates returned to normal levels. Necrosis remained low in all concentrations. The cell death of neutrophils was then tracked over the course of 24 h in the presence of 0.1ng/ml of PMA. Figure 8 shows that the PMA exerted a pro-survival effect on human neutrophils over a full 24-h period through apoptosis and necrosis. The treatment accounted for 68% of the variability seen and time and treatment were both significant ($p<0.0001$), but the interaction between the two was also significant. Bonferroni post tests were done at each time point and all time points were significant ($p<0.001$) except T=4h.

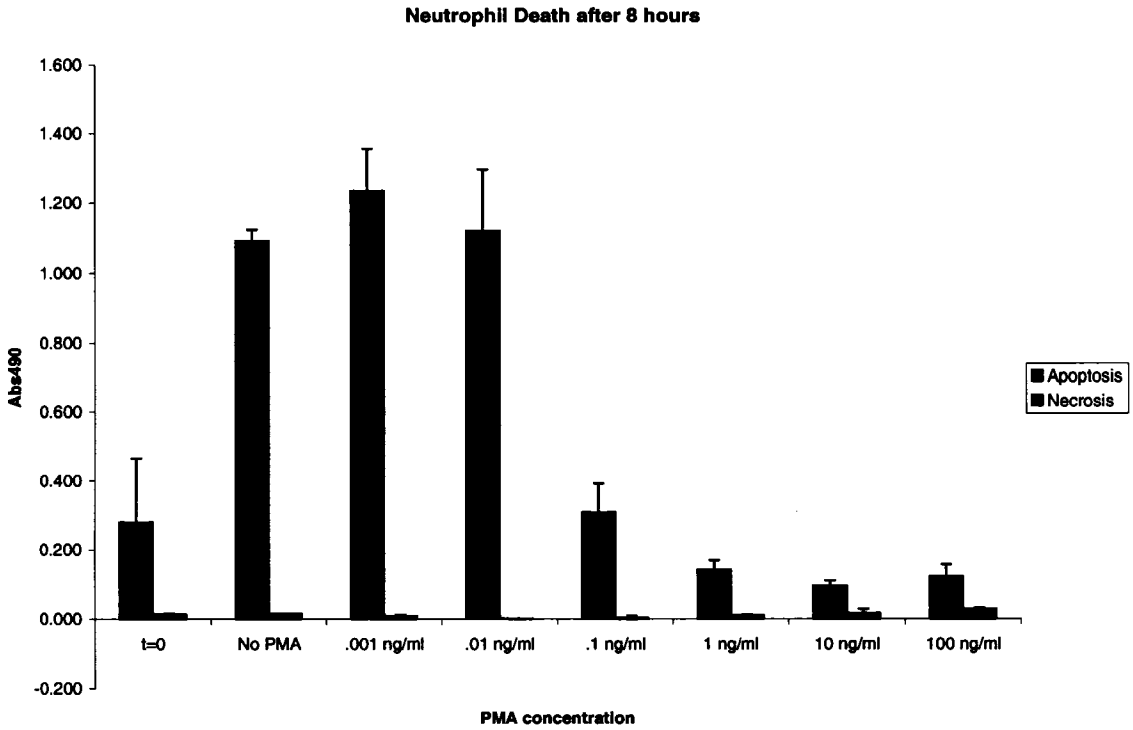


Figure 7: Effect of PMA concentration on the cell death rate of human neutrophils. PMA had a pro-survival effect on neutrophils down to a concentration of 0.1 ng/ml after 8 h. After 0.1ng/ml apoptosis rises to control (no PMA) levels. Cell death levels at the time of the start of the assay are shown for initial values (t=0)

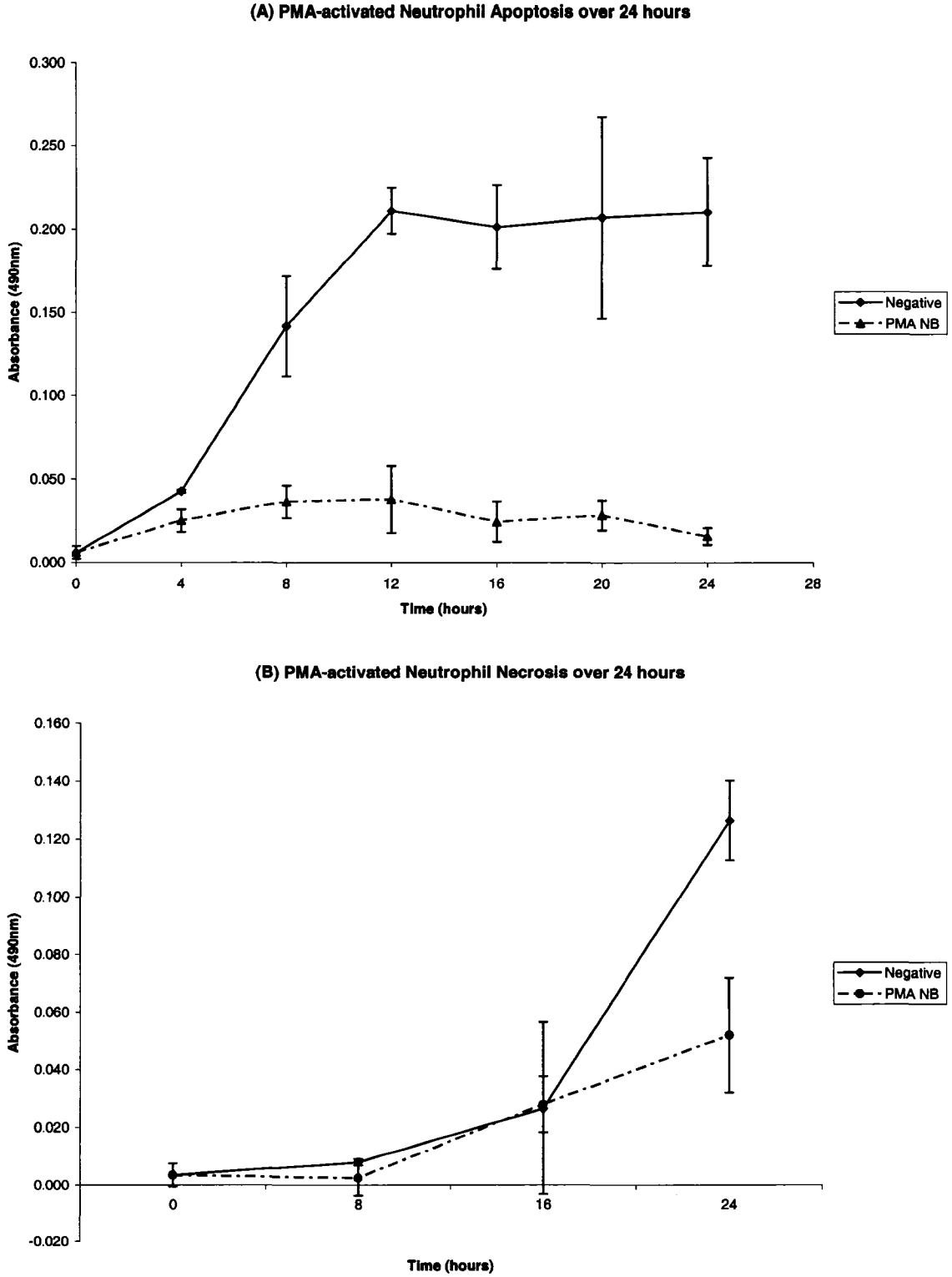


Figure 8: The effect of 0.1 ng/ml of PMA on human neutrophils over 24 h. The PMA exerted a pro-survival effect on the neutrophils over the full 24-h period in both apoptosis (A) and necrosis (B).

_S._ Typhimurium grown anaerobically significantly increases the cell death rate of PMA-activated neutrophils over a 24-h period.

Based on the previous optimization experiments, a ratio of 1 neutrophil to 10 or 100 bacteria was used with a PMA concentration of 0.1 ng/ml. Figure 9 shows that in the presence of anaerobically grown *S.* Typhimurium activated neutrophils initiated apoptosis, which peaks at about 24 h. This was best seen at a neutrophil:bacterial ratio of 1:100 ($p<0.0001$), although an increase in apoptosis was also seen at a ratio of 1:10 ($p=0.0027$). Time was not a significant factor ($p=.461$) at 1:10, but was at 1:100 ($p<0.0001$) as was the interaction between the two. Subsequent post-tests indicated that the differences were significant at all time points except T=4 h.

Necrosis in PMA-activated neutrophils was similar to the negative control up to 16 h, but after 24 h was significantly lower. In the presence of *S.* Typhimurium at a ratio of 100 bacteria to 1 neutrophil, these same neutrophils had a much higher necrosis rate than did the negative control until the 24-h time point at which time they were almost the same.

_S._ Typhimurium grown aerobically slightly increases the cell death rate of PMA-activated neutrophils over a 24-h period.

Figure 10A shows that aerobically grown *S.* Typhimurium also increased the apoptosis rate of PMA-activated human neutrophils, but did so significantly less compared to anaerobically grown *S.* Typhimurium. At a ratio of 1:10, treatment was significant ($p=0.012$) as was time ($p=0.006$). When bacteria were

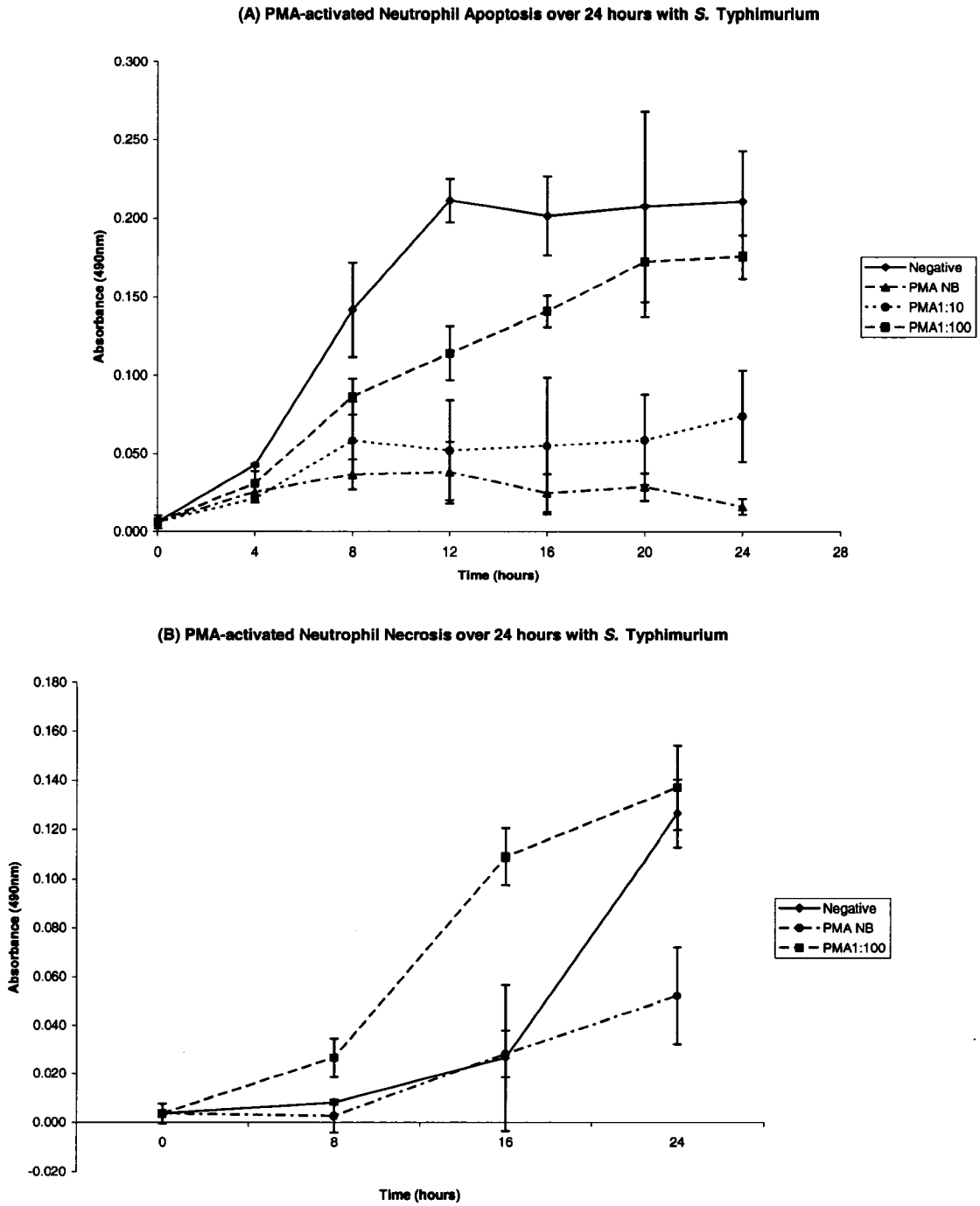


Figure 9: Effect of anaerobically grown *S.* Typhimurium on PMA-activated neutrophils. There was an increase in apoptosis of neutrophils at a neutrophil:bacterium ratio of 1:10, it was more pronounced at a ratio of 1:100 where apoptosis rose and peaked at near-normal unactivated levels at about 20 h. Necrosis was highest in PMA-activated neutrophils in the presence of *S.* Typhimurium. Necrosis was lowest in the absence of *S.* Typhimurium after 24 h.

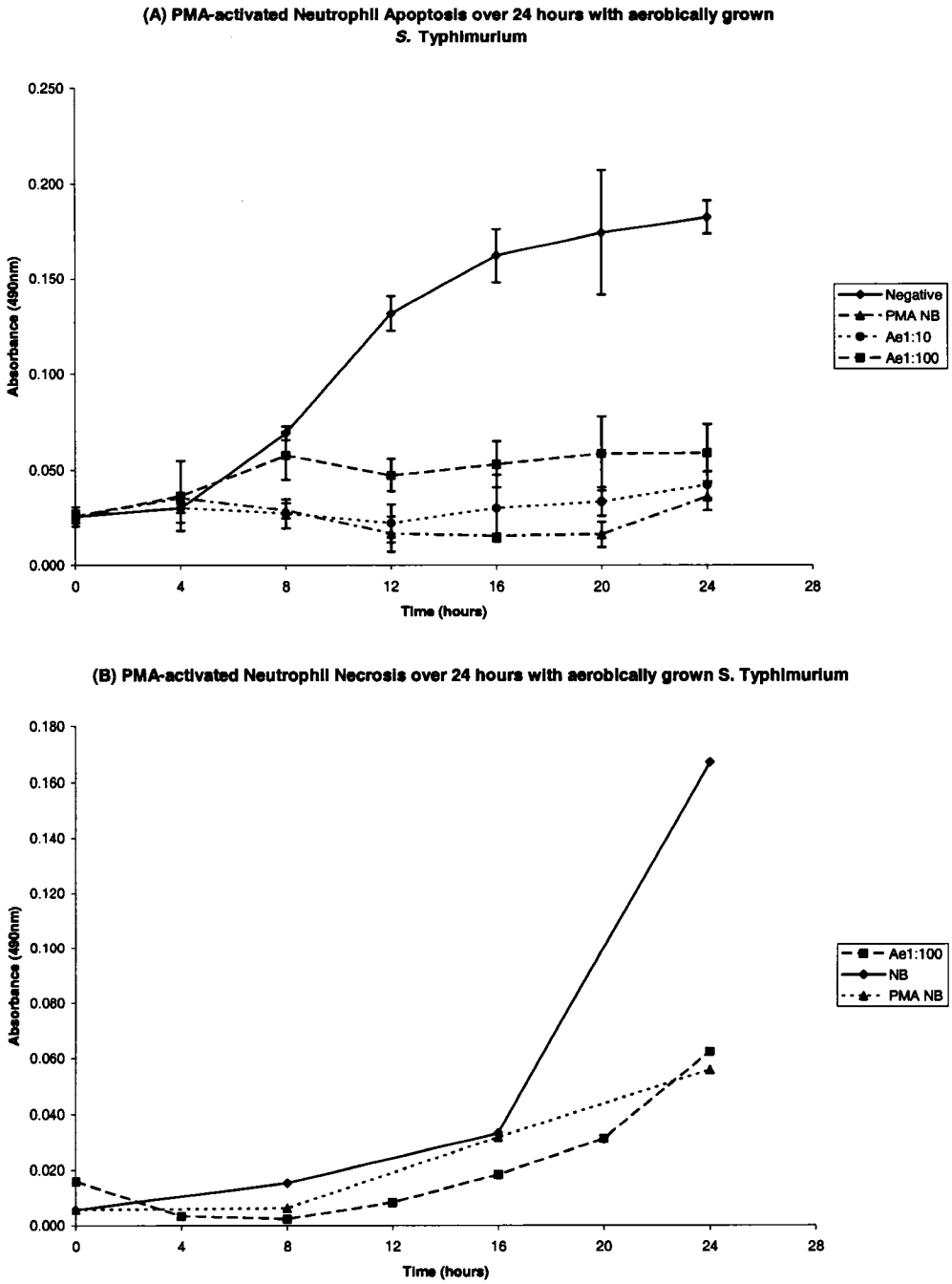


Figure 10: Effect of aerobically grown *S.*Typhimurium on the cell death rate of PMA-activated human neutrophils. (A) Apoptosis was increased at both 1:10 (Ae1:10) and 1:100 (Ae1:100) neutrophil to bacteria ratios compared to the PMA (PMA NB) control. This is level is lower and less significant than seen with anaerobically grown bacteria (See Figure 9). (B) Necrosis levels of bacterial treated PMN remained basically the same across the 24 h assay compared to the PMA (PMA NB) control.

added at a 1:100 ratio both treatment ($p<0.0001$, 30.05% of the variation) and time ($p<0.0001$, 42.25% of the variation) were significant, but the interaction was also significant ($p<0.0001$). In this instance post-tests showed that only times 12 h through 24 h were significant. Necrosis (Figure 8B) in bacterial treated PMN was unchanged over 24 h compared to the PMA (PMA NB) control.

Activating neutrophils through transmigration delays apoptosis significantly, and raises necrosis slightly.

To be sure that transmigration is responsible for the results seen, other variables needed to be examined. A baseline was established by analyzing PMN in HBSS(+) w/ 10mM HEPES. Since transmigration is initiated by the potent chemotactic peptide fMLP, a second control was included to expose neutrophils in the transmigration solution with the fMLP. The final control involved incubating neutrophils on inserts coated with collagen but without T84 cells. As seen in Figure 11A, over the course of 12 h there was no noticeable difference between neutrophils that had not been activated in any way, those that had been exposed to fMLP ($p=0.702$), and those that had migrated across an acellular membrane ($p=0.111$). Figure 11B shows the same cells results for necrosis. Although all samples started at the same point, after 12 h the amount of DNA fragmentation began to diverge among each of the controls. The untreated neutrophils had the highest levels of necrosis, which was to be expected, but unlike the apoptosis assays, neutrophils in the presence of fMLP showed decreased levels of necrosis and the acellular transmigrated neutrophils showed even lower levels.

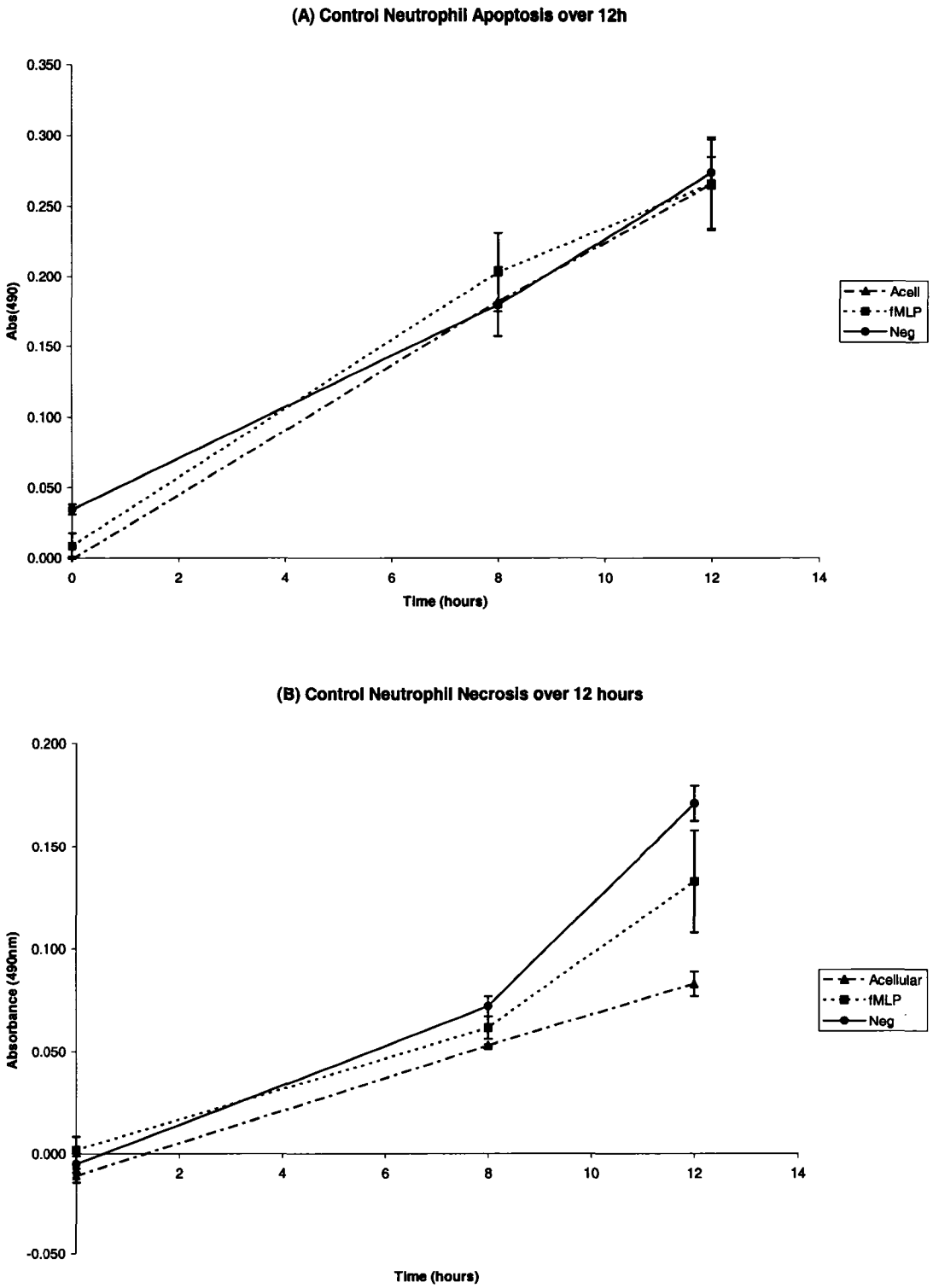


Figure 11: Transmigration controls for the assay. (A) Negative control neutrophils (Neg) showed no difference in apoptosis compared to fMLP (p=.702) and acellular (p=.111) controls . (B) Necrosis was highest in negative control neutrophils followed by fMLP and acellular, respectively.

Thus, factors involved in neutrophil transmigration, other than passage through the cellular monolayer, had no effect on apoptosis. Next, the effect of neutrophil transmigration through the T84 intestinal epithelial monolayer on the death rate of these cells was assessed. Figure 12 shows that after neutrophils had transmigrated across a model T84 intestinal epithelial cell culture monolayer the level of apoptosis was significantly decreased. The effect of transmigration was extremely significant ($p=<0.0001$, 60.33% of the variation), and time was also significant ($p<0.0001$, 25.56% of the variation). The interaction was also significant ($p=0.03$) and subsequent post-tests indicated that all time points were significant except T=24 h. Necrosis on the other hand, started at higher levels in transmigrated neutrophils, rose slowly over the course of 12 h. and ended at approximately the same level as inactivated neutrophils.

_S._ Typhimurium slightly raises the apoptosis and necrosis rates of transmigrated neutrophils as compared to inactivated neutrophils.

Figure 13 shows the results obtained when transmigrated neutrophils were incubated in the presence of *S.* Typhimurium. *S.* Typhimurium increased the apoptosis (A) and necrosis (B) rates of the transmigrated neutrophils over the course of 24 h. Bacterial treatment at both ratios (1:10 and 1:100) was significant ($p=0.002$ and 0.012 respectively) compared transmigrated neutrophils with no bacteria (TrNB). Transmigrated neutrophils in the presence of *S.* Typhimurium follow the same trend as transmigrated neutrophils not in the presence of the bacterium over the course of 24 h, but do so at higher levels.

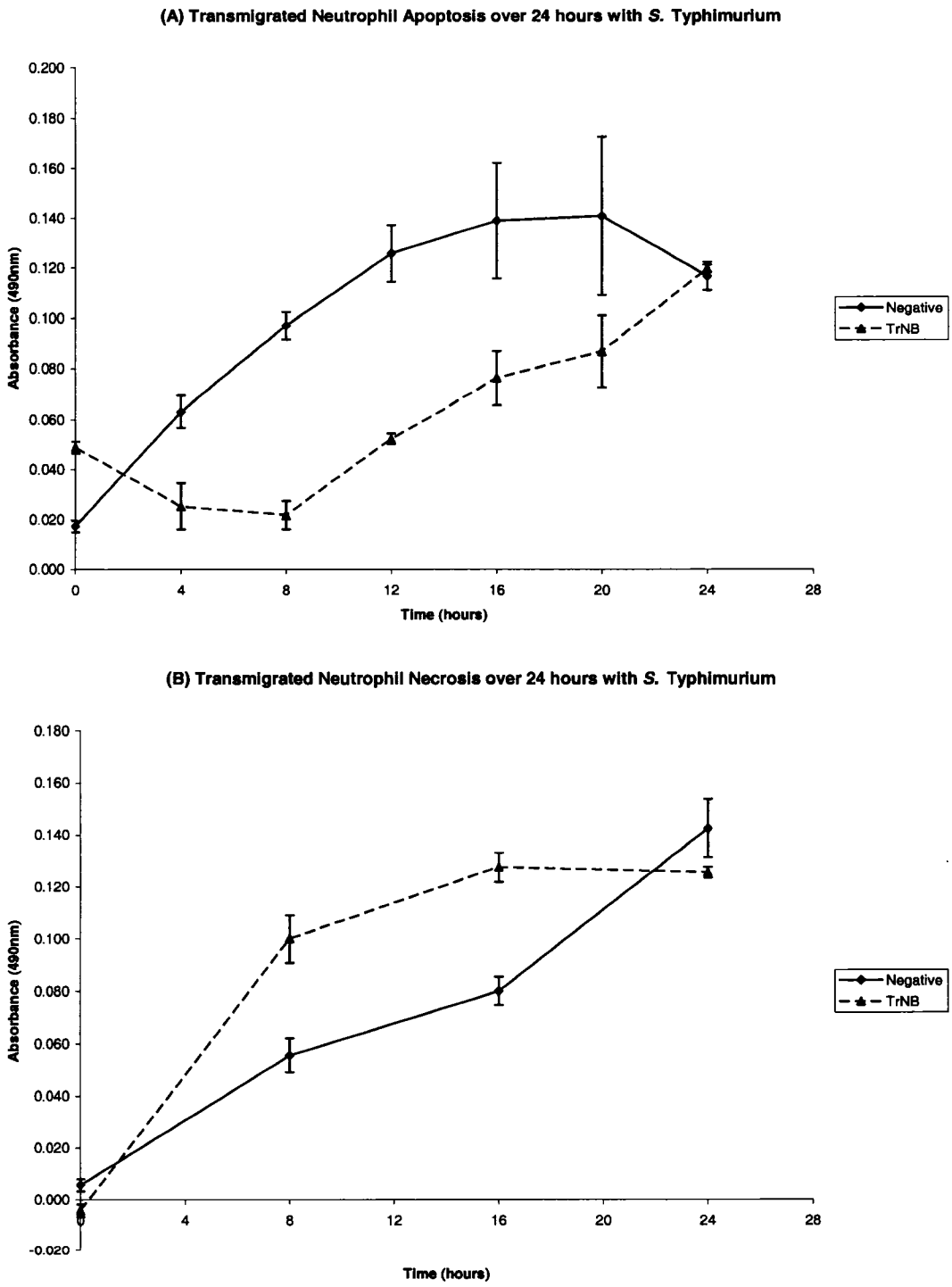


Figure 12: Effect of transmigration on the cell death rate of human neutrophils. (A) *S.* Typhimurium induced more transmigrated neutrophils to undergo apoptosis compared to transmigrated neutrophils not in the presence of the bacterium. (B) *S.* Typhimurium also induced more necrosis compared to transmigrated neutrophils not in the presence of *S.* Typhimurium.

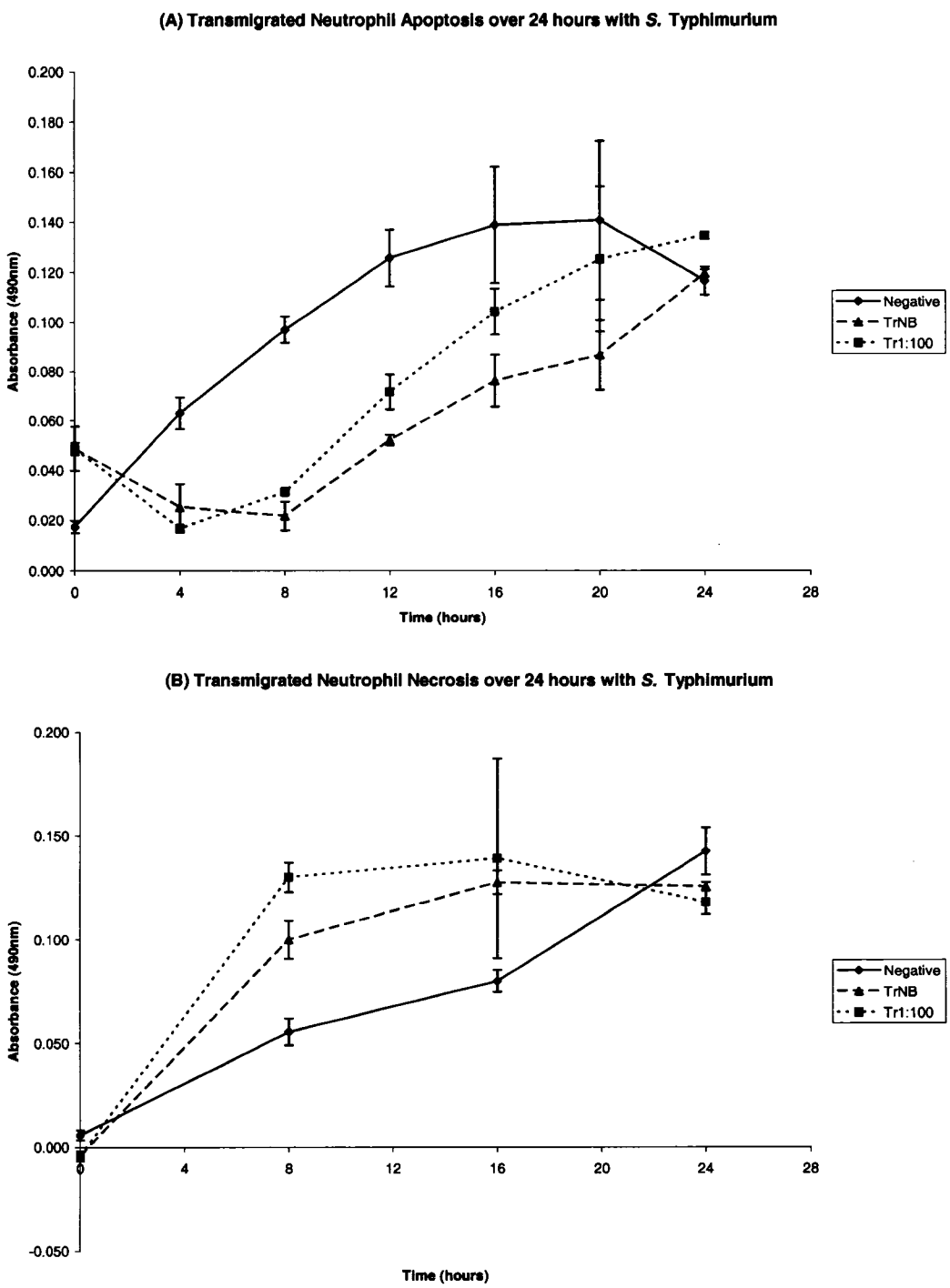


Figure 13: Effect of *S.* Typhimurium on the cell death rate of transmigrated PMN. (A) *S.* Typhimurium induced more transmigrated neutrophils to undergo apoptosis compared to transmigrated neutrophils not in the presence of the bacterium for both ratios 1:10 (p=0.002) an 1:100 (p=0.012). (B) *S.* Typhimurium also induced more necrosis compared to transmigrated neutrophils not in the presence of *S.* Typhimurium.

Caspase activity in human neutrophils

These studies were designed to detect the presence of three different caspases involved in eukaryotic cell death: Caspase-3, -8 and -9. To identify key proteins involved in this system, these caspases were examined in these previously studied conditions: (1) PMN alone, (2) PMN +1 pg/ml PMA, and (3) PMN +1 pg/ml PMA +*Salmonella*. Figure 14 shows that after 3 h caspase-8 does not play a significant role ($p>0.05$) in neutrophil death in this system; neither time nor treatment affected its levels. Caspase-3 activity, on the other hand, was significantly increased ($p=0.002$), but time accounted for most of the variability ($p<0.0001$, 95.4% of the variability) across all conditions after 3 hours, although it was slightly depressed in the presence of *S.* Typhimurium. Caspase-9 activity also exhibited a significant difference in time after 3 hours ($p<0.01$), but the bacterial treatment was not significant. At time 0 there was no significant difference in any group treated with *Salmonella* compared to its control.

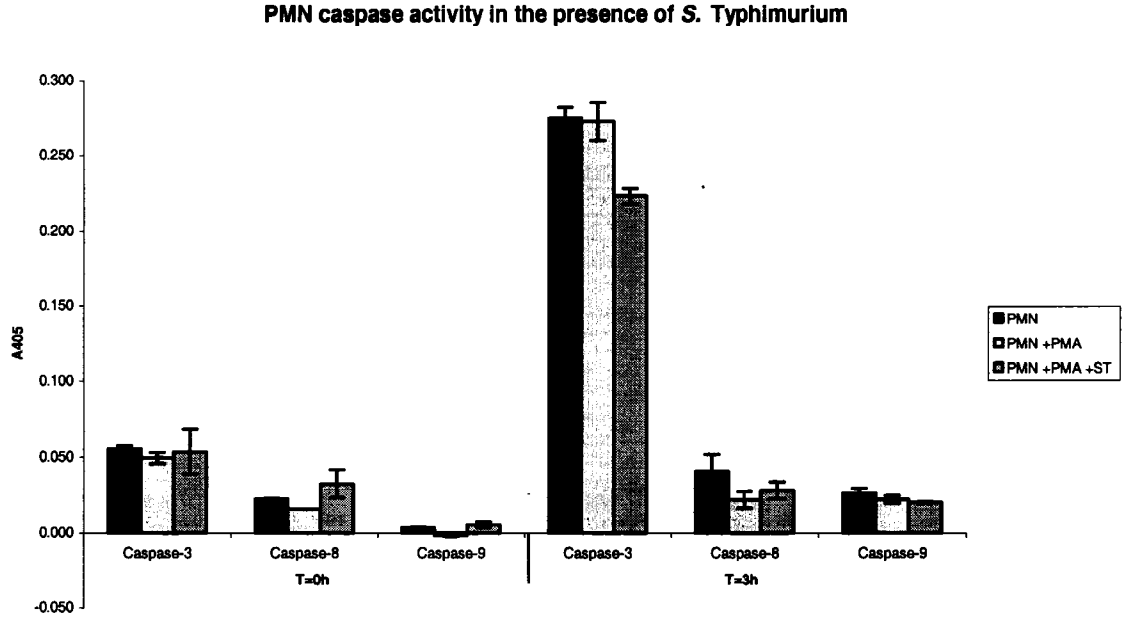


Figure 14: Various caspase activities in human neutrophils. After three hours bacterial presence had little to no affect in caspase-8 ($p=0.778$) activity throughout all conditions. Caspase-9 was affected by time ($p<0.0001$), but not bacterial treatment ($p=0.065$). Caspase-3 increased significantly after three hours ($p<0.0001$) and was affected by the presence of *S.* Typhimurium ($p=0.0129$).

CHAPTER IV

DISCUSSION

The goal of this work was to examine the interaction between human neutrophils and *Salmonella enterica* serovar Typhimurium. *S.* Typhimurium has already been shown to affect the cell death rate of macrophages (Vazquez-Torres, 2000) and dendritic cells (Kiama, 2006), but these are not the first immune cells that *S.* Typhimurium encounters during infection. The first immune cell to arrive at the site of infection is the neutrophil. Previous work in our laboratory examined how neutrophil transmigration across a model intestinal epithelial crypt cell layer influences their ability to kill *S.* Typhimurium (Nadeau, 2002). The work presented here sought to determine how the bacterium affects the neutrophil, by examining the natural cell death rate of these cells, through both apoptosis and necrosis. The cell death rate of neutrophils is intimately linked to their immune function (Haslett, 1999) and many pathogens have been shown to modulate the cell death rates of phagocytes (DeLeo, 2004). *S.* Typhimurium has been previously shown to induce lysis in human neutrophils (Chui, 1999), and in the murine model *S.* Typhimurium has been shown to survive intracellularly inside PMN (Dunlap, 1992), but was not linked with typical characteristics of cell death until now. This work shows that *S.* Typhimurium can,

and does, increase the apoptosis rate of human neutrophils, but does so only in activated neutrophils.

An important part of this work was the measurement of apoptosis. Cells in late stage apoptosis cleave their DNA into tiny fragments, effectively killing the cell. An ELISA was used to detect these DNA fragments. Interpretation of the ELISA results assumes that the more cells that have undergone apoptosis the more DNA fragments will be produced and hence the ELISA will produce a darker/stronger signal.

This is also the case with the caspase production assay. Caspases are the mediators of apoptosis. There are many different caspases that appear at many different points in the apoptotic signal cascade. Here, three different caspases were chosen based upon the type of apoptosis in which they are involved and the stage in which they first appear in the cascade. The more caspases present, the darker/stronger the signal will be, indicating more cells are undergoing apoptosis.

These studies show that S. Typhimurium affects the survival of human neutrophils in a dose-dependent manner. This was first seen in preliminary studies (Fig. 2), but was also clearly seen in later experiments (Fig. 7 and 8). Bacterial dose is important in all studies; modifying the conditions in an assay will affect how the bacterium and the host cell respond. Murine neutrophils that are unable to produce an oxidative burst lower the number of *S.* Typhimurium the mouse's immune system can fight off (Alam, 2002). The results here suggest that human neutrophils may not be able to mount an effective defense until a

critical number are present. It is debatable whether or not this would be beneficial; a lack of response would prevent host tissue damage—clearly beneficial—but might also compromise the host's ability to counter the infection especially since *S.* Typhimurium has been shown to survive inside murine PMN (Dunlap, 1992). Once the bacterial levels are high enough, the state or condition of the neutrophil itself affects how it responds to *S.* Typhimurium.

This work initially focused on inactivated neutrophils. As seen in Figures 5 and 6, in most cases *S.* Typhimurium had little effect on the viability of the neutrophils over 24 h. An exception was the study using anaerobically grown *S.* Typhimurium, which resulted in significantly lower levels of apoptosis. Thus, when *S.* Typhimurium was anaerobically grown to log phase, which is generally considered to be more representative of how the bacterium would be growing *in vivo* (Lee, 1990), and was added to inactivated neutrophils, there actually seemed to be a depressed apoptotic response (Figure 5). This suggests that the neutrophil at this point is unable to mount effective immune defenses, which is what Nadeau et al. (2002) previously showed. In fact, it was not until the neutrophil was activated through transmigration that an increased killing ability was seen. The "pro-survival" effect is puzzling, but may be due to the fact the neutrophil has yet to be primed to actually fight infection. It could also be caused by the microorganism itself as several pathogens have been shown to use granulocytes as protection from the host immune response (Laskay, 2003). Since *S.* Typhimurium is a well-known intracellular pathogen that can modulate reactive oxygen species production in macrophage it is possible that this

pathogen may gain entry to the neutrophil and disrupt is normal function. This is supported by other work, which showed *S.* Typhimurium's ability to survive inside murine PMN (Dunlap, 1992) and which found prolonged survival in neutrophils exposed to various bacteria including *Salmonella enteritidis* (Baran, 1996). Work done in this laboratory also supports this by showing that the enhanced killing ability of transmigrated neutrophils occurred in the absence of an oxidative burst, indicating that *S.* Typhimurium may be able to modulate host defenses (Nadeau, 2002).

Since inactivated neutrophils showed very little activity in the conditions set forth, and previous work done in this laboratory had shown activation to have an effect on neutrophil activity (Nadeau, 2002), apoptosis was examined in activated neutrophils, Initially we studied neutrophils that were activated chemically with PMA. Somewhat surprisingly, neutrophils activated with PMA showed an extremely strong pro-survival effect across the full 24-h period of the assay. This is seen in Figure 8, where the level of apoptosis for non-activated neutrophils peaked at 12 h, while PMA-activated neutrophils show no DNA fragmentation during the 24-h time period. Previous work with murine neutrophils found an increase in chromatin condensation and other apoptotic characteristics in the presence of PMA (Saito 2005), which is in contrast to the DNA fragmentation data presented here. DNA fragmentation however, is a later step in the apoptotic program and might not be initiated as early as other characteristics of apoptosis (Maianski, 2004). Also, the host cell may not be initiating this fragmentation of its DNA until the correct signal is presented (Fig. 8

and 12). When that signal is present, in this case *S.* Typhimurium, apoptosis is upregulated and neutrophils undergo cell death readily in both chemically (PMA) activated (Fig. 7) and biologically (transmigration) activated neutrophils (Fig. 11).

Previous studies found that three different strains of *S.* Typhimurium of different virulence levels in mice each induced lysis in human neutrophils (Chui, 1999). The researchers examined cells microscopically and only at 24 h, so they had no way of knowing how, when, what type or through what mechanisms these strains induced lysis in the PMN. In the present study we found that, after chemical activation of PMN, exposure to *S.* Typhimurium significantly increased apoptosis compared to the PMA control. This occurred in a dose-dependent manner, indicating that the more anaerobically grown *S.* Typhimurium were present, the more the apoptosis rate of the neutrophils increased (Figure 9). These data showed that only activated neutrophils were able to respond to *S.* Typhimurium.

Since chemical activation seemed to have a profound effect on the interplay between *S.* Typhimurium and neutrophils the next step was to examine this relationship in a true biological system to confirm the previous results. For this study, transmigration of freshly isolated human neutrophils across a human epithelial crypt cell monolayer was chosen as the model. What was observed was surprisingly similar to the chemically-activated neutrophils. Specifically we found a delay or pro-survival effect on the activated neutrophils (Figure 12). Furthermore, once the neutrophils were activated, they showed a significant increase in apoptosis when exposed to anaerobically grown *S.* Typhimurium

(Figure 13). These findings support the hypothesis that *S.* Typhiurium can affect the apoptosis rate of human neutrophils, but only once the neutrophils have been activated.

The growth conditions of *S.* Typhimurium also have an effect on how the neutrophil responds. Data shown in this study show that the same population of neutrophils, activated or inactivated, responds differently to anaerobically or aerobically grown *S.* Typhimurium. As noted above, in the case of inactivated neutrophils, anaerobically grown S Typhimurium had a significant pro-survival effect on the neutrophil (Figure 5). A sub-set of these same neutrophils were also exposed to aerobically grown *S.* Typhimurium; there was no significant effect on the amount of neutrophil apoptosis at any ratio of bacteria:PMN (Figure 6).

The difference was even more pronounced with activated neutrophils. As noted above, the results shown Figure 9 indicate how anaerobic *S.* Typhimurium increased apoptosis significantly compared to the PMA control. Again, as with the inactivated neutrophils, a subset of these same neutrophils was exposed to aerobically grown *S.* Typhimurium (Figure 10). *S.* Typhimurium did produce a significant increase in apoptosis compared to the PMA control, but the level of apoptosis in the neutrophil was much lower over the 24-h period compared to the same conditions with the anaerobically grown bacteria.

There are likely benefits for the host being able to differentiate between higher and lower virulent forms of a bacterium and to respond accordingly. In a recent study that looked at the interaction of *Salmonella* and dendritic cells

(Kiama,2006), different strains of *S.* Typhimurium induced different levels of apoptosis in the dendritic cell. In fact, the researchers concluded that the more virulent strains of the bacterium induced more membrane ruffling, or apoptosis. In the present studies a comparable trend was observed when comparing the same strain of bacteria grown under conditions generally considered to enhance virulence. These data suggest that neutrophils are able to distinguish bacteria with differing levels of virulence and support previously reported studies in which anaerobically grown *S.* Typhimurium was found to be more invasive and adherent than the same strain grown aerobically (Lee, 1990) and studies that more virulent *S.* Typhimurium induce apoptosis in host cells to a higher degree (Kiama, 2006). Other work (Chui, 1999), found that virulence did not have an effect, although this determination of virulence was based only upon a plasmid linked to virulence.

Other factors besides the oxygen level during bacterial grown may affect the rate of host cell death. For example, bile salts in the gastrointestinal tract have been shown to mediate bacterial virulence (Ramos-Morales, 2003). Another possible factor is the growth phase of the microorganism. While every attempt was made to synchronize the aerobic and anaerobic growth phases, it is possible that aerobic bacteria, which grow much faster than anaerobic bacteria, had entered the stationary growth phase while the anaerobic cultures were still in exponential growth. *E. coli*, another enteric pathogen, has been shown to have altered attachment and effacing activity due to growth phase and even temperature (Rosenshine, 1996). As previously discussed, both immune cells

and epithelial cells release factors that tightly regulate the lifespan of a neutrophil (Canny, 2006). The altered biological activity of neutrophils that have transmigrated through an intestinal epithelial monolayer seen in these studies supports this statement. The biological activation of the neutrophils may stimulate them to release factors that influence apoptosis in addition to factors that may be released by the T84 intestinal epithelial cells themselves (McCormick, 1998; Yu, 2001).

There are a variety of genes and factors that *Salmonella* possesses that have been shown to regulate immune cell apoptosis. Genes such as PhoP have been shown to alter immune cell function (Valle, 2005). Proteins that have been implicated include SipB (using SPI-1 and the type III secretion pathway in general) and SpvB (using SPI-2). SipB has been shown to increase caspase-1 production (Guiney, 2005), but also has been shown to have no actual effect on macrophage apoptosis (Browne, 2002). While seemingly contradictory, these findings may be explained by the fact that caspase-1 is not an "effector" protein of apoptosis and may not play a direct role in apoptosis, but rather an indirect one the requires other stimuli to induce apoptosis. SpvB and the SPI-2 gene cluster are widely accepted as being important effectors of immune cell apoptosis (Guiney, 2005; Browne, 2002). The genes in SPI-2, as previously discussed, are known to play roles in the intracellular survival of the bacterium, so this would be expected. On the other hand, SPI-1 has also been shown to be essential in macrophage apoptosis (Takaya, 2005; van der Velden, 2000) showing that the

modulation of apoptosis by *Salmonella* species is very complex and needs to be further examined.

Also examined was the role of caspases in the bacterial induced death of neutrophils. Three different caspases were examined: caspases-3, -8 and -9. Only caspase-3 seems to be a major player in this system (Fig. 14). This is not surprising considering that caspase-3 is the major effector protein of apoptosis and plays a role in most apoptotic programs (Woo, 1998). Caspase-9 activity was also increased in these studies, but the presence of *Salmonella* did not influence this increase even though caspase-9 had been deemed important in previous neutrophil-bacterial studies (Genestier, 2005). Although *Salmonella* did not affect the levels of caspase-9, they did increase over time, indicating that this caspase may play a minor role in neutrophil cell death. Caspase-8 showed no significant changes in any part of this system. This is somewhat surprising as caspase-8 is a precursor to caspase-3 (Maianski, 2004). However, caspase-8 may only be present in very early events of apoptosis. The time points examined here may have been too late to detect caspase-8 activity. These three caspases were also examined in a human macrophage-like cell line with *S.* Dublin (Valle, 2005) and the same results were observed as in this work: upregulation of caspase-3 and no change in caspases-8 and -9. Comparing those observations to the work presented here, it seems that *S.* Typhimurium may affect human neutrophils and macrophages in a similar manner. Although these three caspases were selected, based on their likelihood of contributing to the altered neutrophil death rates seen in these studies, many other members of this family

of proteins have been identified. A key caspase previously seen in *Salmonella* – macrophage interactions is caspase-1 (Takaya, 2005) and should be further explored in this system.

Death due to necrosis was also detected in these studies. In general the level of necrosis in neutrophils followed the same pattern as seen in the studies on apoptosis. One exception was seen in the transmigrated samples, where apoptosis was depressed, while necrosis was increased (Figure 13). This may be due to the T84 epithelial cells used in the transmigration assays. These cells were grown for 3-4 weeks and while every effort was made to wash the transmigrated neutrophils thoroughly, some exogenous DNA fragments from the T84 cells may have been retained. It is also possible that the transmigrated neutrophil preparations may have contained dead T84 cells that had detached from the monolayer grown on the membrane. T84 cells are anchorage-dependent, meaning they need to attach to a surface to grow and multiply. When they die, the cells detach, and could have contaminated the neutrophil suspension. In either case these exogenous DNA fragments would contribute to the necrosis samples, providing a falsely inflated value.

Future work should focus on examining which caspases are involved in this system to better understand how the human immune system fights off this pathogen. Also, the strict conditions under which both the bacteria and neutrophils must be grown and maintained should be further explored.

Conclusions

The work presented here shows for the first time that *Salmonella enterica* serovar Typhimurium can and does modulate the apoptosis rate of human neutrophils. This response occurs only in activated neutrophils and is much more pronounced when the bacteria are grown anaerobically and hence in their more virulent form. This work also shows that when human neutrophils are activated by either chemicals (*e.g.,* PMA) or transmigration across an intestinal epithelial cell monolayer, they exhibit a delayed apoptotic response compared to the inactivated control, which peaks at approximately 12 h. When activated, neutrophils are "primed" to fight infection and live longer, waiting for a second stimulus, one that the neutrophils see as foreign and dangerous and that needs to be fought. Once this stimulus is present, shown here with virulent *S.* Typhimurium, the activated neutrophil may initiate its apoptotic program in conjunction with its various and potent defense mechanisms. Caspase-3 seems to be a major player in this system, while caspase-8 and -9 do not seem to play a role in the apoptosis of human neutrophils in the presence of *S.* Typhimurium after 3 h.

LIST OF REFERENCES

Alam MS, Akaike T, Okamoto S, Kubota T, Yoshitake J, Sawa T, Miyamoto Y, Tamura F, Maeda H. 2002. Role of nitric oxide in host defense in murine salmonellosis as a function of its antibacterial and antiapoptotic activities. *Infect Immun.* **70**:3130-42.

Armant M.A. and M.J. Fenton. 2002. Toll-like receptors: a family of pattern-recognition receptors in mammals. *Genome Biol.* **3**:REVIEWS3011.1-3011.6.

Babior B.M., Kipnes R.S., Curnutte J.T. 1973. Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest.* **52**:741-44.

Baran J, Guzik K, Hryniewicz W, Ernst M, Flad HD, Pryjma J. 1996. Apoptosis of monocytes and prolonged survival of granulocytes as a result of phagocytosis of bacteria. *Infect Immun.* **64**:4242-8.

Bowe F., Lipps, C.J., Tsolis, R.M., Groisman, E., Heffrom, F. and J.G. Kusters. 1998. At least four percent of the *Salmonella typhimurium* genome is required for fatal infection of mice. *Infect Immun.* **66**:3372-77.

Brinkmann V, U Reichard, C Goosmann, B Fauler, Y Uhlemann, DS Weiss, Y Weinrauch, and A Zychlinsky. 2004. Neutrophil extracellular traps kill bacteria. *Science.* **303**:1532-5.

Browne SH, Lesnick ML, Guiney DG. 2002. Genetic requirements for salmonella-induced cytopathology in human monocyte-derived macrophages. *Infect Immun.* **70**:7126-35

Canny G, A Swidsinski, and BA McCormick. 2006. Interactions of intestinal epithelial cells with bacteria and immune cells: methods to characterize microflora and functional consequences. *Methods Mol Bio.* **341**:17-35.

Center for Disease Control and Prevention (CDC). Salmonellosis. Updated October 13, 2005.
<http://www.cdc.gov/ncidod/dbmd/diseaseinfo/salmonellosis_t.htm>

Cheminay C., D. Chakravortty, and M. Hensel. 2004. Role of neutrophils in murine salmonellosis. *Infect Immun.* **72**:468-77.

Chiu, C H; Ou, J T. 1999. Intracellular Salmonella typhimurium induce lysis of human polymorphonuclear leukocytes which is not associated with the Salmonella virulence plasmid. *Microbiol Immunol.* **43**:9-14.

Collazo C.M., Zierler, M.K. and J.E. Galán. 1995. Functional analysis of *Salmonella typhimurium* invasion genes *invI* and *invJ* and identification of a target of the protein secretion apparatus encoded in the *inv* locus. *Mol Microbiol.* **15**:25-38.

Collazo C.M. and J.E. Galán. 1997. The invasion-associated type III system of *Salmonella typhimurium* directs the translocation of Sip proteins into the host cell. *Mol Microbiol.* **24**:747-56.**DeLeo, F.R.** 2004. Modulation of phagocytes by bacterial pathogens. *Apoptosis.* **9**:399-413.

Detmers P.A., S.D. Wright, E. Olsen, B. Kimball, and Z.A. Cohn. 1987. Aggregation of complement receptors on human neutrophils in the absence of ligand. *J Cell Biol.* **105**:1137-45.

Dunlap NE, Benjamin WH Jr, Berry AK, Eldridge JH, Briles DE. 1992. A 'safe-site' for Salmonella typhimurium is within splenic polymorphonuclear cells. *Microb Pathog.* **13**:181-90.

Engelich G., M. White, K.L. Hartshorn. 2001. Neutrophil survival is markedly reduced by incubation with influenza virus and Streptococcus pneumoniae: role of respiratory burst. *J Leukoc Biol.* **69**:50-6.

English D., B.R. Anderson. 1974. Single-step separation of red blood cells. Granulocytes and mononuclear leukocytes on discontinuous density gradient of ficoll-hypaque. *J. Immunol. Meth.* **5**:249.

El-Benna J., Dang P.M., Gougerot-Pocidalo M.A., Elbim C. 2005. Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. *Arch Immunol Ther Exp.* **53**:199-206.

Finlay B.B. and J.H. Brumell. 2000. *Salmonella* interactions with host cells: *in vitro* to *in vivo*. *Phil Trans R Soc Lond.* **355**:623-31.

Frierer J., and D.G. Guiney. 2001. Diverse virulence traits underlying different clinical outcomes of *Salmonella* infection. *J Clin Invest.* **107**:775-80.

Fu Y., and JE Galán. 1999. A Salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature.* **401**:293-97.

Galán JE. 1999. Interaction of *Salmonella* with host cells through the centrisome 63 type III secretion system. *Curr Opin Microbiol.* **2**:46-50.

Galán JE and A. Collmer. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science.* **284**:322-28.

Galán JE. 2001. *Salmonella* interactions with host cells: type III secretion at work. *Annu Rev Cell Dev Biol.* **17**:53-86.

Gallois A., J.R. Klein, L.A. Allen, B.D. Jones, and W.M. Nauseef. 2001. Salmonella pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane. *J Immun.* **166**:5741-8.

Galyov E.E., M.W. Wood, R. Rosqvist, P.B. Mullan, P.R. Watson. 1997. A secreted effector protein of *Salmonella Dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. *Mol Microbiol.* **25**:1903-12.

Genestier AL, MC Michallet, G Prevost, G Bellot, L Chalabreysse, S Peyrol, F Thivolet, J Etienne, G Lina, FM Vallette, F Vandenesch, and L Genestier. 2005. Staphylococcus aureus Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. *J Clin Invest.* **115**:3117-27.

Gresham H.D., I.L. Graham, D.C. Anderson, and E.J. Brown. 1991. Leukocyte adhesion-deficient neutrophils fail to amplify phagocytic function in response to stimulation. Evidence for CD11b/CD18-dependent and -independent mechanisms of phagocytosis. *J Clin Invest.* **88**:588-97.

Guiney DG. The role of host cell death in Salmonella infections. 2005. *Curr Top Microbiol Immunol.* **289**:131-50.

Hampton M.B., A.J. Kettle, and C.C. Winterbourn. 1998. Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing. *Blood.* **92**:3007-17.

Hardt W.D., L.M. Chen, K.E. Schuebel, X.R. Bustelo and J.E. Galán. 1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell.* **93**:815-26.

Haslett, C. 1999. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med.* **160**:S5-S11.

Hayward R.D., and V. Koronakis. 1999. Direct nucleation and bundling of actin by the SipC protein of invasive *Salmonella. EMBO J.* **18**:4926-34.

Hersh D., D.M. Monack, M.R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky. 1999. The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc Natl Acad Sci USA.* **96**:2396-401.

Hofman V., V. Ricci, B. Mograbi, P. Brest, F. Luciano, P. Boquet, B. Rossi, P. Auberger, and P. Hofman. 2001. Helicobacter pylori lipopolysaccharide hinders polymorphonuclear leucocyte apoptosis. *Lab Invest.* **81**:375-84.

Hueffer K., and J.E. Galan. 2004. Salmonella-induced macrophage death: multiple mechanisms, different outcomes. *Cell Microbiol.* **6**:1019-25.

Janeway, C.A., P. Travers, M. Walport, and M. Shlomchik. 2005. After entering tissues, many pathogens are recognized, ingested, and killed by phagocytes. *Immunobiology: The immune system in health and disease 6th edition.* New York: Garland Science Publishing:42-5.

Jones B.D., N. Ghori and S. Falkow. 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J Exper Med.* **180**:15-23.

Kiama SG, Dreher D, Cochand L, Kok M, Obregon C, Nicod L, Gehr P. 2006. Host cell responses of Salmonella typhimurium infected human dendritic cells. *Immunol Cell Biol.* **84**:475-81.

Klebanoff S.J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J Bacteriol.* **95**:2131-8.

Kobayashi S.D., J.M. Voyich, F.R. DeLeo. Regulation of the neutrophil-mediated inflammatory response to infection. *Microb Infect.* **5**:1337-44.

Köhler H., B.A. McCormick, and W.A. Walker. 2003. Bacterial-Enterocyte crosstalk: cellular mechanisms in health and disease. *J Pediatr Gastroenterol Nutr.* **36**:175-85.

Kothakota S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T.J. McGarry, M.W. Kirschner, K. Koths, D.J. Kwiatkowski, and L.T. Williams. 1997. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science.* **278**:294-8.

Laskay T, van Zandbergen G, Solbach W. 2003. Neutrophil granulocytes--Trojan horses for Leishmania major and other intracellular microbes? *Trends Microbiol.* **11**:210-4.

Lee, C.A. and S. Falkow. 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc Natl Acad Sci U S A.* **87**:4304-8.

Lehrer R.I. and T. Ganz. 1990. Antimicrobial polypeptides of human neutrophils. Blood. **76**:2169-81.

Lekstrom-Himes J.A., and J.I. Gallin. 2000. Immunodeficiency diseases caused by defects in phagocytes. N Eng J Med. **343**:1703-14.

Liles W.C., A.R. Thomsen, D.S. O'Mahony, and S.J. Klebanoff. 2001. Stimulation of human neutrophils and monocytes by staphylococcal phenol-soluble modulin. *J Leukoc Biol.* **70**:96-102.

Liu C., E. Gelius, G. Liu, H. Steiner, and R. Dziarski. 2000. Mammalian peptidoglycan recognition protein binds peptidoglycan with high affinity, is expressed in neutrophils, and inhibits bacterial growth.

Lotz S., E. Aga, I. Wilde, G. van Zandbergen, T. Hartung, W. Solbach, and T. Laskay. 2004. Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2. *J Leukoc Biol.* **75**:467-77.

Maianski N.A., A.N. Maianski, T.W. Kujipers and D. Roos. 2004. Apoptosis of neutrophils. *Acta Haematol.* **111**:56-66.

McCormick B.A., S.P. Coigan, C.D. Archer, S.I. Miller, J.L. Madara. 1993. *Salmonella typhimurium* attachment to human intestinal epithelial monolayers: transcellular signalling to subepithelial neutrophils. *J Cell Biol.* **123**:895.

McCormick B.A., C.A. Parkos, S.P. Colgan, D.K. Carnes, and J.L. Madara. 1998. Apical Secretion of a Pathogen-Elicited Epithelial Chemoattractant Activity in Response to Surface Colonization of Intestinal Epithelia by *Salmonella typhimurium. J Immun.* **160**:455-66.

Miller R.M., J. Garbus, and R.B. Hornick. 1972. Lack of enhanced oxygen consumption by polymorphonuclear leukocytes on phagocytosis of virulent Salmonella typhi. *Science.* **175**:1010-1.

Moulding D.A., C. Walter, C.A. Hart, and S.W. Edwards. 1999. Effects of staphylococcal enterotoxins on human neutrophil functions and apoptosis. *Infect Immun.* **67**:2312-8.

Nadeau W.J., T.G. Pistole, and B.A. McCormick. 2002. Polymorphonuclear leukocyte migration across model intestinal epithelia enhances *Salmonella typhimurium* killing via the epithelial derived cytokine, IL-6. *Microb Infect.* **4**:1379-87.

Nath R., K.J. Raser, D. Stafford, I. Hajimohammadreza, A. Posner, H. Allen, R.V. Talanian, P. Yuen, R.B. Gilbertsen, and K.K. Wang. 1996. Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. *Biochem J.* **319**:683-90.

Neish AS. 2002. The gut microflora and intestinal epithelial cells: a continuing dialogue. *Micro Infect.* **4**:309-17.

Omann G.M., W.N. Swann, Z.G. Oades, C.A. Parkos, A.J. Jesaitis, L.A. Sklar. 1987. N-formylpeptide-receptor dynamics, cytoskeletal activation, and intracellular calcium response in human neutrophil cytoplasts. **139**:3447-55.

Porter A.G., and R.U. Janicke. 1999. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* **6**:99-104.

Ramos-Morales F., A.I. Prieto, C.R. Beuzon, D.W. Holden, and J. Casadesus. 2003. Role for Salmonella enterica enterobacterial common antigen in bile resistance and virulence. *J Bacteriol.* **185**:5328-32.

Rosenshine I, S. Ruschkowski, B.B. Finlay. 1996. Expression of attaching/effacing activity by enteropathogenic Escherichia coli depends on growth phase, temperature, and protein synthesis upon contact with epithelial cells. *Infect Immun.* **64**:966-73.

Rosen, H. 2004. Bacterial responses to neutrophil phagocytosis. *Curr Opin Hematol.* **11**:1-6.

Saito, T., H. Takahashi, H. Doken, H. Koyama, and Y. Aratani. 2005. Phorbol myristate acetate induces neutrophil death through activation of p38 mitogen-activated protein kinase that requires endogenous reactive oxygen species other than HOCl. *Biosci Biotechnol Biochem.* **69**:2207-12.

Sakahira H., M. Enari, and S. Nagata. 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature.* **391**:96-9.

Santos R.L., S. Zhang, R.M. Tsolis, A.J. Bäumler and L.G. Adams. 2002. Morphologic and molecular characterization of *Salmonella typhimurium* infection in neonatal calves. *Vet Path.* **39**: 200-215.

Takaya A., A. Suzuki, Y. Kikuchi, M. Eguchi, E. Isogai, T. Tomoyasu, and T. Yamamoto. 2005. Derepression of Salmonella pathogenicity island 1 genes within macrophages leads to rapid apoptosis via caspase-1- and caspase-3-dependent pathways. *Cell Microbiol.* **7**:79-90

Tang D, and V.J. Kidd. 1998. Cleavage of DFF-45/ICAD by multiple caspases is essential for its function during apoptosis. *J Biol Chem.* **273**:28549-52.

United States Department of Agriculture (USDA). Foodborne illness disease calculator. Updated April 15, 2003. <http://www.ers.usda.gov/data/foodborneillness/>

Valle E and DG Guiney. 2005. Characterization of Salmonella-induced cell death in human macrophage-like THP-1 cells. *Infect Immun.* **73**:2835-40.

van der Velden AW, Lindgren SW, Worley MJ, and Heffron F. 2000. Salmonella pathogenicity island 1-independent induction of apoptosis in infected macrophages by Salmonella enterica serotype typhimurium. *Infect Immun.* **68**:5702-9.

Vazquez-Torres A., Y. Xu, J. Jones-Carson, D.W. Holden, S.M. Lucia, M.C. Dinauer, P. Mastroeni, F.C. Fang. 2000. Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science.* **287**:1655-8.

Walzog B., F. Jeblonski, A. Zakrzewicz, and P. Gaehtgens. 1997. β_2 integrins (CD11/CD18) promote apoptosis of human neutrophils. *FASEB.* **11**:1177-86.

Weiss, S.J. 1989. Tissue destruction by neutrophils. *New Engl J Med.* **320**:365-76.

Woo M, R Hakem, MS Soengas, GS Duncan, A Shahinian, D Kagi, A Hakem, M McCurrach, W Khoo, SA Kaufman, G Senaldi, T Howard, SW Lowe, and TW Mak. 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev.* **12**:806-19.

World Health Organization (WHO). Drug resistant Salmonella. Updated April, 2005. <http://www.who.int/mediacentre/factsheets/fs139/en/>

Yu Y, C De Waele, and K Chadee. 2001. Calcium-dependent interleukin-8 gene expression in T84 human colonic epithelial cells. *Inflamm Res.* **50**:220-6.

Zhang B., J. Hirahashi, X. Cullere, and T.N. Mayadas. 2003. Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation. *J Biol Chem.* **278**:28443-54.

Zhang S., R.A. Kingsley, R.L. Santos, H. Andrews-Polymenis, M. Raffatellu, J. Figueiredo, J. Nunes, R.M. Tsolis, L.G. Adams, and A.J. Bäumler. 2003. Molecular Pathogenesis of *Salmonella enterica* Serotype Typhimurium-Induced Diarrhea. *Infect Immun.* **71**:1-12.

Zhou D., M.S. Mooseker, and J.E. Galán. 1999. An invasion associated *Salmonella* protein modulates the actin-bundling activity of plastin. *Proc Natl Acad Sci.* **96:**10176-81.

Zhou D., L.M. Chen, L. Hernandez, S.B. Shears and J.E. Galán. 2001. A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host-cell actin cytoskeletal rearrangements and bacterial internalization. *Mol Microbiol.* **39**:248-59.

APPENDIX

UNIVERSITY of NEW HAMPSHIRE

Institutional Review Board Annual Continuing Review Questionnaire

August 19, 2004

Wrocklage, Christopher
Microbiology, Rudman Hall

IRB #: 2609
Study: Effects of Transepithelial Migration on the Killing Ability of Human Neutrophils Amongst Different Strains of Salmonella enteritidis
Review Level: Expedited **Approval Expiration Date:** 10/18/2004

The Institutional Review Board (IRB) is obligated to conduct at least annual reviews of ongoing projects. In order to meet this obligation, the IRB asks you to answer the following questions and submit a report of findings to-date (attach) for this project. If the project is CLOSED, please submit a final report (copies of abstracts, articles, and/or publications specific to the project are acceptable).

1. Is this project still active (see question #4)? If YES, please read the NOTE below. Yes ✓ No ___
2. Please give date of termination if project has ended. —
3. Please give proposed date of termination if project is still active, and refer to the NOTE printed below. 10/18/05
4. At what stage is your research: a) subject recruitment, b) data collection, c) data analysis, d) interpretation, e) other [specify]? [Research projects in stages a - d are considered active, thus you need to request a time extension.] b
5. How many months have you actually performed the proposed investigation or activity? 11
6. How many subjects have been studied or involved to-date? 1
7. Have you conducted the research in accordance with the procedures reviewed and approved by the IRB? yes
8. Have any problems emerged or serious unexpected adverse subject experiences been observed? If YES, please describe on a separate sheet. Yes ___ No X

Principal Investigator/ Advisor Signature: Thomas G. Pistole Date: 24 Sept 2004

Please attach a BLANK copy of EACH INFORMED CONSENT DOCUMENT YOU ARE CURRENTLY USING, and return the completed form and report to the address below.

NOTE: IRB approval is granted for a maximum period of one year. Approval for your project ends on the date stated above. If your project will continue beyond this date, you must request a time extension at least two weeks prior to the approval expiration date. To do this, complete and return this form along with a written request for a time extension. If you have any questions, please call Julie Simpson at 862-2003, or Kathleen Stilwell at 862-3536.

cc: Thomas G. Pistole

Research Conduct and Compliance Services, Office of Sponsored Research, Service Building, 51 College Road, Durham, NH 03824-3585 * Fax: 603-862-3564