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[beta]-hemolysin and the pathogenesis of Serpulina hyodysenteriae infection

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β-hemolysin and the pathogenesis of *Serpulina hyodysenteriae* infection

by

David Leonard Hutto

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology
Major Professors: Joseph S. Haynes and Michael J. Wannemuehler

Iowa State University
Ames, Iowa
1997
This is to certify that the Doctoral dissertation of
David Leonard Hutto
has meet the dissertation requirements of Iowa State University

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For the Major Program
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For the Graduate College
I dedicate this work to my beloved children, Nathan David and Kyla Reneé.
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ABSTRACT

A murine model of *Serpulina hyodysenteriae* infection was utilized to explore the pathogenesis of lesion formation induced by that organism. Detailed characterization of gross and microscopic lesions in that model demonstrated that mice fed Teklad diet 85420 experienced the onset of severe gross and histologic lesions much sooner (within 24 hours) following infection with *S. hyodysenteriae* than did mice fed conventional rodent chow. However, gross and microscopic features of the induced lesions were no different in mice fed either diet.

The model was then used to document the progression of morphologic changes that occur in the cecal mucosa of mice infected with *S. hyodysenteriae*. Mice were necropsied hourly following infection and examined by transmission electron microscopy. Early ultrastructural changes consisted of microvillus effacement, intercellular fluid accumulation and increased internalization of luminal bacteria. These changes progressed to include epithelial cell degeneration and necrosis, translocation of massive numbers of bacteria to perivascular lamina proprial phagocytic and stromal cells, and changes in lamina propria capillaries indicative of increased permeability.

Experiments were then performed in order to assess the role of the β-hemolysin of *S. hyodysenteriae* in the development of the described ultrastructural changes. Purified hemolysin was introduced intraluminally into surgically created murine cecal loops and tissues were examined by transmission electron microscopy. It was shown that the hemolysin induced pronounced microvillus loss and vacuolation of superficial epithelial cells. Cells became detached, often contained bacteria and were occasionally necrotic. Many detached cells appeared apoptotic.

Studies were undertaken to purify the hemolysin and identify its gene. Crude hemolysin was purified by electrophoresis and electroelution and submitted for N-terminal amino acid sequencing. A single sequence was obtained indicating the presence of a single peptide. A degenerate oligonucleotide probe was constructed based on the amino acid sequence and used to screen a genomic library of *S. hyodysenteriae* in
Lambda Zap II. Positive plaques were obtained, restriction mapped and sequenced. An open reading frame that encodes the hemolysin amino acid sequence was identified and was found to have sequence homology with numerous acyl carrier proteins.
CHAPTER 1: GENERAL INTRODUCTION

Swine dysentery is a diarrheal disease of growing and finishing swine caused by *Serpulina hyodysenteriae* that was recently estimated to cost the swine-producing industry 180 million dollars annually (11). Swine dysentery was initially described as a unique disease entity by Whiting in 1921 but it was not until 1971 that two independent groups (Taylor and Alexander in the United Kingdom and Harris in the United States) simultaneously described the identification of a large spirochete, *Treponema hyodysenteriae*. This organism could be isolated in pure culture from pigs with clinical dysentery and could then be used to infect and reproduce the disease in susceptible pigs, thus fulfilling Koch's postulates (7, 30, 32). Since that time much basic and applied research has been performed regarding swine dysentery and its etiologic agent, now known as *Serpulina hyodysenteriae*.

The typical host response to *S. hyodysenteriae* is well characterized and described in swine and consists of necrosis of superficial epithelial cells in the large intestine, with resultant edema and inflammation in the intestinal wall (8). Clinical disease and the variable mortality associated with *S. hyodysenteriae* infection is attributed to severe dehydration and electrolyte imbalances associated with colonic malabsorption (3, 5, 28). Neither the actual mechanisms by which *S. hyodysenteriae* elicits these changes nor the sequence of events that occurs during early lesion development are well understood. A fuller understanding of these mechanisms would do much to advance research concerning the basic pathogenesis of swine dysentery.

Mice can also be infected with *S. hyodysenteriae* and develop similar, although less severe, lesions as swine. Mice infected with *S. hyodysenteriae* do not suffer mortality associated with infection and lesions are confined to the cecum (12, 24). This murine model of swine dysentery has been used repeatedly to demonstrate enteropathogenicity of field isolates and to perform studies relative to *S. hyodysenteriae* pathogenesis (13, 14, 24, 33). Other small animal
species have been used as models of *S. hyodysenteriae* but mice remain the most popular and widely used model (1, 15, 20).

An enhanced murine model for *S. hyodysenteriae* infection has been described (25). In that report a defined basal diet used in micronutrient studies was shown to greatly increase susceptibility to infection and lesion formation. The exact reasons for the increased susceptibility are not clear. Further observations have indicated that mice fed this diet experience severe lesions as soon as 24 hours following infection with *S. hyodysenteriae*. This is in contrast to mice that have been fed conventional rodent chow that are subsequently infected with *S. hyodysenteriae*. In those animals the time of onset of lesion formation is variable and is usually not assumed to occur until at least 5 days following infection. Clearly the diet-enhanced infection model offers advantages over conventional murine or porcine models in that infected subjects develop characteristic, acute lesions in a highly uniform manner. Given the proper conditions, it is feasible in such a system to document very early alterations in host tissue, thereby indicating possible mechanisms of *S. hyodysenteriae* pathogenesis. Such studies have not been previously reported.

Virulence factors that have been attributed to *S. hyodysenteriae* include a β-hemolysin, lipopolysaccharide, NADH oxidase, mucin chemotaxis and a bacteriophage (4, 6, 9, 16, 23, 29). Despite this accrued knowledge, the basic mechanisms by which *S. hyodysenteriae* causes disease in its host species have not been elucidated.

Of all the above mentioned virulence attributes of *S. hyodysenteriae* the β-hemolysin has been studied the most extensively. There have been numerous reports regarding methods of hemolysin collection and purification, the physical and chemical properties of the hemolysin and the nature of erythrocyte-hemolysin interactions (10, 19, 21, 22, 26, 27). The interaction of hemolysin with nucleated cells has also been described (2, 17, 18). In addition, there have been two reports describing studies in which porcine ileal and colonic loops created by surgical ligation were filled with solutions containing various amounts of purified hemolysin (4, 23). Marked necrosis of superficial epithelial cells and villous collapse was the primary change seen by
transmission electron microscopy (TEM) in those studies. TEM studies were not reported on the large intestine. Since lesions associated with *S. hyodysenteriae* infection are confined to the large intestine, ultrastructural changes that occur in the epithelial cells following exposure of that organ to hemolysin would be of great interest. It is proposed that the enhanced murine model described above, subsequent to further refinement and characterization, provides the appropriate conditions to investigate, *in vivo*, the role of the B-hemolysin of *S. hyodysenteriae* in the formation of lesions in animals infected with that organism.

Recently, three separate genes (*tlyA, tlyB, tlyC*) from *S. hyodysenteriae* have been cloned based upon their ability to induce a hemolytic phenotype in a nonhemolytic host strain of *E. coli* (31). None of these genes, however, have been linked directly to an *S. hyodysenteriae*-specific gene product that displays hemolytic activity. While these are usually referred to as hemolysin genes it is not widely accepted that any one of their translation products is the protein traditionally referred to as the B-hemolysin of *S. hyodysenteriae*. Identification and cloning of the hemolysin gene would obviously represent a significant step further understanding of the role of the B-hemolysin in swine dysentery.

**Objectives Of The Dissertation**

The general goal of this dissertation research was to investigate the role of the B-hemolysin of *S. hyodysenteriae* in the disease swine dysentery. The specific aims addressed in accomplishing this goal were: a) refinement and pathologic characterization of a murine model of *S. hyodysenteriae* infection; b) use of the murine model to allow description of the early ultrastructural changes that occur in mice infected with *S. hyodysenteriae*; c) use of the murine model to allow description of ultrastructural changes that occur in mice exposed intraintestinally to hemolysin; d) comparison of the findings in b) and c), above; e) to develop methods of hemolysin purification that allowed
for amino acid sequence determination of the purified protein; and, f) identification and cloning of the hemolysin encoding gene.

**Dissertation Organization**

The dissertation is presented in an alternate format with inclusion of manuscripts. The dissertation contains a general introduction, a literature review, manuscripts, and a general summary. References for each chapter are separate and immediately follow the text of that chapter. The first manuscript is prepared for submission to the Journal of Medical Microbiology and describes refinement and pathologic characterization of the enhanced murine model of *S. hyodysenteriae* infection. The second manuscript is prepared for submission to Veterinary Pathology and describes electron microscopic findings in mice either infected with *S. hyodysenteriae* or exposed intraintestinally to its β-hemolysin. The third manuscript has been submitted to Infection and Immunity and describes the identification, cloning and analysis of a β-hemolysin gene of *S. hyodysenteriae*. The Ph.D. candidate, David L. Hutto, is the first author of each paper and was the principal investigator on the first two papers. The third paper was a collaborative research effort between the listed authors. Dr. Tsungda Hsu performed all of the gene cloning and sequencing described in that paper.

**Literature Cited**


CHAPTER 2: GENERAL LITERATURE REVIEW

Swine Dysentery

Swine dysentery is a diarrheal disease of growing and finishing swine caused by *Serpulina hyodysenteriae*. A recent estimate placed the cost of this disease to the swine-producing industry at 180 million dollars annually (77). During the twenty-five years since the identification of the etiologic agent, considerable effort has been expended in research directed at this disease. In this section the historical and current literature relevant to swine dysentery will be reviewed.

Historical aspects of swine dysentery

Swine dysentery was initially described as a unique disease entity by Whiting in 1921; he also published two subsequent papers that investigated the epidemiology and transmission of the disease (179, 180, 181). In 1944 and 1947, Doyle reported finding a number of different organisms associated with clinical swine dysentery, including a *Vibrio*-like organism; he was able to reproduce clinical dysentery by feeding pure cultures of this organism to susceptible swine (30, 31, 72). In 1968 and 1971, the likelihood of spirochetes as the etiologic agent was recognized by Terpstra and Glock, respectively (45, 167). Finally, in 1971, two independent groups (Taylor and Alexander in the United Kingdom and Harris in the United States) simultaneously described the identification of a large spirochete, *Treponema hyodysenteriae*, which could be isolated in pure culture from pigs with clinical dysentery and which could then be used to infect and reproduce the disease in susceptible pigs, thus fulfilling Koch's postulates (59, 162). In 1991, *Treponema hyodysenteriae* and the closely related, non-pathogenic *Treponema innocens* were reclassified to the genus *Serpula* after it was shown by ribosomal RNA sequence analysis and comparison that they were only distantly related to the type treponemal species, *Treponema pallidum* (154). These organisms were later reclassified into the genus
Serpulina after it was discovered that the name Serpula was already used to name a fungus.

Epidemiology and diagnosis of swine dysentery

Swine dysentery is a disease of worldwide distribution with variability in prevalence rates likely dependent on movement of susceptible swine, types of control measures in use and surveillance instruments available. Data reported by Egan in 1982 indicated that 40% of slaughter animals tested in the midwest United States were seropositive for \textit{S. hyodysenteriae} (35). At approximately the same time Taylor reported that 27% of herds attended by veterinarians in the United Kingdom were affected with clinical swine dysentery. In the United States the prevalence appears to be decreasing; a 1989 survey indicated that 11% of US herds were seropositive for \textit{S. hyodysenteriae} (109).

While \textit{S. hyodysenteriae} is a fastidious organism and difficult to cultivate in the laboratory, it is apparently capable of infecting numerous animal species and surviving varied environmental conditions, thus accounting for the highly transmissible nature of the resultant disease. When outbreaks occur in previously uninfected herds they can often be attributed to the introduction of presumably infected swine from outside sources. Swine free of clinical signs of swine dysentery for 70 days have been shown to be capable of transmitting the disease (149). In addition, mice, rats, birds, dogs and flies have all been shown to be capable of either being infected with or carrying \textit{S. hyodysenteriae} for varying lengths of time (20, 47, 54, 60, 76, 80, 148). Feces from mice infected with \textit{S. hyodysenteriae} has been used to transmit the disease to susceptible swine as has lagoon water effluent from a herd affected with swine dysentery (49, 76, 128). Similarly, \textit{S. hyodysenteriae} can be shown to survive in dysenteric feces and soil for days to weeks, depending on the ambient temperature, with colder temperatures favoring longer survival times (21, 60).

Clinical swine dysentery is most often observed in growing and finishing pigs, with suckling and adult animals much less commonly, or even rarely affected. In herds that are endemically infected the course
of a disease outbreak may last for several weeks, depending on the size of the herd and the success of treatment protocols in use. Individual cases may occur in a cyclical manner and recovered pigs are generally assumed to be resistant to reinfection although they will, in all likelihood, shed spirochetes for some period of time following recovery. Depending on the particular circumstances, morbidity in outbreaks may range from 50-90% with mortalities from 10 to 30% (60). Diarrhea in affected animals ranges from watery to catarrhal to hemorrhagic including all combinations thereof. Secondary clinical signs are dehydration, anorexia, variable pyrexia, weight loss, abdominal pain, weakness, ataxia and death. Some animals may die peracutely, with few premonitory signs.

Lesions of swine dysentery are limited to the cecum and colon. Gross lesions, which are often segmental but occasionally diffuse in distribution, include hyperemia and edema of the mucosa of the large intestine and cecum. The mucosal surface in affected areas is overlain by an exudate containing mucus, fibrin, blood or some combination of those with ingesta often intermixed with the exudate. In severe cases or in severely affected segments of the colon or cecum there is often a fibrinonecrotic (pseudomembranous) exudate adherent to the mucosal surface. Colonic submucosal lymphoglandular complexes are often prominent on the serosal surface, so-called "colitis cystica profundus".

The most prominent microscopic change is necrosis of superficial enterocytes, which are often elevated in groups from the underlying edematous and congested lamina propria. In agreement with the gross findings, the lumen usually contains a mixture of exudative material, primarily mucus, fibrin and blood, the latter two of which are presumed to come from superficial lamina proprial capillaries damaged following exposure to luminal contents. In response to the accelerated loss of superficial epithelial cells, basilar glandular epithelial cells undergo hyperplasia and are often enlarged and basophilic with increased numbers of mitotic figures present. Increased depth of colonic glands results from this basal cell hyperplasia. Spirochetes, which can be demonstrated by silver staining techniques, are present within the
lumen, colonic glands and within the cytoplasmic mucin vacuoles of goblet cells.

Clinical swine dysentery in an individual animal may undergo a protracted course and therefore be referred to as chronic; however the microscopic lesions do not vary much in character in long term infections, although they may increase in severity as time proceeds. The primary microscopic lesion, epithelial cell necrosis, is by definition, an acute process that occurs in an ongoing fashion in animals that remain clinically affected for any significant period of time. The lamina propria of normal pigs contains significant numbers of leukocytes, primarily lymphocytes and plasma cells. Therefore, while neutrophil numbers within the lamina propria will be increased in areas of tissue damage, changes in inflammatory cell populations are difficult to quantify and attach significance to. Electron microscopic lesions associated with *S. hyodysenteriae* will be discussed later.

Clinical or pathologic diagnosis of swine dysentery can be made on the basis of clinical signs (mucohemorrhagic diarrhea), gross necropsy findings (catarrhal to fibrinonecrotic typhlocolitis) and demonstration of the presence of large spirochetes, consistent in appearance and cultural and biochemical characteristics with *S. hyodysenteriae*. Dark field microscopy performed on wet mounts or tissue impression smears can be used to identify large spirochetes, as can Victoria Blue staining of similar material. Many swine are also colonized by another large spirochete *Serpulina innocens*, that is morphologically similar to *S. hyodysenteriae* but non-pathogenic. These two organisms can be distinguished by cultivation on selective media and biochemical testing. Blood agar that includes five selected antibiotics (vancomycin, spectinomycin, colistin, rifampin and spiramycin) will support the growth of both organisms, but restrict the growth of almost all other common microorganisms with the exception of some fungi (100). Furthermore, when grown on that medium, *S. hyodysenteriae* is strongly β-hemolytic while *S. innocens* is weakly β-hemolytic. Biochemical tests utilizing commercial enzyme detection strips (API ZYME and An-Ident) and indole production tests can also be used to differentiate *S. hyodysenteriae* and *S. innocens* (10, 67). More recently,
oligonucleotide probes complementary to 16s ribosomal RNA sequences have been shown to be very specific for the detection of *S. hyodysenteriae* in the feces of infected animals or fecal material spiked with *S. hyodysenteriae* (36, 57, 74). Other reported methods for the identification of *S. hyodysenteriae* and its differentiation from other spirochetes include RFLP analysis, enteropathogenicity studies in mice, genomic DNA analysis by Southern blotting, and immunoblotting, microagglutination and enzyme-linked immunosorbant assay using antibodies to various antigens of *S. hyodysenteriae* (1, 27, 36, 75, 85, 147, 150, 151).

Differential diagnoses in instances where swine dysentery is suspected should include trichuriasis due to *Trichuris suis*, colitis due to *Salmonella cholerasuis* or *Salmonella typhimurium*, ileitis/colitis due to *Lawsonia intracellularis* and intestinal volvulus or torsion. These diseases can be differentiated with relative ease.

**Prevention and treatment of swine dysentery**

Prevention of swine dysentery necessitates great care in the introduction of new swine into an established herd. New additions to a herd should either be young and surgically derived, from a herd known to be free of swine dysentery or quarantined for a satisfactory period of time. Immunization against *S. hyodysenteriae* has traditionally been at best, of little value and at worst fraught with problems. One product, a whole cell, killed bacterin, has been on the market for some time and is thought to confer partial protection (60). There have been reports that such products can actually exacerbate lesion severity and financial losses associated with *S. hyodysenteriae* infection (129). There are numerous other reports of immunization studies using whole, inactivated bacterins either alone or in combination with oral exposure to attenuated, live *S. hyodysenteriae* (28, 39, 48, 56, 62, 63, 132).

While, in some of these studies, protection was conferred, immunization protocols were often complex and entailed repeated exposure by multiple routes, thus limiting their usefulness in routine prophylaxis. Recently two vaccines composed of bacterial components have been
patented and are apparently efficacious in reducing the severity of disease (78).

There are numerous antimicrobials approved for use in the treatment and prevention of swine dysentery. Tiamulin enjoys the most popularity but other compounds that were or are efficacious include various arsenicals, carbadox, lincomycin and bacitracin MD (60). Treatment of groups of sick pigs in which an outbreak has occurred is usually done by medication of drinking water. *S. hyodysenteriae* is rarely eliminated from a premises in this way but is often successfully kept in check. In some European countries eradication has been attempted, with variable success, through a combination of mass treatment of all swine on a premises or depopulation, coupled with cleaning and disinfecting of buildings and reduction or elimination of rodent populations (46).

**Experimental Infection Of Animals With *Serpulina hyodysenteriae***

Experimental studies regarding swine dysentery were performed prior to the identification of *S. hyodysenteriae* as the etiologic agent of that disease. In those studies large intestinal scrapings or homogenates were often used as the source of infectious material. This section will review reports of the experimental infection of animals with cultivated *S. hyodysenteriae* and the described host response.

**Characteristics of *Serpulina hyodysenteriae***

*Serpulina hyodysenteriae* is a large, weakly Gram-negative spirochete that is roughly 0.35 microns by 7 microns in size (59). Individual organisms consist of a protoplasmic cylinder which is surrounded by seven to nine axial filaments, both of which are encased in an outer sheath. While it is known that cholesterol and phospholipids are required for growth by *S. hyodysenteriae* and that it can utilize a number of different carbohydrate substrates as energy sources, the exact nutrient requirements of the organism are not well defined and enriched media, such as trypticase soy broth, brain heart infusion (each
supplemented with serum) or blood agar, are generally used for cultivation (155). Optimal conditions for growth have been shown to include brain heart infusion broth supplemented with fetal bovine serum, vigorous stirring and an atmosphere of 99% N2: 1% O2 (155). For purposes of animal infection described in subsequent sections, organisms are grown in trypticase soy broth supplemented with 2-5% equine serum, to a density of approximately $1 \times 10^9$ cells per milliliter. High degrees of active motility (>70%) are desired and utilized as an indicator of cell vigor.

**Experimental infection of swine with *Serpulina hyodysenteriae***

Since swine are the apparent natural host species of *S. hyodysenteriae* it is not surprising that most early experimental work involving *S. hyodysenteriae* was done in that species. Gross and histologic lesions are described in an earlier section and are well known by diagnosticians and experimentalists whom work with *S. hyodysenteriae*. Those now familiar changes were documented by several workers in the 1970's as being the consistent result following experimental infection of swine with *S. hyodysenteriae* (17, 59, 64, 65, 182).

Ultrastructural changes in the large intestine of pigs infected with *S. hyodysenteriae* have been described, both by scanning and transmission electron microscopy (4, 45, 90, 163, 164). Scanning electron microscopic studies demonstrated the first visible changes as roughening or corrugation of the mucosal surface. This was followed by exudation of fibrin, mucus and blood onto the mucosa, within which spirochetes were enmeshed. Spirochetes were present in tangled masses associated with the colonic glands and gland openings. Transmission electron microscopic studies demonstrated early changes in superficial epithelial cells, which included intercellular edema, mitochondrial swelling and loss of microvilli as well as superficial lamina proprial vascular congestion. Spirochetes, in addition to being present within the lumen and the colonic glands, were often present beneath or on the lateral borders of exfoliated epithelial cells, the
presumption being that they migrated there after detachment of the epithelial cells.

Soon after the identification of *S. hyodysenteriae* as the etiologic agent of swine dysentery, studies of infection of germ-free pigs with that agent were undertaken to allow further clarification of the pathogenesis of the disease. Studies by Meyers et. al. demonstrated that germ-free pigs inoculated with *S. hyodysenteriae* did not develop clinical signs or gross lesions typically associated with swine dysentery in spite of the facts that colonization of the large intestine of germ-free pigs by *S. hyodysenteriae* did occur and conventional pigs similarly treated did develop clinical and pathologic features typical of swine dysentery (114). Mild microscopic lesions, consisting of colonic submucosal edema and inflammatory cell infiltrates were present in some infected germ-free pigs. Those findings led the authors to believe that *S. hyodysenteriae* is an opportunistic pathogen that requires the presence of some other organisms to cause disease. In two subsequent reports, that same group demonstrated that germ-free pigs inoculated with *S. hyodysenteriae* and either *Escherichia coli*, *Lactobacillus*, *Campylobacter coli*, or *Clostridium* did not develop disease, while germ-free pigs inoculated with the combination of *S. hyodysenteriae* and four different anaerobes, characterized as either fusiforms or *Bacteroides*-like, did develop clinical and pathologic changes typical of swine dysentery (113, 115). Numerous other reports verified the finding that gram positive anaerobes fulfill the apparent required role of a secondary, somehow symbiotic bacterial species, necessary for *S. hyodysenteriae* to exert its full pathogenecity. These other reports also concurred with the original finding that certain other species, such as *Peptostreptococcus* or *Vibrio coli*, could not fulfill that role (17). Specifically, infection of germ-free pigs with *S. hyodysenteriae* and either *Bacteroides vulgatus* or *Fusobacterium necrophorum* was shown to be sufficient to induce lesions of swine dysentery (58, 178). Furthermore, infection of germ-free pigs with *S. hyodysenteriae* and both of the anaerobes resulted in clinically more severe disease than infection with *S. hyodysenteriae* and only one of the anaerobes (58, 178). Finally, to confuse the issue, there is a single report of induction
of disease in germ-free pigs inoculated only with *S. hyodysenteriae* (177).

The mechanism by which these secondary bacteria act in concert with *S. hyodysenteriae* to cause disease is not known. As speculated upon by the above authors and others in the field, at least two or three possibilities seem apparent and have been proposed as hypotheses: 1) the secondary bacteria have some effect on *S. hyodysenteriae* which renders it more virulent, 2) the secondary bacteria, in the presence of *S. hyodysenteriae*, have effects on the host that contribute to or account for lesion production or 3) some combination of #1 and #2 (178).

According to the first hypothesis, secondary organisms are responsible for some direct or indirect enhancing effect on *S. hyodysenteriae*. This might occur through alteration of the colonic microenvironmental pH or redox potential, or through the direct effect of bacterial metabolites of the secondary organisms on *S. hyodysenteriae*, any of which may enhance spirochetal virulence. Such virulence enhancement could manifest itself in the form of increased colonization levels, increased toxin production, increased mucin chemotaxis and motility or other mechanisms. The second hypothesis, regarding the effect of secondary bacteria on the host response, is based on the idea that *S. hyodysenteriae*, while unable to induce disease by itself, does cause some damage to the intestinal mucosa and, in doing so allows interaction of the secondary bacteria with the host inflammatory and immune response. According to this hypothesis, it is the interaction of host and secondary bacteria that initiates and perhaps more importantly, perpetuates the inflammatory response that occurs in swine dysentery. Evidence collected from experimental infection in swine supporting either of these hypotheses is lacking. Studies addressing these issues in mice are discussed in a later section. Finally, it is worth considering the possibility that the reason the lesions induced by *S. hyodysenteriae* occur only in the large intestine may be due to the presence there of flora specifically selected for by the specialized conditions in that environment.

The primary lesion in swine dysentery is necrosis of superficial epithelial cells of the colon with secondary inflammation and
hemorrhage involving the lamina propria. However, much of the decreased production and associated costs, as well as mortality attributable to the disease are due to diarrhea and associated dehydration. Prior to the identification of *S. hyodysenteriae* as the etiologic agent of swine dysentery, Glock documented hematologic and biochemical changes in pigs affected by clinical dysentery (45). Inflammatory leukograms were common in these pigs and consisted of neutrophilia, often with a degenerative left shift; these changes were attributed to increased demand for neutrophils within the intestine. Hypoalbuminemia was also commonly found, presumably due to hemorrhage and albumin rich fluid (serum) exudation into the intestinal lumen. Electrolyte and acid/base changes that were documented were associated with the onset of diarrhea. Hemoconcentration, hyponatremia and hypochloridemia were present in most affected pigs. Hyperkalemia also commonly occurred, often in the time shortly before death. HCO$_3^-$ levels were consistently low but acidemia was not a consistent finding. Dehydration, hyponatremia, hypochloridemia and base deficit can all be attributed to loss of body fluids rich in these components, i.e. failure to absorb them from the large intestine, and diarrhea.

Possible mechanisms of the colonic dysfunction and diarrhea seen in swine dysentery have been investigated. Argenzio et. al. utilized cecal-cannulated swine to demonstrate that *S. hyodysenteriae* infected swine had marked net decreases in Na$^+$ and Cl$^-$ absorption (intestinal lumen to blood) compared to controls, but were no different from controls in secretion of those ions (blood to lumen) (7). Schmall et. al. later verified these findings and further showed that cyclic nucleotides were not operative in a secretory diarrhea in cannulated pigs infected with *S. hyodysenteriae* (144). In fact, in those studies isolated colonic mucosa of diseased swine was partially refractory to the effects of theophylline, an agonist of cyclic nucleotide activity. Given that swine infected with *S. hyodysenteriae* have widespread necrosis of colonic epithelial cells and that there is associated hyperplasia of immature, presumably less functional cells, these findings would indicate that the diarrhea that occurs in swine infected with *S. hyodysenteriae* is due to
colonic malabsorption associated with a loss of functional colonic epithelial cells. The primary role of the colon is reabsorption of water and electrolytes, most of which is secreted into the lumen in the small intestine. It has been shown that in the pig up to 50% of the extracellular fluid volume arrives in the colon for reabsorption each day (29). Given these facts, it is evident that the simple lack of colonic absorption is sufficient in pigs to cause serious fluid and electrolyte and acid/base abnormalities.

Intestinal loops created by surgical ligation in swine have been utilized for various experimental purposes. Whipp et. al. utilized that method to demonstrate that isolates of \textit{S. hyodysenteriae} that were deemed pathogenic under conditions of natural or experimental infection induced lesions of swine dysentery in ligated colonic loops, while nonpathogenic isolates did not (176). The same method was used by Joens et. al. to demonstrate that convalescent antisera to homologous \textit{S. hyodysenteriae} strains protected colonic loops from lesion formation following instillation of inoculum from those virulent strains into such loops (79, 88). Finally, ligated ileal loops were utilized by Lysons et. al. in two related studies to examine the effect of the \( \beta \)-hemolysin on the ultrastructural morphology of the mucosa; those results will be discussed in detail in a later section (14, 107).

**Experimental infection of non-swine species with \textit{Serpulina hyodysenteriae}**

Several other species of animals have been infected with \textit{S. hyodysenteriae} and used as models of swine dysentery. One paper described infection of guinea pigs with \textit{S. hyodysenteriae} in which a high mortality was noted, thereby limiting its usefulness (87). Chicks can also develop lesions following infection with \textit{S. hyodysenteriae}. (2, 158, 159, 160). In the most well described of those studies one day old chicks infected with \textit{S. hyodysenteriae} were found to have gross and microscopic lesions at 21 days postinfection similar to those seen in infected swine, including fibrinonecrotic typhlitis with epithelial erosions and resultant epithelial hyperplasia (158). Transmission electron microscopic examination of tissues from those animals
demonstrated effacement of microvilli and the terminal web, intercellular fluid accumulation, passage of spirochetes into the lamina propria and increased numbers of membrane bound vesicles at apical cell surfaces (158).

One report describes the use of ligated ileal loops in rabbits infected with *S. hyodysenteriae* as a model of swine dysentery (97). In that model intestinal fluid accumulation and mucosal necrosis were present in loops infected with pathogenic strains, but not in those infected with nonpathogenic strains.

Infection of mice with *S. hyodysenteriae* is the most frequently reported and well characterized small animal model of swine dysentery. In the first such report, CF-1 mice were fasted for 72 hours and inoculated twice with spirochetes. Gross and microscopic findings included increased cecal luminal mucus, superficial epithelial cell erosions, mucosal edema and epithelial cell hyperplasia, similar to findings in swine infected with *S. hyodysenteriae* (80). The time of onset of such lesions was not specified. A subsequent study, also utilizing CF-1 mice, demonstrated that the murine model could be used to differentiate pathogenic from nonpathogenic isolates (81). Emulating a similar course of experiments performed in swine, gnotobiotic CF-1 mice infected and colonized with *S. hyodysenteriae* did not develop gross lesions unless they were coinfected with *Bacteroides vulgatus* (86). Such mice infected with *S. hyodysenteriae* alone did, however, develop very mild microscopic lesions, consisting of vacuolar degeneration of crypt basilar epithelial cells. As that finding is not reported in conventional mice infected with *S. hyodysenteriae* its significance is unknown. Similar findings regarding the necessity of secondary flora for lesion production have also been reported in germ-free Ta:CF#1 mice (61). In another study germ-free CD-1 mice were infected with either *S. hyodysenteriae* or *S. innocens* then subsequently infected with *Bacteroides vulgatus* (121). Both serpulinal species colonized but only those mice infected with *S. hyodysenteriae* and coinfectected with *B. vulgatus* developed lesions. In another experiment demonstrating the need for secondary cecal flora, a combination of antibiotics was used to abrogate lesions in mice previously infected with
S. hyodysenteriae (121). Such antibiotic treated, lesion free mice were further shown to have similar numbers of colonizing S. hyodysenteriae as nontreated mice with lesions, again supporting the role of secondary flora in lesion induction (43).

Murine models of S. hyodysenteriae have also been used to investigate a number of other factors regarding cecal lesion formation. Such studies include the demonstration of the role of thymus derived lymphocytes in athymic nude mice infected with S. hyodysenteriae, the lack of lesion enhancement by the inclusion of streptomycin in the drinking water, and the protection against lesion formation afforded by dietary zinc supplementation (118, 119, 157, 185).

Variation in susceptibility to S. hyodysenteriae infection and subsequent lesion formation in mice was first shown to be related to genotype in a study in which C3HeB/FeJ mice, which are normoresponsive to lipopolysaccharide, were shown to be more susceptible to lesion formation than C3H/HeJ mice, which are hyporesponsive to lipopolysaccharide, thus implicating the innate host inflammatory response to lipopolysaccharide as a major factor in lesion development (125). In a later paper several inbred mouse strains were compared in their inherent susceptibility to colonization and lesion formation (123). That study confirmed the positive correlation of lipopolysaccharide responsiveness to susceptibility, and also identified some other genetic factors related to susceptibility, among them the H-2 haplotype.

An enhanced murine model for S. hyodysenteriae infection has been described (124). In that report a defined basal diet used in micronutrient studies was shown to greatly increase susceptibility to infection and lesion formation. The exact reasons for the increased susceptibility are not clear. However, in mice maintained on the diet and infected with S. hyodysenteriae numbers of cecal aerobic gram-negative and gram-positive organisms increased by 4 log units and 2 log units, respectively. This would seem to indicate that the diet induced lesion enhancement occurs associated with changes in cecal flora, further supporting the role of secondary bacteria in lesion development.
Virulence Attributes Of *Serpulina hyodysenteriae*

Proposed virulence factors of *S. hyodysenteriae* are β-hemolysin, lipopolysaccharide, NADH oxidase, mucin chemotaxis and a bacteriophage. These factors will be discussed individually in this section.

**β-hemolysin**

One of the phenotypic characteristics of *S. hyodysenteriae* that distinguishes it from the nonpathogenic *S. innocens* is strong β-hemolysis during culture on blood agar. In the first report of hemolysin production from broth cultures of *S. hyodysenteriae* it was reported that the addition of sodium ribonucleate to those cultures greatly increased hemolysin production (134). There have been many subsequent reports on the β-hemolysin of *S. hyodysenteriae* describing its chemical, physical and biological properties. This hemolysin is categorized in the oxygen-stable, heat labile category of bacterial exotoxins. The type toxin for that group is Streptolysin-S (SLS), which is produced by *Streptococcus pyogenes* group A. Methods of collection and purification of the β-hemolysin of *S. hyodysenteriae* are based on similar methods reported for SLS.

The β-hemolysin of *S. hyodysenteriae* has been shown to be heat labile, active over a wide range of pH values, and sensitive to both proteases and lipase, indicating it may be a complex of lipid and protein (98, 142). Hemolysin production is maximal at the logarithmic growth phase and preformed hemolysin is not present within viable spirochetes (102). Zinc has been shown to specifically inhibit the production of hemolysin by interacting with viable spirochetes in some unknown way (33). Numerous methods have been reported for hemolysin production and partial purification, most involving traditional protein purification techniques (94, 98, 102, 143). These studies have been at variance in their reports on the molecular weight of the isolated hemolytic factor, ranging from 19 kiloDalton (kDa) to 74 kDa.

The most frequently cited report of β-hemolysin isolation and partial purification described the necessity for the use of a toxin
extraction buffer containing glucose, magnesium and RNA-core, a purified form of ribonuclease A-digested ribosomal RNA from *Torula spp* (94). RNA-core apparently serves as a carrier or inducer molecule for the hemolysin. Without it (or some other negatively charged molecule) minimal amounts of toxin could be collected from broth cultures of *S. hyodysenteriae*. In that report, logarithmic growth phase spirochetes were suspended in concentrated form in the described buffer, incubated for 30 minutes at 37° C up to 5 times and those supernatants pooled and collected. Pooled supernatants were reduced in volume by dialysis and ultrafiltration and then purified by a combination of ion exchange and gel filtration chromatography. These procedures resulted in a loss of total activity but a marked increase in specific activity (hemolytic units/mg protein) (94). (Hemolysin quantification is expressed in terms of hemolytic units (HU), an arbitrary unit of activity that is, in most cases, defined as the amount of hemolysin required to cause 50% lysis of a 1% solution of sheep erythrocytes in a volume of 2 milliliters of phosphate buffered saline). Comparison of the crude and partially purified hemolysin preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that the purification procedures removed many protein bands, but that the staining pattern of the purified hemolysin was no different than that of the similarly purified and concentrated RNA-core containing buffer. Use of nondenaturing PAGE coupled with blood agar overlays demonstrated the presence of hemolytic activity in the hemolysin preparation which was associated with a band present in both RNA-core buffer preparations and purified hemolysin. This supports the supposition that the RNA-core serves as a carrier molecule and that the hemolysin is in close physical association with it. All bands present in both the purified hemolysin and RNA-core preparations were present at or in front of the dye front, indicating very low molecular weights and/or negative charges for the RNA-core carrier species and the associated hemolysin (94). In addition, hemolysin activity was seen to pass through ultrafiltration membranes with a molecular weight of 5 kDa (94). It is apparent that the hemolysin is present in the same location in the gel as some RNA-core carrier bands but that it does not
have distinctive staining characteristics based on traditional protein stains such as Coomassie Blue and ammoniacal silver stains. It is possible that the amino acids responsible for binding to those respective staining chemicals are inaccessible due to the association of the hemolysin with the RNA-core carrier.

Due to the mucosal environment inhabited by *S. hyodysenteriae*, the possible role of the β-hemolysin in the pathogenesis of swine dysentery is probably not due to its actions on erythrocytes; hemolysis is likely a phenotype manifested during culture on blood agar. However, due to the ease of their collection and manipulation, erythrocytes have been the cell most often used in the few studies on the mechanism of cytolysis induced by the serpulinal hemolysin. Early work indicated that cell binding of hemolysin is temperature independent while cell lysis requires physiologic temperatures and that cell swelling occurs prior to lysis (142). The latter feature, shared with Streptolysin-S, is referred to as the colloid-osmotic mechanism of lysis and is thought to be associated with cytolytic mechanisms other than the formation of discrete, membrane penetrating, complement-like pores (32). However, hemoglobin release was shown to occur prior to cell swelling, indicating another cytolytic mechanism involving membrane perturbation and leakage (142). Enzymatic digestion of cell membrane proteins or phospholipids has not been supported in these studies, however. (142, 143). Osmoprotectants can be used to provide indirect evidence regarding possible pore size in that solutes of a particular diameter will prevent the entry of water into the cell under study through pores of a similar diameter. Such studies regarding the hemolysin of *S. hyodysenteriae* have indicated a possible pore diameter of 1.0 to 1.1 nm (68).

The hemolysin of *S. hyodysenteriae* has been shown to have *in vitro* cytotoxicity for a number of cell types, including fibroblasts and porcine lymphocytes. (92, 93). A continuous colonic epithelial cell line, Caco-2, has been used experimentally to determine the effect of serpulinal hemolysin on epithelial cells (3). Monolayers of those cells treated with hemolysin exhibited decreases in transcellular resistance
and increases in intracellular calcium ion concentration, both indicative of membrane damage.

Given that *S. hyodysenteriae* is a mucosal pathogen and that its hemolysin is a secreted toxin, the presumption would be that the initial interaction between toxin and host would be at the epithelial surface, specifically involving superficial colonic epithelial cells. Since ongoing necrosis of those cells is the underlying primary lesion of swine dysentery, it is tempting to speculate that the hemolysin creates that lesion and is therefore critical in the pathogenesis of swine dysentery. Two reports utilizing one to two month old germ-free pigs have described studies in which ileal and colonic loops created by surgical ligation were filled with solutions containing various amounts of partially purified hemolysin (14, 107). Following incubation periods of variable lengths tissues were examined by scanning and transmission electron microscopy. Instillation of 50,000 to 100,000 HU of hemolysin for 3 hours was required to induce lesions in ileal and colonic loops. The most extensive and well characterized lesions occurred in the ileum; lesion occurrence was highly variable in the colon due to inactivation of the hemolysin by ingesta. In the ileum, degeneration and necrosis of apical villus enterocytes was extensive. By scanning electron microscopy affected villi were shortened and plicated, with marked exfoliation of mature apical enterocytes; goblet cells were less susceptible to damage. Transmission electron microscopy confirmed susceptibility of mature vacuolated enterocytes at villus tips, which exhibited mitochondrial condensation, dilatation of endoplasmic reticulum and nuclear swelling. Affected villi were collapsed due to loss of enterocytes and subsequent folding of the basement membrane due to myofibroblast contraction. This basement membrane folding and villus collapse brought adjacent enterocytes into apposition to allow epithelial restitution, which occurred by extension of cellular lamellododia across the bare basement membrane. Goblet cells and microfold (M) cells were not susceptible to toxin induced damage, although myofibroblast within the lamina propria were occasionally necrotic (14, 107). Complete resolution of lesions had not occurred by 18 hours. Results from colonic loops similarly treated were reported for
scanning electron microscopy only. Colonic epithelial cells within the crypts and those immediately surrounding crypt orifices were not damaged following exposure to hemolysin. However, surface epithelial cells within intercryptal areas, which are the most mature cells, exhibited rounding, individualization and increased extrusion, with cells detaching in groups (14, 107).

Thus, it appears from these studies that mature epithelial cells of the large and small intestine are the most susceptible to toxic actions of the β-hemolysin of \textit{S. hyodysenteriae}. The relevance of the findings regarding villus damage, collapse and restitution is unclear since lesions resulting from infection with \textit{S. hyodysenteriae} only occur in the large intestine, both in pigs and mice. Furthermore, it is not known if the amounts of hemolysin used in these studies are biologically relevant, since it is unknown how much, if any, hemolysin is secreted by \textit{S. hyodysenteriae} in the lumen of the large intestine of host animals.

All of the above information concerns what is sometimes referred to as the "native" hemolysin of \textit{S. hyodysenteriae}. That term has become necessary since the reports of cloning of three separate hemolysin genes from \textit{S. hyodysenteriae} (165). In the report describing the first of these, a plasmid library of \textit{S. hyodysenteriae} DNA was used to transform a nonhemolytic host strain of \textit{E. coli} to a hemolytic phenotype (117). Such hemolytic recombinants were found to contain a 1.5 kilobase (kb) fragment of \textit{S. hyodysenteriae} DNA that contained an open reading frame capable of encoding a 26.9 kDa protein. This recombinant protein could be collected from host \textit{E. coli} and was found to be, like the native hemolysin, heat labile and cytotoxic for various cell lines. Furthermore, it was demonstrated that the gene, identified as the \textit{tly} gene, is present in the pathogenic \textit{S. hyodysenteriae} but not the nonpathogenic \textit{S. innocens}. This gene was not found to have sequence homology with any other known proteins. Knowledge of the \textit{tly} gene allowed construction of \textit{tly} negative \textit{S. hyodysenteriae} mutants (\textit{tly}-) via electroporation and homologous recombination (166). These mutants were shown to have reduced hemolysis, which was partially regained upon repeated passaging. \textit{tly}- mutants were also shown to
colonize but be less pathogenic in mice, although the numbers of mice used were small.

Similar methods were subsequently utilized to identify two other genes from *S. hyodysenteriae* that induced a hemolytic phenotype in nonhemolytic *E. coli* host strains (165). The three hemolysin genes are now referred to as *tlyA*, *tlyB* and *tlyC*. The predicted molecular weights of the *tlyB* and *tlyC* genes are 93.3 kDa and 30.8 kDa, respectively. The protein products of these genes are also heat labile and cytotoxic. The *tlyB* gene has homology with the Clp proteins, which are a group of intracellular proteolysis regulators. The *tlyC* gene, like the *tlyA* gene, has no sequence homology with other known proteins.

Swine were subsequently shown to be susceptible to infection and colonization with *tlyA*- mutants from two strains of *S. hyodysenteriae*, B204 and C5, although no clinical signs occurred in those animals (69). Swine previously infected with the *tlyA*- mutants were then reinfected with wild type *S. hyodysenteriae* of the same strain. These animals were susceptible to colonization by wild type spirochetes but refractory to lesion development. Lesions did occur in animals infected twice with wild type *S. hyodysenteriae* (a finding that is at odds with other reports).

The above data indicate that *S. hyodysenteriae* contains at least three genes, *tlyA*, *tlyB* and *tlyC*, that can influence the hemolytic phenotype in *E. coli*. Furthermore, at least one of those genes, *tlyA*, appears to be necessary for the full expression of virulence in animals infected with *S. hyodysenteriae*. There has been no evidence presented, however, that would indicate that any of these proteins are the "native" hemolysin of *S. hyodysenteriae*. None of the unique physicochemical characteristics described for the native hemolysin such as carrier dependency, apparent small molecular weight and lack of normal protein staining characteristics have been demonstrated to be associated with these recombinant proteins. In fact, very little information at all has been presented concerning the characteristics of these proteins other than that predicted by their nucleotide sequences. Thus, while they may be important in normal metabolic function and/or
virulence, their relationship to the native hemolysin or their true role in the pathogenesis of swine dysentery is not clear.

**Lipopolysaccharide**

Since *S. hyodysenteriae* is a gram negative bacteria, it possesses a lipopolysaccharide moiety (LPS) in its outer membrane. Examination of LPS extracted from *S. hyodysenteriae* by (SDS-PAGE) and silver staining has demonstrated that there are 2 to 4 primary LPS bands in such preparations (52, 53, 55). LPS can be prepared by two different methods: hot phenol/water extraction produces a pure LPS product while butanol/water extraction results in a preparation called endotoxin containing LPS and associated proteins. The biological properties generally associated with LPS and endotoxin have been well described in many common gram negative bacterial species, with *Escherichia coli* and *Salmonella spp.* serving as archetypal examples. *In vivo* properties include fever, endothelial damage, activation of macrophages with subsequent release of inflammatory mediators, shock, release of acute phase reactants from the liver and interaction with contact activation and coagulation cascades, all of which may result in lethality. *In vitro* properties of LPS and endotoxin include polyclonal B cell activation and activation of macrophages resulting in interleukin-1 (IL-1) and tumor necrosis factor (TNF) release. LPS and endotoxin preparations from *S. hyodysenteriae* and *S. innocens* do possess these properties, but in order for them to be observed, 5 to 50 times as much LPS or endotoxin is required when compared to *E. coli* or *Salmonella spp.* (51, 141). Furthermore, the dermal Shwartzman reaction was not elicited in rabbits treated with serpulinal endotoxin. Endotoxin preparations from *S. hyodysenteriae* and *S. innocens* were shown to elicit elevations in serum TNF when given intraperitoneally to mice, but such responses were not seen with systemic administration of such preparations to swine (121). In a recent study LPS and endotoxin preparations from *S. hyodysenteriae* were shown to be capable of eliciting secretion of IL-1β and IL-8 from porcine alveolar macrophages but not IL-6 or TNF (141). As with the other similar findings mentioned, the degree of this response was much less than that elicited by *E. coli.*
Responsiveness to LPS has been implicated as a factor in lesion development in mice infected with *S. hyodysenteriae*. Mice that are less responsive to LPS were less susceptible to *S. hyodysenteriae*-induced lesion formation (123, 126). These conflicting data do not help to clarify the potential role of serpulinal LPS in the pathogenesis of swine dysentery. Since infection by *S. hyodysenteriae* occurs only on the large intestinal mucosa, conclusions drawn from the systemic or *in vitro* effects of serpulinal endotoxin or LPS preparations may be misleading. On the other hand, the correlation between LPS responsiveness status and susceptibility to lesion formation that is evident in mice must also be interpreted with care as LPS activates a cascade of responses in sensitive mice. Decreased susceptibility to lesion induction may be due to a generalized decrease of inflammatory processes at the mucosal surface and not to the lack of an *S. hyodysenteriae*-LPS specific response. Furthermore, there are numerous gram-negative species present within the colon, the LPS of which would be more likely to induce inflammation in the intestine than that of *S. hyodysenteriae*.

**NADH oxidase**

As mentioned in a previous section, optimal conditions for growth of *S. hyodysenteriae* include an atmosphere of 1% oxygen; hence the description of this organism as an aerotolerant anaerobe (155). It has been shown that *S. hyodysenteriae* possesses an NADH oxidase, thus allowing it to reduce molecular oxygen (152, 153). Oxygen delivered to the colonic lumen by erythrocytes derived from hemorrhagic lesions might therefore be available for metabolism and growth by *S. hyodysenteriae*.

**Mucin chemotaxis**

In animals infected with *S. hyodysenteriae* organisms are found within the mucus layer immediately overlying the colonic epithelial cells (91). In fact two separate reports have detailed the chemotactic properties of *S. hyodysenteriae* for mucin, specifically gastric and colonic mucin from swine (91, 116). Virulent *S. hyodysenteriae* strains were shown to be chemotactic toward mucin in contrast to nonvirulent
S. innocens. Conventional views concerning the function of the intestinal mucosal mucus layer assume that bacteria are trapped in the mucus and prevented from coming into contact or close association with epithelial cells, thereby decreasing colonization or the effect of elaborated toxins on the epithelium. In the case of S. hyodysenteriae it would appear that whatever mechanisms result in mucin induced chemotaxis serve to increase the survivability and growth of S. hyodysenteriae in proximity to colonic epithelial cells, thereby increasing the likelihood of necrosis of those cells, which is the characteristic and fundamental lesion of swine dysentery.

**Bacteriophage**

A bacteriophage has been identified in cultures of S. hyodysenteriae and a recent report describes mitomycin C induction of bacteriophage release from viable S. hyodysenteriae (66, 139). A possible role for the phage in pathogenesis has not been suggested.

**Immune Responses To Serpulina hyodysenteriae**

Swine infected with S. hyodysenteriae have been shown to be resistant to secondary infection, in some cases for up to 17 weeks following initial exposure (82, 127). This resistance correlated with prolonged fecal shedding of S. hyodysenteriae and with an anti-S. hyodysenteriae microagglutination titer that lasted up to 8 weeks (82). It is not clear from these findings if protection is related to persistent infection or serum antibody response.

Other studies have demonstrated a lack of correlation between serum antibody response and protection from disease (39, 138). Intestinal immunoglobulin G (IgG) and IgA as well as IgG and IgA memory cells have been shown to be induced by infection with S. hyodysenteriae although correlation with protection was not demonstrated (138). As previously mentioned, immunization with some whole-cell killed bacterins can actually exacerbate lesion
formation, presumably due to a heightened immune response to *S. hyodysenteriae* antigens (129).

Immune responses to specific antigens of *S. hyodysenteriae* have been examined. A 16 kDa outer membrane lipoprotein has been shown to be a consistently immunodominant protein in convalescent sera from swine immunized with or recovered from infection with *S. hyodysenteriae* (84, 145, 168, 169, 170). Antibodies against this protein agglutinate and inhibit the growth of *S. hyodysenteriae* but its role in virulence is unknown (145, 168). Immune responses to the endotoxin of *S. hyodysenteriae* have also been demonstrated. Antisera from pigs following immunization or infection with *S. hyodysenteriae* have been shown to recognize 14 kDa and 19 kDa antigens (174). Both of these antigens were shown to be endotoxin in nature by abrogation of reactivity following treatment with sodium meta-periodate.

Another antigen of *S. hyodysenteriae* commonly recognized by convalescent swine sera is the periplasmic flagella or axial filament (83, 95). Endoflagellar genes have been cloned and expressed in *E. coli* (16). Mice immunized with partially purified recombinant protein were protected against oral challenge with homologous and heterologous strains of *S. hyodysenteriae* and antisera from immunized mice killed *S. hyodysenteriae* in vitro (16). A different group was unable to prevent disease in swine challenged with *S. hyodysenteriae* by previous immunization with recombinant endoflagellar protein (42).

Cell mediated immune responses to *S. hyodysenteriae* have not been examined in as thorough a manner as humoral responses. Lymphocytes collected from swine infected with *S. hyodysenteriae* were shown to be capable of undergoing blastogenesis *in vitro* following exposure to *S. hyodysenteriae* antigens, and some infected animals were capable of developing delayed type hypersensitivity reactions following intradermal injection of serpulinal antigen (73). Mucosal mast cell deficient mice were utilized to demonstrate the lack of a role for mucosal mast cells in lesion development in mice infected with *S. hyodysenteriae* (122). Most recently, examination of surface molecule expression and cytokine profiles of lymph node and lamina proprial lymphocytes from mice infected with *S. hyodysenteriae* on either a
short or long term basis indicated that T-lymphocytes from infected mice secreted more interferon-gamma, especially in response to *Bacteroides vulgatus* antigens, as well as to *S. hyodysenteriae* antigens, early in infection (43). Furthermore, surface expression of CD69 and CD25, markers of activation in T-lymphocytes, was increased in mice infected for 10 days. These data again provide evidence for the possible role of secondary flora in the host response to *S. hyodysenteriae* infection and indicates the role that cytokine expression may play in that response. Severe combined immunodeficient (SCID) mice, which are nearly devoid of functional T or B lymphocytes, that were infected with *S. hyodysenteriae*, however, developed lesions qualitatively and quantitatively similar to normal mice (121). Thus, while cells of the immune system undoubtedly play a role in the immune response to *S. hyodysenteriae* it does not appear that lymphocytes are instrumental in lesion formation.

### Mechanisms Of Cell Death

Cell death can occur as a response of exposure of that cell to a variety of etiologic factors. However, the cellular mechanisms by which cell death occurs are few in number, at least as far as current understanding allows. The following sections will review current knowledge concerning general mechanisms of cell death.

Although there is not complete agreement within the field of pathology that there is more than one type of cell death, mechanisms of cell death are currently divided by most researchers into two broad categories: cell death due to "suicide" (apoptosis) or cell death due to "murder" (necrosis) (38). The last ten years has seen an explosion in publications concerning apoptosis and so much of the section below is devoted to that subject.

### Apoptosis

The term apoptosis is used in this section to refer to instances in which cell death appears to occur due to some type of active suicide.
process by the cell or cells in question. There is a lack of agreement by workers in the field as to the appropriate terminology to be used in this instance; the term "programmed cell death" has been also used to describe such events. Most would prefer that the latter term be reserved for use in reference to the death of cells that occurs during normal embryonic development or tissue involution.

The term apoptosis, then, refers to instances of cell death which require RNA and protein synthesis and which have characteristic biochemical and morphologic features. Biochemical features characteristic of apoptosis, which precede the characteristic morphologic features, are related to activation of a calcium ion dependent endonuclease that cleaves nuclear chromatin into 185 base pair fragments (nucleosomes), a change which can be detected due to the laddered appearance of such DNA on agarose gel electrophoresis (23, 171, 183). Endonuclease mediated endonucleosome cleavage results in the production of a 3' overhang on the remaining DNA fragment. This 3' overhang, which is relatively specific for apoptosis as opposed to necrosis, can be identified by incorporation of labeled nucleotides into the fragments by way a terminal deoxynucleotidyl transferase end labeling reaction (TUNEL reaction) (44, 50). These and other authors have also noted that this method may label necrotic as well as apoptotic cells and that results should be interpreted with caution and the proper controls (50, 184).

Other intracellular events associated temporally with apoptosis are activation and/or inhibition of intracellular proteases, cytoplasmic microtubular dysfunction and, most consistently, prolonged elevations of intracellular calcium ion concentrations (104, 112, 131, 133). Initial elevations in calcium ion concentration apparently arise from intracellular stores while extracellular calcium ions are responsible for later, more prolonged elevations (131). Given the central role of intracellular calcium ions in many important physiologic reactions and pathways, including those involving the enzymatic and contractile proteins mentioned above, it is not surprising that perturbations in calcium ion homeostasis may be associated with cell death. Indeed, calcium ion elevation has long been viewed as the terminal event
resulting in complete cellular dysfunction that precedes what is described as necrosis, discussed below. How elevations of calcium ion in those instances differs from instances in which apoptosis, rather than necrosis, is the result, is not clearly evident. However, it is assumed that apoptotic events that may be due to calcium ion elevation are associated with the function of calcium binding proteins, such as calmodulin, or by way of calcium regulated phosphorylation (functional) states of intracellular proteases (131). These proteases might be involved in DNA degradation via proteolytic activation of endonucleases or digestion of histone proteins, allowing access of endonucleases to target DNA. Alternatively, it has been argued that the actual activator of the apoptotic event is not the elevation of cytoplasmic calcium ion concentration but the depletion of endoplasmic reticular (ER) stores of calcium ion, which are the source of the subsequent cytoplasmic elevation. In this scenario, apoptosis occurs due to release of nucleases and proteases from their storage sites in the ER subsequent to depletion of ER calcium ion (8).

Immunologically mediated apoptosis, such as occurs due to cytotoxic T lymphocyte action, natural killer cells and T lymphocyte deletion during lymphocyte maturation, has been shown to be preceded by elevations in cytoplasmic calcium ion concentrations (5, 111, 120). Another mechanism of intracellular calcium ion concentration elevation that results in apoptosis is that associated with oxidative stress resulting in the generation of reactive oxygen species (18, 34, 37). These reactive species apparently damage membrane associated calcium transport systems that normally maintain intracellular calcium ion concentrations at much lower levels than that in the extracellular fluid (130). Two other proposed mechanisms of apoptosis are intracellular acidification leading to activation of pH dependent nucleases and plasma membrane phospholipid hydrolysis leading to ceramide production which, by some unknown pathway, leads to apoptosis (9, 136).

The morphologic features that characterize apoptosis are: cell shrinkage and cytoplasmic condensation, pyknosis and margination of chromatin into crescentic bodies applied very closely to the nuclear
membrane, production of cytoplasmic buds which subsequently "pinch off" and are phagocytosed as apoptotic bodies, endocytosis of cell membrane resulting in cytoplasmic vesicles and vacuoles (inward blebbing) and normal appearance of mitochondria and other cellular organelles, which often cluster within the cytoplasm (19, 96, 108, 173). Margination of nuclear chromatin into clumps is presumed to be associated with endonuclease mediated cleavage of DNA, although some have suggested that the two events may occur independently (156, 161).

A variety of stimuli can lead to cells acquiring the morphologic and biochemical features of apoptosis, including ionizing radiation, hyperthermia, toxins, cytokines, viral infection and immunologic mediated cytotoxicity such as that mediated by perforin derived from cytotoxic T lymphocytes (6, 22, 103, 173). While this might seem to suggest that different pathways of cell death may result in common morphologic features typical of apoptosis and therefore represent a morphological and not a functional phenomenon, it is argued that the idea of a final common pathway is supported by the ability of anti-apoptotic genes, such as bcl-2, to protect against apoptosis instigated by a variety of stimuli (137). In addition, some stimuli, notably tumor necrosis factor and calcium ionophore, can induce both apoptotic and necrotic phenotypes within different cells (101, 110).

Loss of integrin mediated epithelial cell attachment to underlying basement membrane peptides can induce apoptosis in the detached cell; this specialized mechanistic form of apoptosis has been referred to as anoikis and is apparently the important mode of effete intestinal epithelial cell removal (40, 135). Interestingly, in the guinea pig, and monkey, effete apoptotic enterocytes are phagocytosed by a large population of macrophages within the superficial lamina propria, whereas in the human, rat and mouse most effete apoptotic cells are sloughed into the lumen (71, 146).

Necrosis

Necrosis refers to non-apoptotic cell death and is characterized morphologically by cellular swelling with blebbing, increased
cytoplasmic eosinophilia and karyorhexis and karyolysis; an inflammatory response is usually induced by necrotic cellular debris. Specific insults that induce necrosis are too numerous to mention but most such insults induce necrosis by one or more of the following mechanisms: damage to the selectivity of membrane permeability or failure of plasma membrane ionic pumps, interference with generation of ATP by way of mitochondrial or mitochondrial membrane damage, increases in intracellular calcium ion concentration which may lead to mitochondrial dysfunction, activation of proteases, nucleases and lipases, non-specific DNA breakdown, and protein denaturation and hydrolysis (19, 140). A recent review suggests the word oncosis to describe non-apoptotic cell death; i.e. there are two types of necrosis (cell death): apoptotic and oncotic (108). In any case, what has always been described as necrosis is the sum of morphologic changes that represents the endpoint of the loss of cellular homeostasis, that loss possibly arising from a wide variety of causes.

**Bacterial Exotoxins: A Selected Review**

Bacterial exotoxins (as well as other toxins) can be classified into a number of different categories based on functional characteristics, mechanism of action, chemical and/or physical characteristics, cellular target sites and perhaps other criteria. Some toxins may fall into more than one of these categories, while others occupy their own category in solitude. For example, toxins may be classified as blockers of protein synthesis, neurotoxins, ion channel blockers, pore-formers, phospholipases and affectors of cytoskeletal functions. The following sections will discuss a few of the more well understood bacterial exotoxins and their unique or shared characteristics that result in the use of a particular system to categorize them.

**Pore-forming toxins**

As is self evident, these toxins cause cellular dysfunction or lysis due to the formation of molecular pores in the plasma membrane of
target cells. Pore-forming toxins produced by different classes of organisms (gram negative compared to gram positive) have vastly different chemical and physical properties but have the same end result: formation of a transmembrane pore of variable diameter within the plasma membrane of the target cell. Commonly cited examples are discussed below.

**RTX toxins.** This class of toxins is so named due to the presence of repeating nonapeptide units within the toxin molecule (Repeats in ToXin). RTX toxins are produced by numerous gram negative organisms, including *Escherichia coli, Pasteurella hemolytica, Actinobacillus pleuropneumoniae, Actinobacillus actinomycetemcomitans, Actinobacillus suis, Bordatella pertussis, Proteus vulgaris* and *Morganella morganii;* other organisms possessing the ability to produce RTX toxins may yet be identified. Due to the number of reports concerning the *E. coli* hemolysin (HlyA), it is generally thought of as the type species for the RTX toxins. Most of the information described below pertains specifically to the HlyA of *E. coli,* but it may safely be assumed that other RTX toxins are similar.

The operons that contain the genes for RTX toxins all share similar characteristics. These operons have four genes: A-D. The A gene encodes for the actual hemolytic protein, B and D encode export function proteins and the C gene encodes for the protein that acylates a portion of the A gene product (175). A hydrophobic portion of the HlyA molecule contains eight membrane-spanning α-helices, i.e. the pore-forming portion of the protein (105). This is followed in turn by a β-turn rich region, an acyl binding site and a calcium binding region; the latter contains the glycine rich repeating nonapeptide units (175). The C protein is apparently an acyltransferase and moves an acyl group from acyl carrier proteins to be covalently attached to lysine residues on the A protein. Acylation of the A protein is necessary for activity as is calcium binding by the calcium binding region (15, 70, 106).

The nature and mechanism of cellular binding and lysis mediated by *E. coli* HlyA have not been conclusively demonstrated. Osmotic protection studies would indicate that a transmembrane pore is formed in cells exposed to HlyA, but other workers feel the toxin, especially at
relatively high concentrations, has a detergent effect on the membrane phospholipids (12, 175).

HlyA, in spite of its name, probably exerts its effects on virulence through some other avenue than that of hemolysis. Indeed, HlyA and other RTX toxins have been shown to bind to and affect the functional state of numerous cell types, notably, leukocytes. The RTX toxins of \textit{P. hemolytica} and \textit{A. pleuropneumoniae} (leukotoxins) have very narrow ranges of specificity in that they bind only to the leukocytes of the host species in which they are pathogens, ruminant and porcine, respectively (175). Both of these leukotoxins have been studied in regards to their effects on neutrophils and have been shown to cause dose related, calcium ion mediated responses ranging from granule release, cytoskeletal alterations and oxidative bursts to cell death and lysis (26, 89). Thus, these leukotoxins may increase virulence by causing the premature activation and release of granules by neutrophils which have been attracted to a site of infection and inflammation, thereby removing those neutrophils from the pool of available effective leukocytes and increasing the tissue damage attributable to neutrophil activity.

\textbf{Staphylococcal \textit{\alpha}-toxin.} The alpha toxin produced by \textit{Staphylococcus aureus} was the first extensively studied bacterial toxin recognized as a pore former (41). Alpha-toxin binds to an unidentified, saturatable, specific receptor and forms a small pore (11). Unlike RTX toxins, pore formation induced by \textit{\alpha}-toxin does not result in alterations in calcium ion concentrations, but appears to affect nucleated cells through alterations in potassium and sodium ion homeostasis (11). For example, lymphocytes treated with \textit{\alpha}-toxin leak potassium ion and undergo apoptosis (11).

\textbf{Thiol activated toxins.} Streptolysin-O is produced by Lancefield Group A \textit{Streptococcus pyogenes}. It is the type species of a group of toxins produced by gram positive organisms that are characterized by their cholesterol binding property and by their possession of a single sulfhydryl bond, which renders them susceptible to inactivation by oxidation and reactivation by thiol reagents such as cysteine (172). Streptolysin-O binds to cell membranes via its interaction with cholesterol where it forms very large pores in the cell membrane which
are visible by electron microscopy as circular or crescentic shaped structures (13). Of the thiol-activated toxins, Listeriolysin O (LLO), produced by *Listeria monocytogenes*, has been shown to have the most direct involvement in pathogenesis. *Listeria monocytogenes* is an intracellular pathogen and, following entry into the cell via phagocytosis, *L. monocytogenes* lyses and escapes the phagosome and then spreads to adjacent epithelial cells by virtue of its ability to produce LLO (172).

**ADP-ribosylating toxins**

There are numerous examples of bacterial exotoxins which function to covalently attach ADP-ribose to intracellular target proteins. The best known of these include cholera toxin of *Vibrio cholerae*, pertussis toxin of *B. pertussis* and diphtheria toxin of *Corynebacterium diphtheriae*. The molecular organization of some of these toxins differ widely but they all have a basic structural motif in common, that is the possession of distinct A and B subunits (99). Diphtheria toxin is a single polypeptide chain with the two subunits covalently linked while pertussis and cholera toxin are examples of multimeric polypeptides with a single A subunit noncovalently associated with 5 copies of the B subunit. In all cases the A subunit possesses the ADP-ribosyl transferase activity and the B subunit(s) deliver the A subunit to its intracellular target (99).

The specific intracellular protein that is ADP-ribosylated and the effect of that on cellular function varies with the toxin. Cholera toxin and pertussis toxin both ADP-ribosylate subunits of GTP-binding proteins, so called G proteins. Many cell membrane receptors are coupled to G proteins and exert their effects on signal transduction by altering the phosphorylation state, through GTP-ase activity, of their associated G proteins (24). By ADP-ribosylating those proteins, pertussis and cholera toxins effectively abrogate cellular responses that operate through G protein associated receptors. Diphtheria toxin, as well as ETA toxin of *Pseudomonas aeruginosa* ADP-ribosylate elongation factor-2, thereby inhibiting protein synthesis (99).
Others

Streptolysin-S (SLS) was discussed in a previous section as the type species of the oxygen stable, heat labile toxins, of which the \( \beta \)-hemolysin of \textit{S. hyodysenteriae} is a member. Many organisms, notably \textit{Clostridium perfringens}, produce and secrete enzymes that function in pathogenesis, such as phospholipase, neuraminidase and sphingomyelinase. \textit{Helicobacter pylori} has been recently shown to produce an uncharacterized exotoxin that induces prominent intracellular vacuolation in treated cells (25).

Literature Cited


necrosis by the combined use of in situ tailing and nick translation techniques. Lab Invest 71:219-225.


75. Jensen, N. S., and T. B. Stanton. 1993. Comparison of *S. hyodysenteriae* B78, the type strain of the species, with other *S. hyodysenteriae* strains using enteropathogenicity studies and restriction fragment length polymorphisms. Vet Microbiol **36**:221-231.


dysentery. I. Oral inoculation of germ-free swine with Treponema
dysentery III. The role of selected gram negative obligate
by Serpulina hyodysenteriae and other porcine spirochetes:
potential role in intestinal colonization. Infect. Immun. 62:4095-
4099.
enteric lesions in C3H/HeN mice inoculated with S. hyodysenteriae
serotypes 2 and 4 with or without oral streptomycin
Morphologic analysis of enteric lesions in conventional and
streptomycin-treated inbred C3H/HeN mice infected with
Serpulina (Treponema) hyodysenteriae. Lab Anim Sci 42:7-12.
120. Nakagama, T., Y. Ueda, H. Yamada, E. W. Shores, A. Singer, and C. H.
receptors that are specific for self ligands. Science 257:96-99.
121. Nibbelink, S. K. 1992. The role of tumor necrosis factor and
secondary bacteria in the pathogenesis of swine dysentery
(Serpulina hyodysenteriae). Ph.D. dissertation, Iowa State
University.
Treponema hyodysenteriae infection on mucosal mast cells and T
inbred mouse strains to infection with Serpula (Treponema)


uncontaminated gnotobiotic pigs. Proc International Pig Veterinary Society, Seventh Congress 7:27.


181. Whiting, R. A., L. P. Doyle, and R. S. Spray. 1921. Swine dysentery. Purdue University Agricultural Experiment Station Bulletin #257:


CHAPTER 3: MORPHOLOGIC AND TEMPORAL CHARACTERIZATION OF LESIONS IN AN ENHANCED MURINE MODEL OF SERPULINA HYODYSENTERIAE INFECTION

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Abstract

This laboratory has previously reported a murine model of Serpulina hyodysenteriae infection in which mice fed a defined diet, Teklad 85420 (TD), develop cecal lesions more consistently than mice fed a conventional rodent chow (CRC). The objectives of the current studies were to characterize and compare a) the time of onset of lesions b) the morphologic nature and severity of lesions and c) the extent of colonization by S. hyodysenteriae in mice fed the two diets. In the first of two experiments, 50 C3H/HeJ and 50 C3H/HeOuJ mice were fed either TD or CRC and then half of each group was infected with S. hyodysenteriae (n=25). Mice (n=5) from each group were necropsied on postinfection days 1, 2, 4, 9, and 17, examined grossly and microscopically and assigned lesion scores based on lesion severity. The second experiment was designed identically to the first but had slightly smaller group sizes (n=20). Mice (n=4) were necropsied at the same five time points post infection and their ceca homogenized and examined by quantitative bacteriology using media selective for S. hyodysenteriae. Group lesion scores over the entire experimental period were significantly higher (p < .005) in mice fed TD (mean total lesion index=13) than in those fed CRC (mean total lesion index=8.8). Lesions were also temporally distributed in a significantly different manner (p < .05) in that they appeared earlier (day 1) and persisted longer in the TD fed mice in comparison to CRC fed mice. In addition,
microscopic lesions of equivalent severity from each treatment group were similar in appearance regardless of diet, time of sampling or mouse strain. Finally, quantitative bacteriologic results indicated there was no significant difference in the number of colony forming units of *S. hyodysenteriae* isolated from mice fed TD or those fed CRC. These results demonstrate that the characteristic severe lesions associated with *S. hyodysenteriae* infection in mice can occur one day following oral challenge in mice fed Teklad diet 85420. Bacteriologic results further indicate that the enhancement of lesion formation induced by TD is not due to any significant effect of the diet on numbers of spirochetes in the ceca of infected mice. These findings add support to the idea that intestinal microenvironmental or other host-related factors are critical in lesion development in animals infected with *S. hyodysenteriae*.

**Introduction**

*Serapulina hyodysenteriae*, a large gram negative spirochete, is the etiologic agent of swine dysentery, a highly transmissible diarrheal disease of growing swine. Swine dysentery is characterized grossly by a mucohemorrhagic cecitis and colitis and microscopically by superficial epithelial erosions, lamina proprial congestion and inflammatory cell infiltration and colonic gland hyperplasia; spirochetes are associated with the mucosa at the luminal surface and within the colonic glands [1-3].

Numerous species of small laboratory animals have been experimentally infected with *S. hyodysenteriae*, including mice, guinea pigs and chicks. The mouse model has been the most extensively used, both in enteropathogenicity testing of different strains of *S. hyodysenteriae* and in studies on pathogenesis [4-11]. Lesions in mice, which are less extensive and severe than those in swine, are confined to the cecum, but are similar microscopically to those observed in swine. The microscopic lesions are characterized by epithelial erosions, crypt elongation and lamina proprial and submucosal inflammatory cell
infiltrates and edema [4, 6, 11]. This laboratory has reported on strain-related differential susceptibility in mice to *S. hyodysenteriae* infection and on the use of a defined diet (Teklad diet 85420) to enhance the susceptibility of mice to the pathogenic effects of *S. hyodysenteriae* infection [8, 12]. We have noted that susceptible mice fed this diet may develop gross lesions within 24 hours of infection. In contrast, in most studies utilizing mice as an infection model for swine dysentery, principals are killed and examined seven to 20 days following infection. The purposes of these experiments were to characterize and compare the changes that occur morphologically and bacteriologically (with specific reference to *S. hyodysenteriae*) over time, in two different strains of mice fed two different diets, conventional rodent chow (CRC) and Teklad 85420 (TD).

**Materials And Methods**

**Mice**

Twelve to 24 week old, male and female, C3H/HeJ (lipopolysaccharide hyporesponsive) and C3H/HeOuJ (lipopolysaccharide responsive) mice were used in this study. These animals were obtained from breeder colonies maintained by the Department of Laboratory Animal Resources, Iowa State University, and were cared for in accordance with the guidelines stipulated by the Animal Care and Use Committee of that institution. Breeding colonies were established with mice originally procured from Jackson Laboratory, Bar Harbor, Maine and were routinely screened and found to be negative for the presence of serum antibodies to Sendai virus, murine hepatitis virus and *Mycoplasma spp.*

**Diet**

Breeding colonies and half of the principals of these experiments were maintained on Mouse Lab Chow 5010 (Purina Mills, Inc., St. Louis, Mo.), subsequently referred to as *conventional rodent chow* (CRC). Seventy-two hours prior to inoculation half of the principals were fed
Teklad Diet 85420 (Harlan Sprague Dawley, Madison, Wis.), subsequently referred to as *Teklad diet* (TD), and were maintained on that diet for the remainder of the experimental period. The approximate composition of TD 85420 is as follows (w/w): dextrose-63.4 %; egg white solids-20.0 %; corn oil-10.0 %; cellulose-3.0 %; micronutrients as required. Food and water were given *ad libitum*.

**Bacteria and infection procedure**

Mice were infected with *S. hyodysenteriae* strain B204, serotype 2, which was grown, as previously described, at 37°C in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD.) supplemented with 5% horse serum (HyClone Laboratories, Logan, Utah), 0.5% yeast extract (BBL), 0.05% L-cysteine (Sigma Chemical Co., St. Louis, MO) and 1% VPI salt solutions [8]. Log phase cultures of *S. hyodysenteriae* (motility 75-90%) were evaluated for bacterial concentration with a Petroff-Hauser counting chamber. Mice were given 1 x 10⁸ bacteria in approximately 0.5 ml of culture broth intragastrically, twice within a 24 hour period; food was withheld six hours prior to and six hours following each of the two infections.

**Experimental design**

In the first experiment, mice were randomly allotted to one of the four treatment groups arising from the factorial arrangement of two diets and two mouse strains, with a total of 25 mice allotted to each group. Five randomly selected mice from each group were necropsied at 1, 2, 4, 9 and 17 days following the second gastric intubation and utilized for pathologic examination. A second experiment, which was similar in design to the first, was performed for bacteriologic studies. In that experiment, there were 20 mice per group with four mice necropsied at each of the same time points. Male and female mice were equally represented within treatment groups in both experiments.

**Pathological examination**

In the first experiment, ceca were evaluated grossly for atrophy, edema and increased mucus in the ingesta, given a gross lesion score (0
- 3), and then placed in 10% neutral buffered formalin until the end of the experimental period. Following fixation, ceca were sectioned longitudinally, routinely processed, and embedded in paraffin. Sequential sections (5 mm) were stained with hematoxylin and eosin or Warthin-Starry (silver) stains. Specimens were randomly assigned an identification code number to insure objectivity in evaluation of histopathological changes. Parameters evaluated microscopically for each specimen and the range of possible scores to be assigned to them were gland depth (2 - 7), degree of lamina proprial cellular infiltrate (0 - 3), submucosal and lamina proprial edema (0 - 3), superficial epithelial erosions (0 - 3), and the presence or absence of spirochetes on Warthin-Starry stained sections (+ or -). A total lesion index score was arrived at by summation of all lesion scores for each specimen (excluding +/- Warthin - Starry staining).

**Bacteriological examination**

In the second experiment, aseptically removed ceca were weighed and suspended 1:100 (wt/vol) with phosphate buffered saline (136.9 mM NaCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, pH = 7.2) solution in sterile WhirlPak bags (Baxter Co., Minneapolis, Minn.) and then homogenized in a stomacher laboratory blender (A.J. Seward Co., London, United Kingdom). Appropriate serial dilutions of the homogenate were added to 5 ml of molten (45° C) trypticase soy agar containing 5% ovine blood and antibiotics selective for *S. hyodysenteriae*; this mixture was poured into 60 mm petri dishes and incubated anaerobically at 37° C for 72 to 96 hours [13]. Zones of β-hemolysis were counted and used to calculate colony forming units (CFU) per cecum. Selected colonies were examined by dark-field microscopy to verify the presence of large, motile spirochetes.

**Statistical analysis**

Numerical data from individual mice were used in analysis of variance procedures in SAS to obtain estimates of overall experimental error and to allow comparison of means of different groups.
Results

Pathologic parameters

The mice fed TD developed more severe lesions than the mice fed CRC as evidenced by the higher scores for those mice in the parameters examined; this is illustrated by the total lesion index score comparison of mice fed TD versus mice fed CRC, shown in figure 1. Gross lesions, gland depth, lamina proprial cellular infiltrate and submucosal edema were individually significantly affected by diet (p < .005), irrespective of strain or day postinfection (data not shown).

Mice fed TD demonstrated an earlier onset and prolonged persistence of more severe lesions in comparison to mice fed CRC; this is illustrated by the total lesion index score comparison of the two diet groups in figure 2. The changes that occurred over time in gland depth and lamina proprial edema were individually significantly different (p < .05) due to diet, with mice fed TD demonstrating an earlier onset and prolonged persistence of more severe lesions in comparison to mice fed CRC (data not shown). Mice fed CRC developed their most severe lesions around day four postinfection after which lesion severity gradually decreased. Mice fed TD demonstrated lesions of greatest severity on the first day post infection, experienced a marked decrease in lesion severity during days two and four and then developed quite severe lesions again by day nine. Lesion severity was maintained at a high level in TD mice for the remainder of the experiment. On days two and four, lesion severity was essentially the same for the two dietary groups. However, on all other days, there was a marked difference in lesion scores between the two groups.

Microscopic observations

Normal, uninfected mice, regardless of strain, diet or day postinfection, had ceca lined with a mucosa in which glands were two to three times as deep as they were wide, shown in figure 3. The lamina propria was approximately one to a few cell layers thick and was occupied by spindle cells and occasional lymphocytes; neutrophils were
rarely present. Gut associated lymphoid tissue (GALT), depending on sectioning, had few lymphoid follicles with small germinal centers, shown in figure 4. In mice infected with *S. hyodysenteriae* common features of severe inflammation included epithelial erosions, lamina proprial and submucosal edema, lamina proprial infiltration by inflammatory cells, gland hyperplasia, and other features such as dilatation of glands and exfoliation of necrotic epithelial and inflammatory cells into the cecal lumen. Precise combinations of these features varied slightly from mouse to mouse and within individual cecal sections.

Severe and mild microscopic lesions could be found in all groups at each time point regardless of strain or diet treatment; the score data presented above demonstrate that mice fed TD experienced more severe lesions than those fed CRC. However, the histological features of severe lesions were essentially the same regardless of diet or day of sampling. In other words, a severe lesion from a TD fed mouse was the same in microscopic appearance as a severe lesion from a CRC fed mouse, irrespective of strain or day postinfection. This is illustrated in figures 5 through 8 in which severe lesions from days one and seventeen, each from a different diet treatment group, are compared. Figures 5 and 6 are photomicrographs of tissues from a mouse fed TD; the mouse was killed one day following infection with *S. hyodysenteriae*. Figures 7 is a photomicrograph of tissue from a mouse fed CRC; it was killed 17 days following infection with *S. hyodysenteriae*. The morphologic features typical of severe lesions, as described above, are present in both specimens. As might be expected, gland hyperplasia was a more prominent finding in those animals infected for 17 days compared to those necropsied 1 or 4 days after infection. Also in animals examined after seventeen days, GALT nodules were often grossly nodular due to the presence of prominent lymphoid follicles with enlarged germinal centers shown in figure 8. These effects, gland and GALT hyperplasia, were not influenced by diet treatment.

In all but one of the mice infected with *S. hyodysenteriae* large spirochetes were demonstrable within cecal lumina and glands of
Warthin-Starry stained sections; intestinal spirochetes were not demonstrated in the ceca of uninfected mice by this technique.

**Bacteriology**

There was no significant difference in colony forming units of spirochetes isolated from animals as affected by strain, day or diet, as shown in figure 9. Animals on the TD had slightly higher mean CFU than those on CRC (7.61 and 7.36 log base ten CFU per cecum, respectively) but the difference was not statistically significant. There was considerable animal to animal variation (range of 6.3 to 8.4 log base ten CFU per cecum).

**Discussion**

In the first report describing the murine model of *S. hyodysenteriae* infection, mice were examined between two and 30 days postinfection; gross cecal lesions and clinical signs (mucoid feces) were seen throughout the experimental period [4]. In another report describing infection of gnotobiotic mice, lesions occurred within three days in mice biassociated with *S. hyodysenteriae* and *Bacteroides vulgatus* [14]. While it has been shown that mice may remain colonized with *S. hyodysenteriae* for up to 70 days following infection, most workers examine experimentally infected mice between 10 and 20 days postinfection [5-8, 11, 12]. As presented in this report, use of the Teklad 85420 diet increases the uniformity and usefulness of the murine model of *S. hyodysenteriae* infection by allowing earlier collection of cecal tissue samples with more consistent lesions from experimental animals.

Two identical experiments were performed to examine the effects of TD, as compared to CRC, in mice infected with *S. hyodysenteriae* with regards to 1) qualitative and kinetic nature of lesion formation and 2) numbers of *S. hyodysenteriae* in the cecum on various days postinfection. Microscopic examination was not performed in the bacteriology experiment; however, gross lesions were recorded in the
bacteriology experiment and were very similar in kinetics and severity to those seen in the experiment dedicated to lesion evaluation (data not shown).

Overall lesion severity, both gross and microscopic, was significantly increased in mice fed TD; total lesion index score is presented as the summative measure of lesion severity. Importantly, the microscopic appearance of lesions of similar severity was not different due to diet. While hyperplasia of gland epithelium and GALT was present in mice of both diet treatment groups examined after 17 days of infection, this expected effect is attributed to the duration of infection. These findings are in agreement with a previous report in which the Teklad diet was shown to increase the mean gross cecal lesion score in three different strains of mice infected with *S. hyodysenteriae* [12]. However, microscopic examination of lesions was not performed in the previous study. As noted, in that study and others utilizing the murine model for swine dysentery, principals were killed and examined for lesions or their tissues utilized for further experimentation at 10 to 20 days. In our studies, the animals fed the two diets exhibited significantly different patterns of lesion development over time; however, in both cases maximal group lesion development occurred much earlier in the course of the experiment than 10 to 20 days. In effect, these studies establish that the use of TD as compared to use of CRC in the murine model of *S. hyodysenteriae* infection results in cecal lesions that a) have the same morphological features as those that occur with CRC, and b) occur at a much earlier time postinfection (day 1) than previously reported.

Any explanation of the differences demonstrated in these experiments in patterns and severity of lesion development due to TD must be based on the idea that TD somehow influences the intestinal microenvironment to allow these differences to occur. The most marked differences in the kinetics of lesion development in the TD and CRC fed mice lie in the first few days post-infection, where lesions were of their greatest and least severity, respectively. One explanation for the pronounced difference at that time might be that changes in the intestinal microenvironment induced by TD allow *S. hyodysenteriae* to
maintain greater numbers, increased viability, enhanced secretion of toxic factors or a combination of the above, in comparison to the intestinal microenvironment in an animal fed CRC. These studies, however, refute the idea that increased spirochetal numbers account for the initial differences in lesion severity between the two groups of mice. Nibbelink reported similar findings, although statistical analysis was not performed in those studies [12]. The lack of evidence supporting increased spirochetal numbers in the ceca of mice fed TD does not rule out the possibility that those that are present may possess enhanced pathogenicity. The present experiments were not designed to test that hypothesis.

Other possible explanations for the more rapid onset of severe lesions in animals fed TD as compared to those fed CRC also relate to alteration of the intestinal environment by the diet, specifically the resident microflora. Early studies on the induction of swine dysentery in gnotobiotic pigs established that colonization with S. hyodysenteriae alone was insufficient to elicit lesion production. Coinfection with other organisms, primarily gram negative rods, such as Bacteroides vulgatus, is required for lesions to occur; the same requirements have been shown to be true in the murine model [14-18]. The role these other organisms play in the pathogenesis of swine dysentery is not clearly established but at least three possibilities seem to exist: a) they result in the "maturation" of the immune and inflammatory responses in the host b) they directly or indirectly induce or increase the virulence of S. hyodysenteriae, or c) they produce cell wall components or metabolites which, in synergy with or subsequent to expression of virulence factors by S. hyodysenteriae, result in lesion formation. If TD in some way selects for the increased growth or metabolic activity of other members of the cecal flora, either of the latter two mechanisms could explain the enhancement of acute lesion formation that occurs in infected mice fed that diet. In the original paper describing the effect of TD on the murine model of swine dysentery, Nibbelink reported increases in the numbers of cecal aerobic gram-positive and gram-negative organisms, although the potentially more significant anaerobic cultures were not
reported [12]. Those findings demonstrate that TD does, in fact, alter non-serpulinal microbial populations in the cecum.

It is also possible that the enhancing effect exerted by TD in this infection model is due to some direct effect on the host by the components of the diet, specifically the glucose and egg white solids of which it is largely composed. Increased luminal glucose levels (20 mM) have been shown to interfere with maintenance of normal functional and structural properties of intestinal epithelial cell tight junctions [19, 20]. If mice fed TD experienced such alterations, mucosal damage following infection with \textit{S. hyodysenteriae} might occur in an accelerated manner. In the previously reported study concerning TD 85420, feeding glucose (250 mM in the drinking water) to mice infected with \textit{S. hyodysenteriae} did not result in increased susceptibility to severe lesion formation [12]. It is unknown, however, if supplying glucose in the drinking water would closely simulate and have the same effect as feeding a diet that is approximately 63% glucose. Protein components of the diet might also have an effect on the host inflammatory or immune response, perhaps through a dietary hypersensitivity, although there is no evidence to support that supposition. Furthermore, uninfected mice fed TD did not demonstrate any cecitis. As in other biological systems, some combination of the above mechanisms may account for the enhancing effect on lesion development exerted by TD in mice infected with \textit{S. hyodysenteriae}.

Model disease systems in alternate animal hosts must meet several criteria in order to prove more useful than the true host species and therefore justify their use. They should have a predictable time course of lesion development such that the pathogenesis and its possible prevention can be studied. Lesion development should be acute and uniform thereby allowing timely, accurate and useful experiments. Finally, the model should allow the development of identical or very similar pathologic changes as those that occur in the true host species. The conventional murine model of \textit{S. hyodysenteriae} infection meets some of these criteria in regards to swine dysentery. Use of the Teklad 85420 diet in that murine model further meets those criteria by allowing acute onset of lesions that have identical morphologic features
in comparison to those that occur in mice fed conventional rodent chow. In addition, lesions occur much earlier and with greater uniformity in TD fed mice thereby providing the investigator a more uniform and efficient model with which to study factors that influence development and resolution of lesions. This uniformity allows studies, currently underway, designed to document the very early morphologic and ultrastructural changes in the colonic mucosa in this model of *S. hyodysenteriae* infection. Furthermore, elucidation of the effects of the diet itself on the host or the host intestinal microenvironment could add to an understanding of the pathogenic mechanisms involved in swine dysentery and possibly other enteric inflammatory diseases.

**Literature Cited**


**Figure 1.** The effect of Teklad diet 85420 compared to conventional rodent chow on gross and microscopic pathologic parameters. Total lesion index score is the sum of gross lesion score, cecal gland depth, submucosal and lamina proprial edema, lamina proprial cellular infiltrate and epithelial erosions. Mice fed TD had higher total lesion index scores, inclusive of strain, sex and day post-infection. * = significant difference with p < .005.
**Figure 2.** The effect of Teklad diet 85420 (TD) compared to conventional rodent chow (CRC) on the temporal distribution of lesion occurrence in mice infected with *S. hyodysenteriae*. Lesion index score is the sum of all individual lesion scores for a particular treatment. Mice fed TD had more severe lesions that occurred much earlier and persisted longer than mice fed (CRC). * = p < .05.
Figure 3. Photomicrograph of cecal tissue from a C3H/HeOuJ mouse fed Teklad diet 85420 (TD). Note the relatively shallow glands, columnar superficial colonocytes, and indistinct lamina proprial and submucosal spaces of low cellularity. HE. Bar = 100 μm.

Figure 4. Photomicrograph of gut-associated lymphoid tissue (GALT) in the cecum of a C3H/HeOuJ mouse fed TD. Note that this GALT nodule has few germinal centers and that the overlying cecal glands are shallow. HE. Bar = 300 μm.
Figure 5. Photomicrograph of cecal tissue from a C3H/HeOuJ mouse fed TD, infected with *S. hyodysenteriae* and necropsied 24 hours later. Note the glandular dilatation and pronounced expansion of submucosal and lamina proprial spaces by edema and inflammatory cells. HE. Bar = 200 μm.

Figure 6. Higher magnification of the superficial mucosa of the tissue shown in figure 5. Note the superficial epithelial erosions (arrow), expansion of the lamina proprial space by edema and inflammatory cells and crowding of dilated glands by tall, hyperplastic epithelial cells. HE. Bar = 50 μm.
Figure 7. Photomicrograph of cecal tissue from a C3H/HeOuJ mouse fed TD, infected with *S. hyodysenteriae* and necropsied 17 days later. Note the expansion of submucosal and lamina proprial spaces with edema and inflammatory cells, superficial epithelial erosions and lengthening of glands with glandular epithelial hyperplasia. HE. Bar = 200 μm.

Figure 8. Photomicrograph of cecal tissue from a C3H/HeOuJ mouse fed TD, infected with *S. hyodysenteriae* and necropsied 17 days later. Note that the cecal GALT has numerous germinal centers and is overlain by mucosa which is thickened due to glandular epithelial hyperplasia. HE. Bar = 300 μm.
Figure 9. A comparison of the effect of Teklad diet and conventional rodent chow on colonization of the murine cecum following infection with *S. hyodysenteriae*. Entire ceca were homogenized, diluted, added to molten blood agar containing antibiotics selective for *S. hyodysenteriae* and poured into plates. Hemolytic zones were counted and used to calculate colony forming units (CFU). There were no significant differences between treatment groups.
CHAPTER 4: A COMPARISON OF THE EFFECTS OF SERPULINA HYODYSENTERIAE OR ITS BETA-HEMOLYSIN ON THE ULTRASTRUCTURE OF THE MURINE CECAL MUCOSA

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Abstract

Two experiments were performed to compare the early ultrastructural changes in the cecal mucosa of mice either infected with Serpulina hyodysenteriae or exposed by intraluminal installation to the β-hemolysin of S. hyodysenteriae. In the first experiment, 65 C3H/HeOuJ were placed on a refined basal diet for 3 days, fasted briefly and then infected with S. hyodysenteriae by gastric intubation. Two mice were necropsied every hour for the subsequent 30 hours post-infection. Serpulina hyodysenteriae was isolated from the cecal contents of each mouse at all time points. Gross lesions were progressive and consisted of edema of the cecal wall, decrease in overall cecal size and mucoid cecal contents. Light microscopic lesions were first apparent at 7 hours PI and included flattening, loss of normal orientation and eventual necrosis and exfoliation of superficial epithelial cells, with edema and inflammatory cell infiltration of the lamina propria and submucosa; these changes were severe by 30 hours PI. Ultrastructural changes were first evident at 6 hours PI. There was disarray and loss of microvilli and terminal web, with dilatation of cytocavitary and intercellular spaces, consistent with accumulation of fluid in those areas. In addition, luminal bacteria were internalized by viable epithelial cells and translocated to cytoplasmic processes of perivascular cells in the lamina propria. Capillaries surrounded by those bacteria-containing cells exhibited changes consistent with increased vascular permeability.
Large numbers of these bacteria accumulated within the cytoplasm of lamina proprial macrophages, heterophils and other cells late in the experimental period. Also late in the experiment there was pronounced necrosis and exfoliation of epithelial cells, with exposure of lamina proprial elements to the lumen. Throughout the experiment *S. hyodysenteriae* cells were usually located at some distance from epithelial cell surfaces. In the second experiment, C3H/HeOuJ mice were fed the basal diet for 3 days, fasted briefly and then surgically manipulated to create a closed cecal loop. Hemolysin containing solutions, as well as various control solutions, were injected into the cecal lumen and incubated for 3 or 6 hours. Representative samples were collected from one of 4 identical experiments. None of the controls exhibited significant pathologic change and there were no differences due to time of incubation. There were no gross lesions in mice treated with hemolysin. By light microscopy, mice treated with hemolysin exhibited pronounced vacuolation and exfoliation of superficial epithelial cells. Ultrastructural changes in these mice consisted of loss of microvilli and terminal web and marked vacuolation and exfoliation of epithelial cells. A large number of detached epithelial cells exhibited apoptosis; other cells were necrotic. Moderate numbers of bacteria were internalized by affected epithelial cells, however bacterial translocation was not evident. These findings demonstrate that the cecal mucosal lesions induced in mice by either infection with *S. hyodysenteriae* or exposure to its hemolysin have common features, and that those features may be attributable to the \( \beta \)-hemolysin of *S. hyodysenteriae*.

Swine dysentery is caused by the large spirochete *Serpulina hyodysenteriae*. Gross and microscopic pathologic changes present in swine infected with this organism have been well characterized and consist of mucohemorrhagic and/or fibrinonecrotic typhlitis and colitis. The characteristic microscopic lesion is necrosis of superficial epithelial cells with subsequent lamina proprial congestion and cellular infiltration, accompanied by luminal fibrin exudation and hemorrhage. The pathophysiologic mechanisms by which these lesions cause diarrhea
with subsequent dehydration and death have been shown to be a colonic malabsorption syndrome, due to a lack of mature absorptive epithelial cells \(^4, 48\). Infection of mice with *S. hyodysenteriae* is the most frequently utilized and well described small animal model of swine dysentery, and has been used in experiments ranging in scope from demonstration of pathogenicity of various isolates to the effects of genetic background on susceptibility of different inbred strains of mice \(^16-18, 35, 37\). While lesions induced in mice by *S. hyodysenteriae* are less severe both grossly and microscopically than those seen in swine, they are fundamentally the same and are characterized by superficial epithelial cell necrosis, lamina proprial cellular infiltration and edema and occasional luminal fibrin exudation and hemorrhage. In both swine and mice, germfree animals infected only with *S. hyodysenteriae* do not develop lesions while those coinfected with *S. hyodysenteriae* and *Bacteroides vulgatus* (or other gram negative anaerobes) develop lesions in the same manner as conventional animals \(^13, 18, 34\). Previously reported electron microscopic studies in swine and mice have reiterated the known light microscopic findings \(^2, 9, 19, 54, 55\).

An enhanced murine model for *S. hyodysenteriae* infection has been described \(^36\). In that report a defined basal diet used in micronutrient studies (Teklad Diet 85420) was shown to greatly increase susceptibility to infection and lesion formation. The rapid onset and highly predictable nature of lesion formation that occurs in this model following infection of mice with *S. hyodysenteriae* makes it ideal for use in the studies reported herein, in which the goal is to identify early ultrastructural changes and document the progression of those changes following infection.

Hemolysis is a well known phenotypic phenomenon associated with the growth of *S. hyodysenteriae* on blood agar, and the \(\beta\)-hemolysin of that organism has long been assumed to have a role as a virulence factor in swine dysentery. Much research involving the hemolysin of *S. hyodysenteriae* has centered on its interactions with and effects on erythrocytes \(^15, 43, 44\). However, if the hemolysin has a critical role in *S. hyodysenteriae* pathogenesis, it is probably via its interaction with host cells other than erythrocytes. In fact, serpulinal
hemolysin has been shown to exhibit in vitro toxicity to a number of primary cells and continuous cell lines.\textsuperscript{1,21,22}

Given that \textit{S. hyodysenteriae} is a mucosal pathogen and that its hemolysin is a secreted toxin, the presumption would be that the initial interaction between toxin and host would be at the epithelial surface, specifically involving superficial colonic epithelial cells. Since ongoing necrosis of those cells is the underlying primary lesion of swine dysentery, it is tempting to speculate that the hemolysin causes that lesion and is therefore critical in the pathogenesis of swine dysentery.

Two reports utilizing one to two month old germ free pigs have described studies in which ileal and colonic loops created by surgical ligation were filled with solutions containing various amounts of purified hemolysin and examined by scanning and transmission electron microscopy.\textsuperscript{5,27} The most extensive and well characterized lesions occurred in the ileum; lesion occurrence was highly variable in the colon due to inactivation of the hemolysin by ingesta. Transmission electron microscopy demonstrated susceptibility of mature vacuolated enterocytes at villus tips to hemolysin induced degeneration and necrosis. Affected villi were collapsed due to loss of enterocytes and subsequent folding of the basement membrane due to myofibroblast contraction. Scanning electron microscopic studies performed on colonic loops similarly treated demonstrated rounding, individualization and increased extrusion of mature surface epithelial cells within intercryptal areas, with cells detaching in groups.\textsuperscript{5,27} These experiments were the first of their kind reported regarding the effect of the hemolysin of \textit{S. hyodysenteriae} on the intact intestinal mucosa. In regards to swine dysentery, however, the relevance of these findings regarding villus damage is unclear since lesions resulting from infection with \textit{S. hyodysenteriae} occur only in the large intestine, and, with the exception of epithelial cell necrosis, have different morphologic features than those induced by the hemolysin in those studies. Furthermore, since secondary bacteria are necessary for the expression of \textit{S. hyodysenteriae} induced disease, the effect of the germ-free status of those animals on the results obtained is not known. It would be desirable to compare the effects of the hemolysin on the large intestinal
mucosa with the known ultrastructural changes that occur in that tissue following infection with *S. hyodysenteriae*.

Therefore, the objectives of the experiments reported herein were to a) utilize a highly controllable and reproducible murine model of *S. hyodysenteriae* infection to document the earliest ultrastructural changes that occur in the cecal mucosa of mice so infected; and b) compare and contrast the above findings to those present in the cecal mucosa of mice exposed *in vivo* to the B-hemolysin of *S. hyodysenteriae*. Comparison of these findings will allow insights into the pathogenesis of *S. hyodysenteriae*, particularly in regards to the role of its B-hemolysin.

**Materials And Methods**

**Mice**

Twelve to 24 week old, male and female C3H/HeOuJ mice were used in this study. These animals were obtained from breeder colonies maintained by the Department of Laboratory Animal Resources, Iowa State University, and were cared for in accordance with the guidelines stipulated by the Animal Care and Use Committee of Iowa State University. Breeding colonies were established with mice originally procured from Jackson Laboratory, Bar Harbor, Maine and were routinely screened and found to be negative for the presence of serum antibodies to Sendai virus, murine hepatitis virus and *Mycoplasma spp.* Seventy-two hours prior to inoculation with *S. hyodysenteriae* or exposure to the B-hemolysin of *S. hyodysenteriae* the principals were placed on Teklad Diet 85420 (Harlan Sprague Dawley, Madison, Wis.) and were maintained on that diet for the remainder of the experimental period. The approximate composition of TD 85420 is as follows (w/w): dextrose-63.4%; egg white solids-20.0%; corn oil-10.0%; cellulose-3.0%; micronutrients as required. Food and water were given *ad libitum*.

**Bacteria and infection procedure**

Mice were infected with *S. hyodysenteriae* strain B204, serotype 2, which was grown, as previously described, at 37° C in trypticase soy
broth (BBL Microbiology Systems, Cockeysville, MD.) supplemented with 5% horse serum (HyClone Laboratories, Logan, Utah), 0.5% yeast extract (BBL), 0.05% L-cysteine (Sigma Chemical Co., St. Louis, MO) and 1% VPI salt solutions. Log phase cultures of S. hyodysenteriae (motility 75-90%) were evaluated for bacterial concentration with a Petroff-Hauser counting chamber. Mice were given 1 x 10⁸ bacteria in approximately 0.5 ml of culture broth intragastrically; food was withheld six hours prior to and one hour following infection.

**Hemolysin preparation**

β-hemolysin of S. hyodysenteriae (subsequently referred to as hemolysin) was prepared by a modification of a procedure described previously. Three liters of S. hyodysenteriae culture, grown as described above, were centrifuged at 10,000 r.p.m. in a high speed centrifuge at 4° C until bacterial cells were pelleted. Bacteria were suspended in hemolysin extraction buffer at 1/20th of the original culture volume and held at 37° C for 30 minutes, with agitation sufficient to maintain suspension. Hemolysin extraction buffer is composed of phosphate buffered saline (136.9 mM NaCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, pH = 7.2) supplemented with 0.05% RNA-core (Sigma Chemical Co., St. Louis, MO), 1 mM glucose and 1 mM MgSO₄. Following incubation at 37° C, bacterial cells were pelleted by centrifugation, the supernatant retained and held at 4° C and the extraction procedure repeated. Supernatants were pooled, vacuum filtered through a .22 μm filter (Gelman Sciences, Ann Arbor, MI) and stored at -70° C until further purification. Throughout all manipulations hemolysin-containing solutions were maintained at 4° C or less and assayed periodically for hemolytic activity. Hemolysin was reduced in volume and desalted by repeated concentration and reconstitution with chilled, sterile, distilled, deionized water utilizing a forced-flow ultrafiltration device with an approximate molecular weight cutoff of 3 kilodaltons (kDa) (Filtron Technologies, Northborough, MA). This concentrated, crude hemolysin was loaded onto preparative, nondenaturing, polyacrylamide slab gels and electrophoresed. Hemolytic activity was localized within the gel by overlay on blood agar.
and electroeluted from polyacrylamide slices into tris-glycine buffer (37.6 mM tris, 50 mM HCl, pH 7.47), then reduced in volume and desalted by ultrafiltration, as described above. The above process was repeated so that hemolysin preparations were electrophoresed and electroeluted twice. Hemolysin activity of crude and purified hemolysin containing solutions was determined and expressed in terms of hemolytic units (HU). One HU is operationally defined as the quantity of hemolysin required to result in lysis of 50% of the erythrocytes in 2 ml of a 1% solution of ovine erythrocytes in PBS, as measured spectrophotometrically by hemoglobin release at 545 nanometers. Purity of hemolysin containing solutions was assessed by nondenaturing and denaturing polyacrylamide gel electrophoresis (PAGE and SDS-PAGE, respectively) followed by ammoniacal silver staining (Accurate Chemical and Scientific Corp., Westbury, N. Y.). Purified hemolysin preparations usually contained approximately 100 HU per microliter.

Surgical manipulation

Prior to intraluminal instillation of hemolysin mice were surgically manipulated in the following manner. Following a 14 hour fast to allow cecal emptying, mice were anesthetized and maintained in a surgical plane of anesthesia with isoflurane (Pitmann-Moore, Washington Crossing, New Jersey). Following ventral paramedian skin and body wall incisions, the cecum was exteriorized and ligatures were placed snugly around the distal ileum and proximal large intestine, thereby creating a closed cecal loop. Care was taken to avoid ligation of mesenteric and antimesenteric vessels supplying the cecum. Hemolysin or control solutions in a volume of 250 μl were injected into the cecal lumen such that the cecum was moderately distended. The surgical incision was closed and the mice allowed to recover for up to six hours.

Experimental design

Sixty-five mice were infected with S. hyodysenteriae. Two mice were necropsied every hour for 30 hours following infection. Control, uninfected mice were also necropsied periodically throughout the experimental period. Following gross observations, cecal contents were
collected and used to inoculate blood agar plates supplemented with antibiotics selective for *S. hyodysenteriae* 24. These plates were maintained anaerobically at 37° C, as described, for 24 to 48 hours and observed for the presence of bacterial colonies surrounded by ß-hemolysis 35. Selected colonies were then examined by dark field microscopy to confirm the presence of large spirochetes. Following collection of cecal contents, cecal lumina were filled with cold glutaraldehyde fixative; entire ligated ceca were then excised and immersed in fixative.

Mice treated with hemolysin were surgically prepared as described above. Solutions containing 2500, 5000, 15000 or 25000 HU were injected into the cecal lumen; mice were allowed to recover for 1 to 6 hours. Control mice were exposed similarly to PBS, 1% RNA-core, 25000 HU of heat inactivated hemolysin or distilled water. Treated mice were then necropsied and their tissues fixed as described above.

**Electron microscopy**

Tissues were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 0° C for several days. Following several washes in cacodylate buffer, tissues were postfixed with 1% OsO4 in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 hours at room temperature. Following a distilled water wash and dehydration in a graded series of acetone, tissues were embedded in plastic (EMbed, Electron Microscopy Sciences, Ft. Washington, PA). One to two micron thick sections were prepared, stained with toluidine blue and examined for selection of areas to be evaluated by electron microscopy. Ultrathin sections (80 to 90 nanometers) were prepared, stained with uranyl acetate and lead citrate and examined with an Hitachi H500 transmission electron microscope at 75 kilovolts.
Results

Bacteriology
Large spirochetes consistent in appearance and growth characteristics with *S. hyodysenteriae* were isolated from all mice infected with that organism beginning one hour after inoculation and continuing throughout the experimental period. Spirochetes were not isolated from uninfected mice.

Mice infected with *S. hyodysenteriae*

Gross observations. Normal ceca were moderately well distended with yellow-tan ingesta that was partially visible through the semitranslucent cecal wall (figure 1). The earliest grossly apparent cecal changes began at 9 hours PI and became progressively more obvious throughout the experimental period. There was diminution of total organ size and increased opacity of the cecal wall due to mural edema with these changes first becoming apparent at the cecal apex and extending centrally (figure 2). There was an increased catarrhal nature to the cecal contents beginning at 10 hours which also progressed throughout the experimental period. By 30 hours ceca of infected mice were approximately half the normal length and demonstrated severe mural edema leading to pronounced opacity of the cecal wall (figure 3).

Light microscopic observations. The cecal mucosa from uninfected mice was lined by an epithelium in which cecal glands were two to four times as deep as they were wide and in which the superficial epithelial cells were tall columnar and regularly arranged (figure 4). The lamina proprial space was minimal but contained occasional lymphocytes and heterophils. Light microscopic changes were first apparent in mice infected with *S. hyodysenteriae* at 14 hours PI. These changes consisted of exfoliation, flattening and disorder of the superficial epithelial cells, dilatation of cecal glands, expansion of the lamina proprial space by heterophils, lymphocytes and macrophages, and submucosal edema (figure 5). By 30 hours PI these same changes were present but were much more pronounced than earlier in the experimental period (figure 6).
Electron microscopic observations. The cecal mucosa of normal mice was characterized by tall columnar epithelial cells which contained a prominent microvillus border, glycocalyx and terminal web (figure 7). The nuclei were most often round to oval and positioned apically within the cell, with mitochondria clustered laterally and subjacent to it. There were moderate amounts of rough endoplasmic reticulum within these cells and numerous unattached ribosomes. Lateral cell borders were interdigitating and intercellular junctions were prominent. Some cells contained multivesicular-type bodies, variably filled with laminated and vesiculated lipid material (figure 8). At the apical intercryptal zone vacuolated, effete epithelial cells were occasionally present, undergoing apparent extrusion. The lumina of these mice contained extremely large numbers of bacteria, most of a single type. These were long and filamentous, contained numerous internal lipid bodies, were somewhat rhomboid in cross-section and had numerous helical ridges or flagellae arising from the cell membrane. These bacteria were rarely present within extruded cells. The lamina proprial space within normal mice was minimal and contained capillaries lined by flattened endothelial cells, perivascular cellular processes and the basal aspects of tightly apposed epithelial cells, all of which were separated by prominent basement membrane material which contained interposed processes of perivascular cells (figure 9).

Ultrastructural changes present in the cecal epithelial cells of mice infected with *S. hyodysenteriae* were first apparent at 6 hours PI and included disarray of and a moderate diminution in number and length of microvilli. There was also decreased prominence and increased electron lucency of the terminal web and dilatation of intercellular spaces and the intracellular cytocavitary network, consistent with the accumulation of fluid within those spaces. In addition, many affected cells contained intracellular bacteria, both individually and in small clusters, identical in appearance to those previously described as being present in large numbers within the cecal lumen (figure 10). Some bacteria appeared to be within a membrane bound vacuole while others did not. At 14 hours PI the above mentioned changes became more pronounced and also included complete loss of microvilli, rounding of
epithelial cells, lateral extension of epithelial cell cytoplasmic processes resulting in loss of normal mucosal architecture, accumulation of intracellular vacuoles filled with an osmophilic substance consistent in appearance with lipid, and expansion and increased electron lucency of perivascular cytoplasmic processes in the superficial lamina propria (figure 11). By 18 hours PI these superficial lamina proprial perivascular cytoplasmic processes were markedly enlarged and electron lucent and contained fragments of electron dense material resembling bacteria (figure 12). Lamina proprial capillary endothelial cells also exhibited significant change at that time, consisting of increased cell volume, increased numbers of pinocytotic vesicles and vacuoles, prominent villous projections and surface undulations which were particularly prominent in the area of marginal ridges; intercellular junctions underlying the affected marginal ridges were increased in electron density (figure 13). The basal laminae surrounding these affected capillaries was indistinct and discontinuous and perivascular collagen fibers were separated by electron lucent space. All of these findings are indicative of edema associated with increased endothelial permeability. At 24 to 30 hours PI heterophils and macrophages were increased in number in the deep lamina propria. Those cells and other unidentified cells in the deep lamina propria were also filled with massive numbers of bacteria (figure 14). Also present between 24 and 30 hours was necrosis of superficial epithelial cells, with movement of spirochetes and other bacteria between detached epithelial cells and lamina proprial elements (figure 15). In instances where degenerate and necrotic cells remained attached, however, spirochetes were usually found at some distance from the cell border, presumably in the overlying mucus layer (figure 16). Fibrin thrombi were also seen in submucosal capillaries, which were lined by endothelial cells that contained surface blebs, increased numbers of pinocytotic vesicles and vacuoles and intercellular junctions of increased electron density (figure 17).
Mice exposed to the hemolysin of *S. hyodysenteriae*

**Gross observations.** There were no gross lesions in the ceca of mice exposed to the hemolysin of *S. hyodysenteriae* or other materials used as control treatments.

**Light microscopic observations.** Control animals in which ceca were exposed to *S. hyodysenteriae* cultures, PBS, RNA-core or water did not exhibit changes detectable by light microscopy.

In mice in which ceca were exposed to hemolysin, the significant microscopic change present was pronounced vacuolation and exfoliation of superficial epithelial cells (figure 18). Empirically, this occurred in a dose dependent manner, with mice exposed to 25000 HU exhibiting more cellular vacuolation and exfoliation than those exposed to 15000 HU, which were in turn more severely affected than those mice exposed to 5000 HU. Mice exposed to 2500 HU demonstrated no lesions by light or electron microscopy.

**Electron microscopic observations.** Control animals in which ceca were exposed to PBS, RNA-core or water did not exhibit significant changes by electron microscopy. Mice in which cecal lumina were instilled with viable *S. hyodysenteriae* exhibited scattered foci of epithelial cell necrosis and extrusion with intermixing of necrotic cells and spirochetes (not shown).

Mice in which ceca were instilled with hemolysin exhibited similar changes regardless of the number of HU used; they are described without regard to dosage. The most pronounced effects were apparent in those animals necropsied after 3 hours. Affected epithelial cells contained multiple, sharply delimited, cytoplasmic vacuoles which appeared in some instances, to arise from the endoplasmic reticulum (figure 19). These vacuoles ranged in size from approximately .1 to 5 micrometers, occasionally displaced or compressed the nucleus and were variably filled with lamellar or amorphous membranous debris. Microvilli in these cells were irregular and decreased in prominence, as was the terminal web. In some instances affected cells remained attached to the mucosa while in other instances affected cells were partially or completely detached, either individually or in groups. These cells often remained attached to each other via intact intercellular
junctions, although lateral interdigitations were often absent (figure 20). Moderate numbers of bacteria were often present within affected cells, both within the described vacuoles and apparently free within the cytoplasm (figure 21). Among the vacuolated and exfoliated cells, some were necrotic, with pronounced cytoplasmic electron lucency and loss of detail, mitochondrial swelling and loss, lipid body accumulation and nuclear fragmentation (figure 22). Other such cells exhibited evidence of apoptosis, characterized by crescentic clumps of electron dense chromatin margined subjacent to the nuclear envelope (figure 23). Typical of apoptotic cells, mitochondria in these cells were normal in appearance but often clustered or more densely distributed than normal due to apparent cell shrinkage (figure 24).

**Discussion**

The purposes of the experiments reported herein were to a) utilize a highly controllable and reproducible murine model of *S. hyodysenteriae* infection to document the earliest ultrastructural changes that occur in the cecal mucosa of mice so infected; and b) compare and contrast the above findings to those present in the cecal mucosa of mice exposed *in vivo* to the β-hemolysin of *S. hyodysenteriae*. In the first portion of the reported studies, it was shown that mice infected with *S. hyodysenteriae* experience demonstrable ultrastructural changes by 6 hours PI, consisting of loss and disarray of microvilli and the terminal web and apparent translocation of resident bacteria through the cecal epithelium. These epithelial cell changes progressed with time to include dilatation of the cytocavitary network and intercellular spaces, lipid body accumulation and extension of lateral cytoplasmic processes. There was also increased electron lucency of cytoplasmic processes perivascular cells surrounding lamina proprial capillaries. Fragments of translocating bacteria accumulated in these perivascular cytoplasmic processes and lamina proprial capillaries exhibited changes consistent with increased permeability with subsequent perivascular edema. Bacteria eventually accumulated in
massive numbers in the lamina propria within the cytoplasm of heterophils, macrophages and other cells. Obvious necrosis of superficial epithelial cells occurred towards the end of the experimental period, with spirochetes present near and between necrotic cells. Earlier in the experimental period (<18 hours), however, spirochetes were rarely present in close association with epithelial cells but were present at some distance luminally, presumably in the mucus layer.

In the second portion of these studies it was shown that, when intact murine cecal mucosa was exposed to the 6-hemolysin of \textit{S. hyodysenteriae}, superficial epithelial cells exhibited pronounced vacuolar change, moderate loss of microvilli and terminal webs and nuclear changes consistent with apoptosis. Some cells were necrotic as well, and both apoptotic and necrotic cells contained moderate numbers of internalized bacteria. In comparing the ultrastructural changes present in mice either infected with \textit{S. hyodysenteriae} or exposed to its 6-hemolysin, it is evident that there are similarities and differences between the two treatments, both qualitatively and quantitatively. Infection and hemolysin both induced apparent membranous effects in epithelial cells, with loss of microvilli and vacuole formation. Vacuole formation was marked in hemolysin treated mice while intercellular fluid accumulation was more pronounced in infected mice. Both treatments rendered cells more susceptible to luminal bacterial internalization, although this was certainly more pronounced in infected mice, where bacteria accumulated within lamina proprial stromal cells and had apparent effects on lamina proprial capillaries. Necrosis of epithelial cells was apparent in both infected and hemolysin treated mice, but apoptosis was observed only in hemolysin treated mice.

The nature of the possible membrane changes that occur in mice infected with \textit{S. hyodysenteriae} is not demonstrable in these studies. Such changes might involve increased membrane fluidity resulting in transient membrane instability or pore formation, thereby allowing bacterial internalization. Dilatation of the cytocavitary and intercellular spaces is probably also due to some perturbation in membrane homeostasis. Since spirochetes were not present in close association
with the affected epithelial cells, it is likely that a diffusable factor, such as the hemolysin, is the agent of the described changes. This is supported by the fact that the hemolysin treatment also elicited ultrastructural changes indicative of membrane effects, specifically loss of microvilli and vacuole formation. Such a likelihood would be in agreement with a previously mentioned study in which cell cultures treated with hemolysin exhibited increased intracellular calcium and decreased transcellular resistance, both changes indicative of membrane damage \(^1\). A recent report describes an *in vitro* model of epithelial cell migration and restitution following wounding of intact guinea pig small intestinal mucosa \(^3\). In that report, loss of microvilli in migrating cells was attributed to disassembly of those microvilli and use of the resultant membrane to form the leading edge of the migrating cell. Cells demonstrating loss of microvilli were morphologically normal cells at the edges of zones of epithelial cell loss from mucosal wounding. In the studies reported herein cellular flattening and extension of lateral cytoplasmic borders occurred later in the study than the initial microvillus and terminal web loss seen after only 6 hours. Therefore, it is likely that some mechanism of microvillus loss other than that due to cell migration is operative in mice infected with *S. hyodysenteriae* or treated with its hemolysin.

Apoptosis has been repeatedly shown to be associated with increases in intracellular calcium ion concentration \(^{26, 28, 38, 39}\). It is likely that calcium ion effects apoptosis through modulation of the numerous intracellular pathways in which it is involved, including calmodulin dependent pathways, intracellular protease and nuclease activity and microtubular function. Cells undergoing necrosis also exhibit cytoplasmic calcium ion elevations, usually as a causal event of mitochondrial dysfunction and ATP depletion \(^8, 41\). Since the \(\beta\)-hemolysin of *S. hyodysenteriae* has been shown to induce changes in intracellular calcium ion concentration, presumably through some effect on membrane calcium excluding ability, high concentrations of hemolysin may induce a degree of calcium ion flux sufficient to induce apoptosis \(^1\). Levels of hemolysin present in mice infected with *S. hyodysenteriae* may, on the other hand, cause calcium ion changes that
result in necrosis of cells due to prolonged exposure to lower levels of toxin. Likewise, the marked epithelial cell vacuolar change present in hemolysin treated animals may represent the structural membrane effects of exposure to large amounts of hemolysin while sustained, lower level exposure may cause membrane changes like those seen in infected mice, specifically, microvillus loss and cytocavitary dilatation. The time limitations of this surgical model do not allow exploration of whether longer duration exposure to hemolysin could induce the internalization and translocation of luminal bacteria seen in infected mice. Translocation of those bacteria appears to be an important component of the ultrastructural changes seen in mice infected with *S. hyodysenteriae*.

It has been shown repeatedly that bacteria other than *S. hyodysenteriae* must be present in the large intestine of experimental swine and mice in order for those animals to acquire typical lesions once infected with *S. hyodysenteriae* 7, 12, 13, 18, 29-31, 34, 58. However, only certain bacterial species satisfy that requirement, the most commonly identified of those being *Bacteroides vulgatus*. Other gram negative anaerobic rods have also been shown to fulfill this requirement.

The mechanism by which these secondary bacteria act in concert with *S. hyodysenteriae* to cause disease is not known. At least two or three possibilities seem apparent and have been proposed as hypotheses: 1) the secondary bacteria have some effect on *S. hyodysenteriae* which renders it more virulent, 2) the secondary bacteria, in the presence of *S. hyodysenteriae*, have effects on the host that contribute to or account for lesion production, or 3) some combination of #1 and #2 58. The findings of these two experiments would support at least the second of the above possibilities. Mice infected with *S. hyodysenteriae*, and to a lesser extent those exposed to its β-hemolysin, exhibited increased internalization of luminal bacteria. In infected mice, those bacteria accumulated in perivascular cellular processes surrounding lamina proprial capillaries, and those capillaries subsequently exhibited changes consistent with increased permeability and perivascular edema. Perivascular cells were macrophages and
heterophils, and presumably pericytes, myofibroblasts or other stromal cells, all of which are capable of secreting a variety of soluble vasoactive cytokines and chemokines following activation. Thus, the role of normal flora bacteria in inducing lesions following infection with S. hyodysenteriae may be that they induce increased vascular permeability by way of vasoactive substances secreted by the mesenchymal cells that internalize those bacteria. Impaired lamina proprial vascular function could result in inefficient nutrient and oxygen delivery to and catabolite removal from the epithelial cells supplied by those vessels. These changes, coupled with the direct cytotoxic effects of the hemolysin, could then be sufficient to result in the lesions typically present in conventional animals infected with S. hyodysenteriae. In germ-free animals, however, bacteria are not available to translocate and induce permeability changes in lamina proprial capillaries, and direct cytotoxicity induced by S. hyodysenteriae and its hemolysin is insufficient alone to cause lesions. Note that in this hypothesis the hemolysin of S. hyodysenteriae is instrumental in lesion formation by way of its role in both bacterial internalization and subsequent translocation, and direct epithelial cytotoxicity.

The morphologic properties of the bacteria present in these mice are similar to those presented in numerous reports describing bacteria isolated from normal murine cecum and colon. Numerous different oxygen-intolerant and anaerobic fusiform bacteria have been identified in mice, as have several different spiral shaped bacteria. Some of the fusiforms have been shown to have flagellae similar to those seen on the bacteria in these studies. These bacteria are present in the large intestine of mice in very high numbers, often between $10^9$ and $10^{11}$ per gram of cecum, and most have been placed into the genera Eubacterium, Fusobacterium, Clostridium or Catenabacterium. Fusobacterium and Eubacterium species have lipopolysaccharide moieties which, in the case of Fusobacterium species, have potent biologic activity, including activation of macrophages. Thus, it is feasible that, in mice infected with S. hyodysenteriae, the bacteria translocated to and internalized by lamina proprial
perivascular cells could cause activation of those cells with subsequent cytokine release and vascular permeability effects.

Findings similar to these described herein have not been previously reported in mice or swine, either infected with *S. hyodysenteriae* or exposed to its hemolysin. Transmission electron microscopic studies of pigs infected with *S. hyodysenteriae* demonstrated early changes in superficial epithelial cells similar to those described in mice in this report, including intercellular edema, mitochondrial swelling and loss of microvilli as well as superficial lamina proprial vascular congestion; bacterial translocation was not reported. Whether or not bacterial translocation can be demonstrated to occur in pigs infected with *S. hyodysenteriae* would depend on whether or not it in fact does occur, the use of representative sampling and the availability of a highly controllable model in which sequential ultrastructural changes can be reliably documented. In this laboratory, colonic specimens derived from acutely ill swine which had been experimentally infected with *S. hyodysenteriae* have been examined following silver staining to determine if luminal bacteria were observable by light microscopy within the lamina propria of pigs with acute colonic lesions of swine dysentery (not shown). While bacteria were often present within the edematous lamina propria, most often underlying areas of superficial epithelial cell necrosis, which may have allowed direct access to the lamina propria. This is as opposed to the results reported in this study in which bacterial translocation to the lamina propria occurred slightly before or concurrently with epithelial cell necrosis.

Another potential virulence attribute of *S. hyodysenteriae* is its lipopolysaccharide-like moiety (LPS). It has been shown that, while the LPS of *S. hyodysenteriae* has biologic activity qualitatively similar to the LPS of *Escherichia coli* or *Salmonella* spp., it is probably of a degree of potency 50 times less that that of *E. coli* or *Salmonella* 11, 34, 42. Thus, while the LPS of *S. hyodysenteriae* may have biologic effects in vivo, it is not thought to be a major factor in pathogenesis of disease. A bacteriophage of *S. hyodysenteriae* has recently been identified but no significance has been attributed to it relative to pathogenic mechanisms.
Chemotaxis of *S. hyodysenteriae* for mucin has been demonstrated and may contribute to the overall pathogenesis of the organism but it is unlikely that such a quality would contribute directly to the necrosis and inflammation seen in infected animals. Similarly, the possession of an NADH-oxidase function by *S. hyodysenteriae* probably increases survivability in the environment of the large intestine, but may not contribute directly to pathogenesis. None of these potential virulence attributes is likely to cause the lesions seen in animals infected with *S. hyodysenteriae*.

Three genes from *S. hyodysenteriae*, *tlyA*, *tlylB* and *tlyC*, have been identified, cloned and sequenced. Due to their ability to transform nonhemolytic host *E. coli* strains to a hemolytic phenotype, these are referred to as hemolysin genes. However, the protein translation products of these genes do not appear to have any features in common with the hemolytic principal described and investigated in this and other reports.

In summary, the effects of either infection with *S. hyodysenteriae* or exposure to its β-hemolysin on the cecal mucosa of mice share many qualitative similarities, but differ quantitatively. Shared similarities include membrane perturbing effects that result in loss of microvilli, vacuolation, cytocavitary dilatation and bacterial internalization. Hemolysin treatment resulted in marked apoptosis of epithelial cells, which was not seen in infected mice, although necrosis was seen in both treatments. In mice infected with *S. hyodysenteriae*, translocation of luminal bacteria to perivascular cells in the lamina propria was pronounced and was associated with increased permeability of lamina proprial capillaries. These findings indicate that the β-hemolysin of *S. hyodysenteriae* may act as a virulence factor through its effects on the membranes of superficial epithelial cells, possibly by facilitating internalization and translocation of luminal bacteria to the lamina propria, where those bacteria effect vascular damage. These findings also provide a possible explanation of the required role of luminal bacteria in lesion formation in the large intestine of animals infected with *S. hyodysenteriae*.
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Literature Cited


15. Hyatt, DR, Muir S, and Joens LA. Biophysical characteristics of the Serpulina hyodysenteriae native hemolysin. in Conference of Research Workers in Animal Diseases. 1992. Chicago, IL:


21. Kent, KA, Haemolytic and adhesive properties of Treponema hyodysenteriae. 1984, Ph.D. dissertation, University of Reading


46. Savage, DC and Blumershine RVH: Surface-surface associations in microbial communities populating epithelial habitats in the


Figure 1. Cecum; normal, uninfected C3H/HeOuJ mouse. Note the semitransparent wall and moderate distention with pale ingesta. Bar = .75 cm.

Figure 2. Cecum; C3H/HeOuJ mouse 9 hours following infection with *S. hyodysenteriae*. Note the increased opacity and wrinkling of the cecal wall due to edema, and overall decrease in size. The cecal apex is more severely affected. Bar = .75 cm.

Figure 3. Cecum; C3H/HeOuJ mouse 30 hours following infection with *S. hyodysenteriae*. Note the marked opacity of the cecal wall and proximal colon, and pronounced overall decrease in size and lack of cecal contents. Bar = .75 cm.

Figure 4. Cecum; normal, uninfected C3H/HeOuJ mouse. Cecal glands are shallow and lined by regularly arranged epithelial cells. The lamina proprial and submucosal spaces are minimal and contain few cells. Plastic embedded, toluidine blue stained. Bar = 50 μm.

Figure 5. Cecum; C3H/HeOuJ mouse 14 hours following infection with *S. hyodysenteriae*. Note exfoliation, flattening and disorder of the superficial epithelial cells, dilatation of cecal glands, and expansion of the lamina proprial space by inflammatory cells and edema. Plastic embedded, toluidine blue stained. Bar = 50 μm.
Figure 6. Cecum; C3H/HeOuJ mouse 30 hours following infection with *S. hyodysenteriae*. Note the pronounced expansion of the lamina propria by inflammatory cells and edema, flattening, disorder and exfoliation of superficial epithelial cells, luminal debris and hyperplasia of gland epithelium. Plastic embedded, toluidine blue stained. Bar = 50 μm.

Figure 7. Electron micrograph. Cecum; normal, uninfected C3H/HeOuJ mouse. Note the prominent microvilli, glycocalyx and terminal web. Some cells contain apical multivesicular bodies. Uranyl acetate and lead citrate. Bar = 2.5 μm.
Figure 8. Electron micrograph. Cecum; normal, uninfected C3H/HeOuJ mouse. Note the prominent microvilli, glycocalyx, terminal web and intercellular junctions. The lumen contains filamentous bacteria with internal lipid bodies and helical ridges. Uranyl acetate and lead citrate. Bar = 1 μm.

Figure 9. Electron micrograph. Cecum; normal, uninfected C3H/HeOuJ mouse. A superficial lamina proprial capillary is separated from surrounding basal aspects of epithelial cells by abundant basement membrane material, which contains interposed cytoplasmic processes of perivascular cells (arrowhead). Uranyl acetate and lead citrate. Bar = 1 μm.
**Figure 10.** Electron micrograph. Cecum; C3H/HeOuJ mouse 6 hours following infection with *S. hyodysenteriae*. Note the diminution in the number of and disarray of microvilli and loss of prominence of the terminal web. There is also dilatation of intercellular spaces. Numerous bacteria are present within the cytoplasm (arrowhead). Uranyl acetate and lead citrate. Bar = 2.5 μm.

**Figure 11.** Electron micrograph. Cecum; C3H/HeOuJ mouse 14 hours following infection with *S. hyodysenteriae*. Note the complete loss of microvilli and terminal webs, rounding and flattening of epithelial cells resulting in loss of normal mucosal architecture, accumulation of intracellular vacuoles filled with lipid, dilatation of intercellular spaces and presence of intracellular bacteria. Uranyl acetate and lead citrate. Bar = 2.5 μm.
Figure 12. Electron micrograph. Cecum; C3H/HeOuJ mouse 18 hours following infection with \textit{S. hyodysenteriae}. Superficial lamina proprial perivascular cytoplasmic processes are markedly enlarged and electron lucent and contain fragments of electron dense material, some of which resemble bacteria (arrowhead). Lipid bodies are present within the surrounding basilar cytoplasm of epithelial cells. Uranyl acetate and lead citrate. Bar = 2 \mu m.

Figure 13. Electron micrograph. Cecum; C3H/HeOuJ mouse 18 hours following infection with \textit{S. hyodysenteriae}. Lamina proprial capillary endothelial cell exhibits increased cell volume, villous projections and surface undulations which are particularly prominent in the area of marginal ridges; intercellular junctions underlying the affected marginal ridges are increased in electron density. There are increased numbers of pinocytotic vesicles and vacuoles. Note the bacterial profiles (arrow) and neurosecretory granules (arrowhead) in cytoplasmic processes of surrounding cells. There is decreased prominence of basement membrane and increased electron lucency and separation of collagen fibrils in the perivascular space. Uranyl acetate and lead citrate. Bar = .75 \mu m.
Figure 14. Electron micrograph. Cecum; C3H/HeOuJ mouse 24 hours following infection with *S. hyodysenteriae*. Macrophages and other cells within the lamina propria have markedly expanded and electron lucent cytoplasm which is filled with bacteria and electron dense debris. Basal aspects of epithelial cells are present in the upper left corner. Uranyl acetate and lead citrate. Bar = 3 μm.

Figure 15. Electron micrograph. Cecum; C3H/HeOuJ mouse 30 hours following infection with *S. hyodysenteriae*. Note the necrotic exfoliated epithelial cells and a heterophil surrounded by abundant and varied bacterial forms. A laminal proprial capillary is filled with platelets and erythrocytes and is separated from the lumen by necrotic perivascular cytoplasmic processes. Uranyl acetate and lead citrate. Bar = 3 μm.
**Figure 16.** Electron micrograph. Cecum; C3H/HeOuJ mouse 30 hours following infection with *S. hyodysenteriae*. Epithelial cells are degenerate and necrotic. Note the swollen and mineralized mitochondria, destruction of microvilli and intra- and intercellular vacuolation. Bacteria are present at some distance from the surface. Uranyl acetate and lead citrate. Bar = 4 μm.

**Figure 17.** Electron micrograph. Cecum; C3H/HeOuJ mouse 30 hours following infection with *S. hyodysenteriae*. Note the fibrin aggregates in the capillary lumen. The endothelium contains numerous bud-like and villous projections. Perivascular collagen fibrils are widely separated by electron-lucent space. Uranyl acetate and lead citrate. Bar = 1.5 μm.
Figure 18. Cecum; C3H/HeOuJ mouse exposed to 25000 HU of the β-hemolysin of *S. hyodysenteriae*. Note the clumps of exfoliated and highly vacuolated superficial epithelial cells. Plastic embedded and stained with toluidine blue. Bar = 20 μm.
Figure 19. Electron micrograph. Cecum; C3H/HeOuJ mouse exposed to 25000 HU of the β-hemolysin of *S. hyodysenteriae*. Note the variably sized, often large intracytoplasmic vacuoles within attached cells, containing small amounts of membranous debris. Partially detached cells exhibit loss of microvilli and remain attached to adjacent cell via intact intercellular junctions. Uranyl acetate and lead citrate. Bar = 2.5 μm.
Figure 20. Electron micrograph. Cecum; C3H/HeOuJ mouse exposed to 25000 HU of the β-hemolysin of *S. hyodysenteriae*. A large clump of superficial epithelial cells is detached and exfoliated from the mucosal surface. Note the numerous variably sized intracytoplasmic vacuoles and intracytoplasmic bacteria. Cells remain attached to adjacent cells via intracellular junctions but have lost microvilli and lateral interdigitations. Uranyl acetate and lead citrate. Bar = 1.5 μm.
Figure 21. Electron micrograph. Cecum; C3H/HeOuJ mouse exposed to 25000 HU of the β-hemolysin of *S. hyodysenteriae*. A single superficial epithelial cell is detached and contains several variably sized vacuoles and lipid bodies. One large vacuole is filled with numerous bacteria. Uranyl acetate and lead citrate. Bar = 2 μm.
Figure 22. Electron micrograph. Cecum; C3H/HeOuJ mouse exposed to 25000 HU of the β-hemolysin of S. hyodysenteriae. Several superficial epithelial cells are exfoliated. Some necrotic cells exhibit cytoplasmic electron lucency, nuclear fragmentation and mitochondrial swelling and mineralization (arrow). Other cells have relatively intact organelles but are highly vacuolated and exhibit loss of microvilli. Note the vacuolated cell with peripheralized, crescentic chromatin (arrowhead). Uranyl acetate and lead citrate. Bar = 2.5 μm.
**Figure 23.** Electron micrograph. Cecum; C3H/HeOuJ mouse exposed to 25000 HU of the β-hemolysin of *S. hyodysenteriae*. A single superficial epithelial cell is detached and exhibits complete loss of microvilli, vacuolation and peripheralized crescentic chromatin clumps, typical of apoptosis. Other organelles are relatively normal. Uranyl acetate and lead citrate. Bar = .75 μm.

**Figure 24.** Electron micrograph. Cecum; C3H/HeOuJ mouse exposed to 25000 HU of the β-hemolysin of *S. hyodysenteriae*. A detached epithelial cell contains crescent shaped clumps of chromatin subjacent to the nuclear envelope. There is diffuse loss of microvilli and terminal web. Mitochondria are tightly packed due to cell shrinkage. Uranyl acetate and lead citrate. Bar = 1.5 μm.
CHAPTER 5: PURIFICATION, CLONING AND SEQUENCING
OF A BETA-HEMOLYSIN GENE FROM SERPULINA
HYODYSENTERIAE

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Abstract

Serpulina hyodysenteriae induces a mucohemorrhagic diarrheal
disease in swine. The production of a β-hemolysin has been considered
a major virulence attribute of this organism. As a first step in
establishing the role of the β-hemolysin in disease, the toxin was
purified from culture supernatants and the N-terminal amino acid
sequence was determined (K-D-V-V-A-N-Q-L-N-I-S-D-K). Using a
degenerate probe, a Lambda S. hyodysenteriae genomic library was
screened by hybridization resulting in the identification of three
overlapping clones. When the three cloned fragments were
transformed into hemolytic negative Excherichia coli, the transformants
were β-hemolytic on blood agar media. DNA sequence analysis of one of
the clones revealed a single open reading frame coding for a putative
8.93 kilodalton polypeptide containing the N-terminal sequence of the
purified protein immediately downstream of a seven amino acid signal
sequence. Based on sequence analysis the translated protein has a pI of
4.3, possesses sequence homology to acyl carrier proteins, has an alpha-
helical structure and a phosphopantetheine binding motif. Hybridization
analysis of genomic DNA indicated that the gene was present in S.
hyodysenteriae and S. intermedia, but was not detected in S. innocens,
S. pilosicoli, or S. murdochii. To distinguish this gene from previously
cloned putative hemolysin genes, it has been designated \textit{hlyA}. The role of this protein in the virulence of \textit{S. hyodysenteriae} remains unclear.

\textbf{Introduction}

\textit{Serpulina hyodysenteriae}, the etiologic agent of swine dysentery, induces a mucohemorrhagic diarrheal disease in susceptible swine and a severe cecitis in mice (12, 15). Virulence attributes associated with \textit{S. hyodysenteriae} include the production of a \(\beta\)-hemolysin, presence of a biologically active lipooligosaccharide, motility, and presence of an NADH oxidase (9, 17, 31-34, 42). \(\beta\)-hemolytic activity has been one of the major phenotypic characteristics used to distinguish \textit{S. hyodysenteriae} from avirulent species of \textit{Serpulina}. Numerous methods have been reported for hemolysin production and purification, most involving traditional protein purification techniques (20, 21, 23, 34). These studies have been at variance in their reports of the apparent molecular mass of the isolated hemolytic factor, ranging from 19 to 74 kilodaltons (kDa). This \(\beta\)-hemolysin has been shown to be physicochemically similar to streptolysin S; it is oxygen-stable, heat labile, active over a wide pH range, requires an appropriate carrier for its isolation (usually RNA-core), and is sensitive to both protease and lipase digestion (21, 33).

The biologic activity of the \(\beta\)-hemolysin recovered from \textit{S. hyodysenteriae} has been studied primarily \textit{in vitro}. Early work indicated that the binding of the hemolysin to erythrocytes was temperature independent while lytic activity required physiologic temperatures, and that cell swelling occurred prior to lysis (33). While proteolytic or phospholipase-like activity has not been associated with the \(\beta\)-hemolysin of \textit{S. hyodysenteriae}, hemoglobin release was detected prior to cell swelling indicating a cytolytic mechanism involving membrane perturbation and leakage (33, 34). Studies with osmoprotectants indicated that a pore with a diameter of 1.0 to 1.1 nm was formed by the \(\beta\)-hemolysin (14).
In addition to erythrocytes, the β-hemolysin of *S. hyodysenteriae* has been shown to have in vitro cytotoxicity for a number of cell types, including fibroblasts and porcine lymphocytes (18, 19). A continuous colonic epithelial cell line, Caco-2, has been used experimentally to examine the effect of the serpulinal β-hemolysin on epithelial cells (1). Monolayers treated with β-hemolysin exhibited decreased transcellular resistance and an increased intracellular calcium concentration, both indicative of membrane damage. Other studies have shown that purified hemolysin placed in the lumen of porcine small intestinal loops resulted in necrosis of mature superficial villous enterocytes (5).

Recently, three separate genes from *S. hyodysenteriae* have been cloned based upon their ability to induce a hemolytic phenotype in a nonhemolytic host strain of *E. coli* (29, 44). These three genes, referred to as *tlyA*, *tlyB* and *tlyC*, encode gene products with apparent molecular masses of 26.9, 93.3 and 30.8 kDa, respectively. Furthermore, it was demonstrated that *tlyA* was detected in the pathogenic *S. hyodysenteriae* but not the nonpathogenic *S. innocens*. The *tlyB* gene product was reported to have homology with Clp proteins, a class of intracellular proteolysis regulators, but the other two sequences have no homology with any known proteins. None of these genes, however, have been linked directly to an *S. hyodysenteriae*-specific gene product that displays hemolytic activity.

In an attempt to identify the gene encoding the β-hemolysin of *S. hyodysenteriae*, the native protein was purified, and the N-terminal amino acid sequence obtained. Unexpectedly, this sequence was not present in any of the three translated *tly* genes identified previously. The subsequent cloning of the gene revealed a sequence with no homology to *tlyA*, *B*, or *C*, but it appeared to have homology with acyl carrier proteins (ACPs). The coding sequence is much smaller than previously reported *tly* genes which correlates directly with the results of studies of the β-hemolysin separated on native polyacrylamide electrophoresis gels. To distinguish between our studies and previous work, the designation *hlyA* has been given. This is the first direct linkage of the β-hemolysin with a specific gene sequence in *S. hyodysenteriae*. 
Materials And Methods

Bacteria

*S. hyodysenteriae* strain B204, serotype 2, was grown as previously described (30). Spirochetes were grown in volumes of three liters to a concentration of approximately $1 \times 10^9$ organisms per milliliter as determined with a Petroff-Hauser bacterial counting chamber. Active motility in greater than 75% of the organisms was desired and interpreted as an indication of cell vigor. *Escherichia coli* strains LE392 (11) was routinely grown in Luria-Bertani (LB) broth or on LB agar media. Phage plates for LE392 consisted of standard LB agar base supplemented with 2.5 mM CaCl$_2$ with LB soft agar overlays.

SOLR™ [e14-(mcrA) Δ(mcrCβ-hsdSMR-mr')171 sbcC recB recJ uvrC umuC::Tn5(kan') lac gyrA96 relA1 endA1 1$^R$ [F' proAB lacI97ZΔM15] Su'] was the Lambda ZAP II recipient for plasmid excision (Stratagene, La Jolla, Calif.).

Hemolysin preparation and purification

Crude hemolysin was prepared by a modification of a method previously described (20). Three liters of *S. hyodysenteriae* culture were centrifuged at 10,000 x g at 4°C until the bacterial cells were pelleted. Bacteria were suspended in β-hemolysin extraction buffer [phosphate buffered saline (140 mM NaCl - 8 mM Na$_2$HPO$_4$ - 1.5 mM KH$_2$PO$_4$ - 2.7 mM KCl, pH 7.2) –0.05% RNA-core (Sigma Chemical Co., St. Louis, Mo.) –1 mM glucose–1 mM MgSO$_4$] at 1/20th of the original culture volume and held at 37°C for 30 min with sufficient agitation to maintain suspension. Cells were pelleted by centrifugation, the supernatant retained and frozen, and the extraction was repeated 3 to 5 times depending on the extent of cell vigor as assessed by motility. Supernatants were pooled, vacuum filtered through a 0.22 μm filter (Gelman Sciences, Ann Arbor, Mich.) and stored at -70°C until further purification. Throughout all manipulations, β-hemolysin-containing solutions were maintained at 4°C or less and assayed periodically for hemolytic activity. The β-hemolysin-containing supernatant was reduced in volume and desalted by repeated concentration and
reconstitution with sterile, deionized water utilizing a forced-flow ultrafiltration device with an approximate molecular weight cutoff filter of 3 kDa (Filtron Technologies, Northborough, Mass.). This concentrated, crude hemolysin was loaded onto preparative non-denaturing polyacrylamide slab gels and electrophoresed until the dye front reached the bottom of the gel. Hemolytic activity was localized within the gel by placing the gel on blood agar. Slices of polyacrylamide gel containing the hemolytic zones were obtained and subjected to electroelution into Tris-glycine buffer (37.6 mM Tris–50 mM HCl, pH 7.5). The supernatant was then reduced in volume and desalted by ultrafiltration, as described above. The process was repeated so that β-hemolysin preparations were electrophoresed and electroeluted twice. This purified hemolysin was submitted to the Protein Center at Iowa State University for N-terminal amino acid sequence by the Edman reaction. The amino acid sequence was then used to construct a degenerate oligonucleotide probe for subsequent hybridization and gene identification.

**Library construction and screening**

The *S. hyodysenteriae* genomic library was constructed by Stratagene in Lambda ZAP II using 5-10 kilobase (kb), randomly sheared DNA fragments whose ends were polished by DNA polymerase. *EcoRI* linkers were added using DNA ligase, and the fragments cloned into Lambda ZAP II *EcoRI*-digested, dephosphorylated DNA. Following packaging, the library was amplified using standard techniques (35).

To screen the library, the phage were grown at a density of 500 plaques per 85 mm petri dish. Nitrocellulose disks were used to lift the plaques, and the DNA denatured with 0.1 M NaOH and baked at 80°C. The lifts were prehybridized in 2X SSC (1x SSC = 150 mM NaCl–15 mM sodium citrate, pH 7.0) – 5X Denhardt’s (1 g Ficoll 400–1 g polyvinyl pyrrolidone–1 g bovine serum albumin, per liter) –200 µg per ml salmon sperm DNA –0.1% sodium dodecyl sulfate (SDS) for 3 h at 47°C. Hybridization was performed at 47°C overnight with 32P-labeled oligonucleotide probe. The blots were washed twice with 2X SSC at 25°C and twice at 47°C prior to exposure to X-ray film. Positive plaques were
purified using standard techniques and rescreened using the above protocol.

DNA manipulations

Plasmids containing cloned *S. hyodysenteriae* chromosomal DNA fragments were excised *in vitro* from the corresponding recombinant Lambda ZAP II plaques with helper phage R408 and introduced into SOLR according to the manufacturer's instructions (Stratagene). Restriction maps of each cloned fragment were constructed by digesting the plasmids with various enzymes and analyzing the resulting fragments on 0.7% agarose gels.

DNA sequencing and analysis

DNA sequence analysis was performed by first determining region of the three recombinant clones that reacted with the degenerate oligonucleotide probe. This was accomplished by DNA-DNA hybridization using plasmid DNAs digested with EcoRI-EcoRV-HindIII and with Clal-HindIII. Following separation of the fragments by agarose gel electrophoresis, blotting to nylon membranes and hybridization with the degenerate oligonucleotide probe, the region containing the amino terminal portion of the hemolysin was identified and the sequence of this region determined in plasmid pISM1236. The reverse primer (5'-CAGGAAACAGCTATGACC-3') and two custom primers designated TV2 (5'-CAAAATAATAGTCGCCCTCAAC-3') and TV7 (5'-TAGCTGTGACGGCGGAATG-3') were used to obtain complete overlapping sequence with an automated Applied Biosystems, Inc. Model 373A Fluorescent DNA sequencer. The DNA sequence data were assembled and analyzed for open reading frames (ORFs) and sequence homologies using MacVector software, version 5.0.2. The sequence was also analyzed for sequence motifs using the PROSITE software located at URL: http://www.genome.ad.jp/SIT/MOTIF.html (2).

DNA-DNA hybridization

Genomic DNA was isolated from six different serpulinal species by a modified Marmur technique (43). The six species were: *S. innocens*
strain B256; an unnamed pathogenic chicken isolate, strain C-1 (proposed name, \textit{S. alvinipulli}); \textit{S. intermedia} strain PWS/A, an isolate from a pig with non-dysenteric colitis; \textit{S. murdochii} strain 155-20, a non-pathogenic isolate from a pig; \textit{S. pilosicoli} strain P43/678, an end-on attaching spirochete isolated from a pig with colitis, and \textit{S. hyodysenteriae} strain B204. All of the strains analyzed, except for \textit{S. hyodysenteriae} B204, are moderately to weakly $\beta$-hemolytic. DNA from each of these spirochetal species was digested individually with four different restriction enzymes: \textit{MboI} (Gibco BRL, Gaithersburg, MD), \textit{HindIII} (Gibco BRL), \textit{AseI} (New England Biolabs, Beverly, Maine) and \textit{SspI} (Gibco BRL). Two $\mu$g of DNA from each reaction mixture were electrophoresed through 1% agarose gels and blotted to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL). DNA was fixed to the nylon membranes by UV crosslinking using a Stratagene 1800 UV crosslinker.

Hybridization was performed to determine if other \textit{Serpulina} species contained homologs of the $\beta$-hemolysin gene identified in this study in \textit{S. hyodysenteriae}. A \textit{ClaI} - \textit{EcoRI} 0.95 Kb fragment from plasmid pISM1236 (Fig. 1) containing the entire open reading frame of the $\beta$-hemolysin gene was used in these hybridization studies. This fragment was isolated by agarose gel electrophoresis following plasmid digestion and was purified using a Qiagen column (Qiagen, Chatsworth, Calif.). The probe was labeled with $^{32}$P by random primer labeling using Klenow fragment of DNA polymerase (Amersham). Prehybridization of the membrane blots was performed for 2 h at 65°C in a solution containing 0.1% SDS-5x SSC-5x Denhardt’s solution-100 mg per ml salmon sperm DNA. Hybridization was performed overnight at 65°C using the labeled probe in the hybridization buffer; final washes were performed with a buffer containing 0.2x SSC-1% SDS, twice for 5 min at room temperature and twice for 15 min at 65°C. Autoradiographs were exposed for 9 h at -80°C.

**Nucleotide sequence accession number**

The nucleotide sequence of the \textit{S. hyodysenteriae} \textit{hlyA} gene has been assigned GenBank accession number U94886.
Results And Discussion

Hemolysin purification and amino acid sequencing

For the purposes of biological and biochemical characterization, the β-hemolysin was purified from extraction buffer by ultrafiltration concentration of supernatant fluid followed by successive separations on native polyacrylamide gels and electroelution. Analysis of this hemolytically active material utilizing size exclusion, capillary electrophoresis revealed a single polypeptide peak with an apparent molecular mass of between 19 and 21 kDa (data not shown). Hemolysin purified by electroelution was then analyzed for its N-terminal amino acid sequence which also indicated that a single polypeptide had been obtained with the sequence K-D-V-V-A-N-Q-L-N-I-S-D-K. From the N-terminal sequence of the purified β-hemolysin, a degenerate oligonucleotide probe was designed (5'-AAAGATGT(A/T)GT(A/T)GC(A/T)AATCA-3') and used to screen the genomic library.

Identification of the S. hyodysenteriae hemolysin gene

Screening of the S. hyodysenteriae genomic library resulted in 8 positive plaques representing three independent clones. Following excision of the DNA into pBluescript plasmids, the three cloned fragments were restriction mapped (figure 1). The three plasmids were designated pISM1235-pISM1237. Each of the cloned fragments contained a Clai-HindIII fragment of approximately 0.45 Kb which reacted positively with the degenerate probe (data not shown). Colonies of the E. coli host strain SOLR were converted from a nonhemolytic to a hemolytic phenotypes when carrying these plasmids (figure 2). Control strains containing either Bluescript pSK- (Stratagene) or a pSK-derivative with an unrelated 4 kb insert of S. hyodysenteriae chromosomal DNA were hemolytically negative on blood agar. They were also negative by DNA hybridization when probed with the degenerate oligonucleotide (data not shown).

Nucleotide sequencing of plasmid pISM1236 yielded an ORF with the sequence shown in figure 3. The amino acid sequence derived from
the purified *S. hyodysenteriae* hemolysin was found immediately downstream of a 7 amino acid signal sequence (figure 3). In order to differentiate between this gene and the putative hemolysin genes identified and designated *tly* by ter Huune et al., this gene has been designated *hlyA* in keeping with more conventional nomenclature (44). Analysis of this sequence indicated that it had homology with numerous acyl carrier protein genes from a variety of organisms (data not shown). The predicted gene product was 8.93 kDa and it included a seven amino acid leader sequence immediately upstream of the amino terminal amino acid sequence obtained from the purified protein (figure 3). The translated product had a pI of 4.3 and a predicted rod-shaped conformation arising from alpha helical structure (figure 4). This rod shape may explain the disagreement between the predicted molecular mass and that estimated by size exclusion capillary electrophoresis (19-21 kDa), i.e., linear proteins tend to appear larger than globular proteins of similar size by size exclusion chromatography. Discrepancies in the reported molecular masses of the β-hemolysin of *S. hyodysenteriae* may also be attributable to the rod shape conformation of this molecule, although multimeric forms of the protein could also explain these differences (20). The translation product contained a conserved phosphopantetheine binding site, common to all acyl carrier proteins, that spans amino acid residues 32 through 47 (figure 3). Sequencing and subsequent analysis of the surrounding open reading frames indicated that these genes have homology with acyl carrier protein synthetases (data not shown).

Acyl carrier proteins function in fatty acid synthesis in a manner analogous to coenzyme A by binding and transferring acyl groups to growing fatty acid chains (25). The acyl groups attaches not to the peptide but to a phosphopantetheine moiety, a prosthetic group that binds to a conserved amino acid sequence within the ACP (Fig. 3). In *E. coli*, phosphopantetheine binds to a serine at residue 36; the analogous serine in HlyA is at residue 37. Acyl carrier proteins also function in acylation of other molecules in *E. coli*, such as the lipid A portion of lipopolysaccharide, various outer membrane proteins and the α-hemolysin (25, 37). This acyl group transfer function is essential and
considered to be ubiquitous, although not all organisms have been shown to possess such activities. Recently, other ACP functions, not related to fatty acid synthesis, have been identified. Two separate ACP functions have been shown to exist in *Rhizobium* spp, in which one is involved in the synthesis of essential lipids and the other functions in host specific nodulation (36). In addition, the genes for invasion related proteins secreted by a type III protein secretion system in *Salmonella typhimurium* have been shown to be located immediately upstream of a gene bearing homology to other known ACP genes thus raising speculation that this ACP gene product may play some role in pathogenesis other than an essential acyl transfer function (16). Thus, it is possible that an organism may have ACP genes that code for functions other than fatty acid synthesis. This may be particularly relevant for *S. hyodysenteriae* where fatty acid synthesis may be limited.

The lipid metabolic capabilities of intestinal spirochetes are not well understood (40). It is known that *S. hyodysenteriae* requires cholesterol and phospholipid for growth (22, 39, 41) which may indicate that *S. hyodysenteriae* is incapable of synthesizing certain fatty acids or phospholipids. Conversely, other studies have shown that *S. hyodysenteriae* contains lipids different from those included in the culture medium, a finding indicative of *de novo* fatty acid synthesis (26-28). The lipid biosynthetic capabilities of *S. hyodysenteriae*, in fact, may be quite limited, as has been shown to be the case with other spirochetes (24). Thus, the function of an ACP in *S. hyodysenteriae* is not clear.

A possible connection between acyl carrier function as suggested by gene sequence analysis and the hemolytic activity demonstrated in purified HlyA preparations from *S. hyodysenteriae* is not immediately apparent. It is unlikely that another protein is involved in the hemolytic activity since HlyA was isolated as a single chromatographic peak from solutions containing high levels of hemolytic activity. The β-hemolysin of *S. hyodysenteriae* causes erythrocyte lysis by membrane perturbation and alters membrane potentials and calcium ion fluxes in nucleated cells which are indicative of damage to the cell membrane (1, 33, 34). Since ACPs bind acyl and lipid groups of various lengths, the β-
hemolysin of *S. hyodysenteriae* may function by the binding to lipids within the erythrocyte membrane resulting in membrane instability.

**Southern blot analysis**

Genomic DNA from six different species of *Serpulina* were digested with four restriction enzymes, electrophoresed and hybridized with the oligonucleotide probe. Results from all four enzymes were similar. A blot performed with one of those enzymes, *AseI*, is shown in Figure 5. *S. intermedia* strain PWS/A, which has an intermediate β-hemolytic pattern, was the only species other than *S. hyodysenteriae* to show significant binding of the probe. This organism is representative of what was formerly known as the "intermediate types" of *S. hyodysenteriae*. The pathogenicity of these isolates, now collectively categorized as *S. intermedia*, has been equivocal in the hands of some researchers; other reports have indicated that these strains are clearly pathogenic, but compared to *S. hyodysenteriae*, induce a different and milder disease in experimentally infected swine called porcine spirochetal colitis (4, 7, 13). In these studies there was positive association between the presence of the ACP-like β-hemolysin gene and pathogenicity of porcine *Serpulina* isolates.

**Summary**

In summary, the β-hemolysin of *S. hyodysenteriae* has been electrophoretically purified and the N-terminal amino acid sequence obtained. Construction of an oligonucleotide probe from that amino acid sequence facilitated identification, cloning and sequencing of the gene encoding the protein. Analysis of that sequence provided information about the physicochemical properties of the hemolytic factor and demonstrated that it had sequence homology with acyl-carrier proteins. It had no relationship to previously identified *S. hyodysenteriae* hemolysin genes suggesting that this gene was unique. It has been designated *hlyA* to distinguish it from *tlyA-C* genes. This study is the first direct linkage between a hemolytic functional activity derived
from *S. hyodysenteriae* and its cognate gene sequence in the *Serpulina hyodysenteriae* chromosome. DNA-DNA hybridization performed on six different serpulinal species indicated that at least one other species, *S. intermedia*, possessed the gene.

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**Literature Cited**


inv and spa loci of Salmonella typhimurium that have homology to the Shigella IpaD and IpaA proteins. J. Bacteriol. 177:7078-7085.


38. Stanton, T. B., personal communication.
Figure 1. Physical maps of the recombinant clones from the Lambda ZAP II genomic library. The plasmid designations are also given. The position of *hlyA* is indicated by the shaded box. The scale at the top is in kilobases. The sequences used for the hybridization probe are indicated by the bar. Symbols: C, *ClaI*; Ev, *EcoRV*; H, *HindIII*; P, *PstI*. Not shown is the *EcoRI* site in the vector multiple cloning site.

Figure 2. Hemolytic activity of SOLR (pISM1236). The strains were streaked for single colonies onto blood agar plates and grown overnight at 37°C. Panel A, SOLR (negative control); Panel B, SOLR (pISM1236). The image was digitized from a color slide, cropped in Adobe Photoshop and labeled in Aldus FreeHand.
Figure 3. Nucleotide and deduced amino acid sequence of the *S. hyodysenteriae* hlyA gene. The structural gene begins at nucleotide 78 at the underlined ATG codon and ends at nucleotide 312 at the bolded TAA codon. The numbers refer to the DNA sequence. The single underlined amino sequence represents the phosphopantetheine binding motif. The double underlined amino acid sequence is the N-terminal sequence derived from purified β-hemolysin.

Figure 4. Predicted hydrophilicity and structural properties of the translated sequence of *S. hyodysenteriae* hlyA. All analyses were performed using MacVector software version 5.0.2. The scale at the bottom of the figure indicates the amino acid residue number. The type of analyses are indicated on the left. Positive numbers indicate high probability for hydrophilic regions. Shown in the bottom panel are the regions of secondary structure that agree using the Chou-Fasman (Cf)(6) and Robson-Garnier (Rg)(8) methods. Hlx, α-helix; Sht, β-sheet; Trn, turn.
Figure 5. Hybridization analysis of chromosomal DNA from different *Serpulina*. Chromosomal DNA from different serpulinal species was digested with *Ase*I, the fragments separated by agarose electrophoresis and transferred to nylon membrane. Hybridization was performed suing a 0.95 kb *HindIII/EcoRI* fragment from plasmid pISM1236 as described in the Materials and Methods. Lane 1, *S. pilosicoli* P43/678; lane 2, *S. hyodysenteriae* B204; lane 3, *S. murdochii* 155-20; lane 4, *S. intermedia* PWS/A; lane 5, strain C-1; lane 6, *S. innocens* B256. Molecular weights (kb) are given on the left. The radiograph of the blot was digitized using a Cohu model 4900 high performance CCD camera (Cohu, Inc., San Diego, Calif.) and a Macintosh IIci equipped with a Scion Corporation (Frederick, Md.) video board. The resulting TIFF file was cropped with and assembled in Adobe Photoshop and labeled in Aldus FreeHand.
CHAPTER 6: GENERAL CONCLUSIONS

In the 25 years since *Serpulina hyodysenteriae* was identified as the etiologic agent of swine dysentery a considerable body of research has been performed in efforts to elucidate the pathogenic mechanisms of that organism and to discover more effective methods of prevention and control of the disease. While significant advances have been made, particularly in the areas of the development of diagnostic tools and the understanding of serpulinal phylogeny, fundamental pathogenic mechanisms of *S. hyodysenteriae* remain elusive. Of the virulence factors attributed to *S. hyodysenteriae* its β-hemolysin remains the one most likely to be crucial in pathogenesis. While numerous studies have demonstrated various biological properties associated with the hemolysin, interpretation of these findings relative to lesions that occur following infection has been difficult. Thus, it was the purpose of some of the studies reported in this dissertation to assess the host response to the hemolysin utilizing a highly reproducible and relevant model. This required initial and detailed assessment of that murine model, in terms of its similarity to both other models and to the naturally occurring disease.

Meaningful study of the virulence attributes of microorganisms cannot occur without a basic understanding of their mode of action, physical and chemical nature and relatedness to other virulence attributes. Such information has been lacking in regards to the β-hemolysin of *S. hyodysenteriae*, significantly slowing meaningful advances in research in that area. Molecular techniques that allow identification of virulence factor genes are essential and commonly used tools in the study of virulence attributes, but have not been widely applied to the study of *Serpulina hyodysenteriae*. Therefore, a second purpose of the studies reported in this dissertation was to identify, clone and characterize the gene encoding the β-hemolysin of *S. hyodysenteriae*.

The first manuscript is a detailed characterization of an enhanced murine model of *S. hyodysenteriae* infection (10). The need for this work arises from the marked difference in this diet-induced enhanced
model and the conventional murine model in terms of the time and rapidity of lesion development. Since the conventional model is widely accepted it was necessary to demonstrate that the lesions that occur in the enhanced model are fundamentally identical to those that occur in the conventional model. To that end, mice were fed either conventional rodent chow (CRC) or Teklad diet 85420 (TD), the enhancing diet, and subsequently infected with \( S. \) hyodysenteriae. Mice from each group were necropsied at intervals throughout a 17 day period. A scoring system was used to assess changes that occurred in the cecum of infected mice and it was shown that mice fed TD developed grossly and histologically severe lesions within 24 hours of infection, much sooner than mice fed CRC. Overall lesion scores were also higher in mice fed TD than in mice fed CRC. Furthermore, the morphologic features of lesions in the two groups were the same. These studies demonstrated that in the enhanced model lesions are more severe and develop much more rapidly but are structurally the same lesions as those that occur in the conventional murine model. Quantitative bacteriology performed in these studies indicated that the enhancing effect of TD was not due to increases in total numbers of \( S. \) hyodysenteriae.

The mechanism by which TD enhances lesion development is not apparent but may be related to alterations in the cecal flora. In the original report describing this model total numbers of gram positive and gram negative organisms were increased in number in animals fed TD (10). This adds support to a substantial body of evidence indicating that the large intestinal microflora play a role in lesion development in animals infected with \( S. \) hyodysenteriae (2, 3, 4, 8, 9).

The second manuscript utilizes the reproducible and well-characterized model described above to demonstrate ultrastructural changes that occur in the ceca of mice following either infection with \( S. \) hyodysenteriae or exposure to its \( \beta \)-hemolysin. The primary goal of these studies centered on the effects of the hemolysin. However it is apparent that in order to determine the relevance of hemolysin induced changes it is necessary to have a pre-existing baseline knowledge concerning similar changes that occur following infection with \( S. \) hyodysenteriae. Such information, derived from highly controlled and
reproducible experimental conditions, has not been previously available. In the first of the described experiments mice were fed TD, infected with *S. hyodysenteriae* and necropsied at frequent intervals. Transmission electron microscopic examination of those tissues demonstrated early damage to superficial epithelial cells in the form of microvillus damage intercellular fluid accumulation. Increased numbers of resident bacteria were present within epithelial cells and accumulated in lamina proprial stromal and phagocytic cells. Lamina proprial capillaries subsequently exhibited changes consistent with increased permeability. There was eventual necrosis and exfoliation of superficial epithelial cells, and accumulation of massive numbers of bacteria within the lamina propria. These studies provide more support for the role of luminal flora in the pathogenesis of lesion development following *S. hyodysenteriae* infection. The pronounced translocation of bacteria from the lumen to the lamina proprial space is clearly an *S. hyodysenteriae* induced phenomenon. The changes that occur in the lamina proprial capillary endothelium subsequent to the accumulation of those bacteria in the cytoplasm of lamina proprial macrophages and stromal cells could very possibly be induced by those cells, activated by the bacteria they have internalized. Macrophages and stromal cells are capable of secreting a wide array of vasoactive substances and it seems likely that large numbers of bacteria within their cytoplasm would stimulate those cells to do so.

In the second of the described experiments surgically created cecal loops in mice fed TD were filled intraluminally with various quantities of β-hemolysin. Examination of those tissues by transmission electron microscopy demonstrated changes in superficial epithelial cells including loss of microvilli, marked cytoplasmic vacuolation and detachment of clumps of apparently viable epithelial cells. There was also necrosis of a few detached cells and apoptosis of large numbers of detached cells. These findings were unexpected but appear to share some features in common with those that are present in mice infected with *S. hyodysenteriae*. In both instances there is apparent damage to apical cytoskeletal structures of the exposed surface epithelial cells. Hemolysin can apparently induce apoptosis in these cells by an
unknown mechanism, and is also capable of causing necrosis of those cells, a change that also occurs in mice infected with *S. hyodysenteriae*. Hemolysin has been demonstrated to cause changes in intracellular calcium ion concentration which is one pathway in the induction of apoptosis (1).

The third manuscript addresses not a specific question but rather the need to better understand the physical and chemical nature of the β-hemolysin of *S. hyodysenteriae*. In the many reports describing collection and characterization of the hemolysin there has been much conflicting information regarding its size and chemical nature (5, 6, 7, 11). It was determined that a molecular approach would yield more definitive results. An electrophoretic method of hemolysin purification was developed which allowed isolation of hemolytic activity associated with a single sequencable peptide. The amino acid sequence of that peptide was used to construct an oligonucleotide probe which was instrumental in identifying and cloning the hemolysin gene. Sequencing of this cloned hemolysin gene yielded the surprising result that the gene (designated *hly*) had sequence and structural homology with numerous acyl carrier proteins, which function in acylation and fatty acid synthesis. The connection between acyl carrier function and hemolytic function is not immediately evident. Since acyl carrier proteins bind lipids of various lengths (via a prosthetic group, phosphopantetheine) it is possible that hemolytic or cytotoxic activity is the result of membrane destabilization induced by lipid binding. Elucidation of this mechanism will require further study, possibly with the purified, recombinant protein. The first step in such studies would be to determine if the recombinant protein has the same biological properties (hemolysis, cytotoxicity, calcium fluxes) as are attributed to the β-hemolysin.

Based on the findings of these experiments, a generalized hypothesis of *S. hyodysenteriae* pathogenesis may be proposed. Following infection of the host by *S. hyodysenteriae* and colonization of the large intestine there is serpulinal growth and secretion of hemolysin. This hemolysin, an acyl-carrier like protein, interacts and binds with lipids in membranes of the superficial epithelial cells with
which it comes into contact. Cell membranes are sufficiently disturbed to allow translocation of resident bacteria through those cells into the lamina propria, where they are phagocytosed by macrophages and other stromal cells. These cells, activated following internalization of bacteria, secrete various cytokines, chemokines and eicosanoids, which have structural and functional effects on the capillary endothelium. Capillary permeability is increased leading to edema of the lamina propria, which in turn causes inadequate exchange of nutrients and metabolites for the superficial epithelial cells. This coupled with continued membrane damaged induced by the hemolysin, is sufficient to cause eventual necrosis of these epithelial cells.

In summary, these experiments demonstrate pathologic changes associated with \textit{S. hyodysenteriae} infection or treatment with its hemolysin in a murine model. These findings provide compelling evidence that the \(\beta\)-hemolysin is an important virulence factor of \textit{S. hyodysenteriae} that likely exerts its effects through interactions with the cell membrane, possibly by binding lipids. These studies also provide evidence that further support and perhaps define the role of the luminal flora in lesion development in animals infected with \textit{S. hyodysenteriae}. An experimental model is useful for studies that require economical and highly controlled conditions. However, at some point the findings of the model must be tested in the true host species or setting. Such is the case with these findings. Until it is demonstrated in swine that luminal bacteria accumulate in the lamina propria, the relevance of these findings in mice cannot be estimated. The development of a comparable porcine model would do much to facilitate studies that verify or refute these murine findings.

\textbf{Literature Cited}


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