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CHARACTERIZATION OF THE ATTACHMENT OF *TREPONEMA HYODYSENTERIAE* TO HENLE INTESTINAL EPITHELIAL CELLS *IN VITRO*

by

Christine Ann Bowden

A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN MICROBIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to the following individuals for the assistance they gave me during the construction of this thesis:

Dr. Lee Kelley, for his statistical expertise and his serene perspective and demeanor;

Dr. William Grimes, for his advice on biochemical matters and his inspiration;

Bronnie Bauer Trumper, for her continual friendship and moral support;

Julie and Kim Joyiens, for their year-long patience and diligence in typing and editing and re-editing this manuscript.

and

Dr. Lynn Joens, for putting up with me for three years and making this all possible.

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ABSTRACT

Properties of the attachment of Treponema hyodysenteriae to Henle intestinal cells (HEI 407) were examined. Frequency of attachment was dependent on motility and viability of the spirochetes. Rabbit hyperimmune and swine convalescent antisera inhibited attachment, but immune colonic secretions did not. Pretreatment of HEI cells with protease, phospholipase C,and sodium metaperiodate decreased attachment. Post-attachment treatment of cells and spirochetes with neuraminidase decreased adherence. Attachment was inhibited by N-acetylneuraminic acid, colominic acid, D-glucuronic acid, N-acetylgalactosamine, and fetuin. Adherence was increased with N-acetylglucosamine or yeast mannan. Surface antigens of T. hyodysenteriae and concentrated culture supernatants competitively inhibited adherence. Sialic acid was isolated from T. hyodysenteriae and culture supernatants, in higher concentrations than from washed spirochetes or HEI cells. Fluorescent wheat germ agglutinin lectin detected N-acetylglucosamine on the HEI cell surface. Our results suggest that the binding adhesins on T. hyodysenteriae contain sialic acid, and the HEI cell receptor is a glycoprotein.

CHAPTER 1

INTRODUCTION

Etiology of Swine Dysentery

Swine dysentery is a mucohemorrhagic diarrheal disease that primarily affects the post-weanling pig. Swine dysentery (SD) has been reported in all major swine-producing countries and causes significant economic losses due to reduced weight gain, expense of treatment, and death of animals (Harris and Glock, 1981). Morbidity may run as high as 90% and mortality 30% in herds, depending on the course of treatment. Swine dysentery was first described by Whiting et al. in 1921. In 1924, Whiting demonstrated the transmissible nature of the disease by feeding infected colonic contents to healthy pigs, which subsequently became ill. Whiting was unable, however, to isolate the caustive organism and propagate it in culture. Doyle, (1944) isolated a curved, gram-negative organism identified as Vibrio coli, which he associated with the production of lesions in SD. It was later shown however, that this organism was not involved in the pathogenesis of

SD (Harris and Glock, 1981). Terpstra et al. identified large spirochetes in the colons of dysenteric pigs in 1968, and in 1971 Taylor and Alexander isolated and cultured anaerobic spirochetes from the colons of infected pigs, and used these microorganisms to reproduce the clinical signs and typical lesions of SD in minimal-disease pigs. That same year Harris and Glock confirmed the findings of Taylor and Alexander, and named the organism Treponema hyodysenteriae (Harris et al., 1972; Glock and Harris, 1972).

T. hyodysenteriae is a gram-negative, oxygen-tolerant anaerobic spirochete. It measures 6-8.5um in length and 320-380nm in diameter, is loosely coiled, and possesses 7-9 periplasmic flagella inserted at each end of the spirochete and overlapping at the middle (Harris and Glock, 1981; Raynaud et al., 1980). T. hyodysenteriae is motile and hemolytic; it is negative in tests for catalase, cytochrome oxidase, H_2S production and urease; and is positive for esculin hydrolysis, and for bile- and iodoacetate-tolerance (Harris et al., 1976). The organism ferments carbohydrates, producing acetic acid, butyrate, H_2 and CO_2, and degrades pyruvate but not lactate (Harris and Glock, 1981; Harris et al., 1976). T. hyodysenteriae is classified as a type 1, or large, spirochete, and is unique

in that it is the only pathogenic treponeme that can be maintained in culture (Harris et al., 1976).

T. hyodysenteriae is isolated from infective feces by streaking plates of trypticase soy agar supplemented with 5% bovine blood and 400ug Spectinomycin (Songer et al., 1976). It is propagated in liquid medium consisting of trypticase soy broth (TSB) with 10% fetal bovine serum, in an atmosphere of deoxygenated H_2 and CO_2 (Kinyon and Harris, 1974). Other liquid media also have been used successfully to propagate the organism: TSB with 10% rabbit serum in N_2: CO_2 (Lemcke et al., 1979); and serum-free media supplemented with bovine serum albumin and cholesterol (Lemcke and Burrows, 1980).

When Taylor and Alexander first isolated T. hyodysenteriae in 1971 they also described another anaerobic spirochete, morphologically similar to T. hyodysenteriae, that was not pathogenic for swine and which produced a weak beta-hemolysis. Kinyon and Harris (1979) proposed that this nonpathogenic strain be named Treponema innocens. T. innocens can be isolated from the intestines of most healthy pigs, as well as in pigs affected with SD; for this reason a reliable method for differentiating T. innocens from T. hyodysenteriae is crucial for both diagnostic and epidemiological purposes. The simplest,

though least accurate, method to distinguish the two organisms is by their patterns of hemolysis on blood agar. T. hyodysenteriae exhibits strong beta-hemolysis, T. innocens a weak beta-hemolysis. Immunologic assays have been devised that can discriminate between the two organisms with much greater accuracy. Baum and Joens (1979a) isolated a surface antigen specific for T. hyodysenteriae using a hot phenol water extraction method. The phenol-phase antigen reacts with its homologous antisera in a double immunodiffusion test, but does not cross-react with anti-T. innocens sera. The water phase of these extractions contains primarily lipopolysaccharide (LPS). Serological examination of the LPS from 13 isolates of beta-hemolytic T. hyodysenteriae with antisera against each isolate demonstrated the existance of seven distinct serotypes (Baum and Joens, 1979b; Mapother and Joens, 1985). Nonimmunologic analysis of the lipids of T. hyodysenteriae and T. innocens can also distinguish the two spirochetes: T. hyodysenteriae contains monogalactosyldiglyceride, while T. innocens contains monoglucosyldiglyceride. In addition, T. hyodysenteriae possesses a galactolipid that has not been found in any other treponeme, and which has been identified tentatively as an acylmonogalactosyldiglyceride (Matthews et al., 1980a;

1980b).

In vivo methods to differentiate T. innocens from T. hyodysenteriae also have been studied. Kinyon and Harris (1979) orally inoculated pigs with a variety of strains of T. hyodysenteriae or T. innocens, and found that 25 of 25 isolates of T. hyodysenteriae were enteropathogenic, while 13 of 13 isolates of T. innocens were not pathogenic. Whipp et al, (1978) used isolated colonic segments to test for enteropathogenicity, and the results were similar to those reported by Kinyon. Knoop (1979) surgically ligated segments of the small intestine in New Zealand white rabbits, and inoculated the segments with spirochetes. Pathogenic T. hyodysenteriae colonized the loops and caused lesions typical of SD, while the nonpathogenic T. innocens did not produce lesions. Joens and Glock (1979) reported the utility of the CF1 mouse to differentiate T. hyodysenteriae from T. innocens. Following oral inoculation of mice with both strains of spirochetes, only mice inoculated with T. hyodysenteriae developed lesions typical of SD (Joens, 1980a).

Pigs are the primary host for T. hyodysenteriae, and transmission of the organism occurs by ingestion of infected fecal matter (Harris and Glock, 1981). Swine that have recovered from infection with T. hyodysenteriae can

shed the organism in their feces for as long as 70-80 days after the last clinical evidence of disease (Fischer and Olander, 1980). Exposure of normal pigs to either acutely ill or convalescent, carrier pigs can result in clinical swine dysentery (Songer and Harris, 1978). T. hyodysenteriae has been isolated from a variety of other sources in the field. Songer et al. (1978) collected samples from three separate farms, and the following sources were positive for T. hyodysenteriae: lagoon water, material from a waste-holding pit, and a dog which had eaten the feces of pigs inflicted with diarrhea. Wild rodents from eight pig-producing farms were trapped and tested for the presense of T. hyodysenteriae (Joens and Kinyon, 1982). T. innocens was isolated from a number of rats and mice, and 3 out of 4 strains of T. hyodysenteriae isolated from mice were pathogenic when inoculated into swine. In an experimental situation, healthy pigs exposed to the feces of mice infected with pathogenic T. hyodysenteriae developed SD (Joens, 1980b). It appears that rodents may act as reservoir hosts and aid in the spread of SD. Since it is apparent that the organism can persist in the farm environment long enough to reinfect swine herds, proper management and waste disposal can help prevent or control outbreaks of dysentery on pig farms.

Several animal models have been developed to facilitate the study of SD. Outbred and inbred (Hartley) strains of guinea pigs inoculated intragastrically with pathogenic T. hyodysenteriae developed some of the clinical signs of SD (ie. depression and/or diarrhea) (Joens et al., 1978). Gross and microscopic lesions were similar to those seen in pigs, and fecal cultures were positive for T. hyodysenteriae as well. Nonpathogenic strains did not produce clinical signs or lesions. CF1 mice inoculated intragastrically with T. hyodysenteriae developed mucoid feces, shed the organism in their stool, and presented colonic lesions at necropsy (Joens and Glock, 1979). Mice that had been challenged with T. innocens did not exhibit any of these pathological changes. The New Zealand white rabbit has also served as a model host for T. hyodysenteriae (Knoop, 1979).

Induction of SD in pigs and in other animals has been attempted by a number of routes, but success has been achieved only via oral, intragastric, or intracolonic inoculation. Intradermal, intramuscular, subcutaneous, intrarectal and intravenous routes of inoculation failed to induce clinical dysentery, enteric lesions, or shedding of T. hyodysenteriae in pigs, mice, guinea pigs or rabbits (Hughes et al., 1975). Olson reported induction of SD in

pigs inoculated intravenously with filtrates of T. hyodysenteriae from infected colonic scrapings (Olson, 1981) but those results have not been confirmed by other reseachers.

Swine dysentery can be produced in conventional and specific-pathogen-free pigs using minced colonic tissue, colonic scrapings, colonic filtrates, feces from dysenteric pigs, or pure cultures of pathogenic T. hyodysenteriae. Initial attempts to infect gnotobiotic pigs with pure cultures of T. hyodysenteriae and/or V. coli were not successful, although inoculation with colonic scrapings from infected pigs did result in clinical dysentery and characteristic lesions (Meyer et al., 1974a, 1974b). These discoveries led researchers to think in terms of a mixed etiology for SD, and a variety of enteric bacteria were surveyed for their synergism with T. hyodysenteriae in the pathology of SD. Meyer et al. (1974b) were not able to induce dysentery in germ-free pigs coinfected with Escherichia coli, lactobacillus, V. coli and Clostridium. However, if these pigs were first colonized with E. coli and then inoculated with T. hyodysenteriae, Clostridium, and four other gram-negative anaerobes, SD developed (Meyer et al., 1975). In addition, germ-free pigs colonized first

with T. hyodysenteriae, and later with the four anaerobes or vice-versa, also developed SD. Brandenburg et al. (1977) could not induce lesions in gnotobiotic pigs using T. hyodysenteriae alone or with V. coli and/or a peptostreptococcus. If gastric acidity was neutralized with sodium bicarbonate prior to inoculation with T. hyodysenteriae, the spirochetes did establish in the colon and were shed in the feces, but little if any pathological changes occurred. Harris et al. (1978) demonstrated that T. hydoysenteriae alone, or coinoculated with F. necrophorum, did not produce lesions of SD in gnotobiotic pigs; whereas T. hyodysenteriae coinoculated with F. necrophorum and B. vulgatus, or with just B. vulgatus, caused lesion production. Mucofibrinous, watery diarrhea and colonic lesions were observed in gnotobiotic pigs inoculated with T. hyodysenteriae strain B204 in a variety of combinations with Fusobacterium necrophorum, Bacteroides vulgatus, Clostridium sp., and Listeria denitrificans (Whipp et al, 1979). Both authors noted a direct correlation between the number of T. hyodysenteriae established in the gut and the pathological changes detected there.

The presense of T. hyodysenteriae was required for lesion development in all the aforementioned cases, supporting the claim that the spirochete is the primary

etiological agent of SD. It appeared, however, that colonization of the gut of gnotobiotic pigs with any one of a number of synergistic bacteria was a prerequisite for subsequent colonization and/or pathogenicity of T. hyodysenteriae. Harris and Kinyon (1974) observed that T. hyodysenteriae could become established in the germ-free gut, but did not express pathogenicity. Others also have postulated that the role of synergistic bacteria is to enhance the pathogenic expression of T. hyodysenteriae (Whipp et al, 1979; Harris et al., 1978). On the other hand, Whipp et al. (1982) discovered that colitis and shedding of T. hyodysenteriae can be induced not only by the establishment of synergistic anaerobes, but also by altering the intestinal environment in other ways. Gnotobiotic pigs inoculated with T. hyodysenteriae alone, followed every six hours with infusions of growth media, were colonized with T. hyodysenteriae and shed the organism in significant numbers; but no lesions were produced. Germ-free pigs fed a diet of autoclaved pig-feed, rather than sterile milk substitute, not only shed T. hyodysenteriae but also developed colitis. Based on these results, the authors concluded that the alteration in the colonic environment brought on by the introduction of synergistic anaerobes, or by the digestion of rough feedstuff, created

an environment more suitable for the colonization and proliferation of T. hyodysenteriae.

Pathogenesis

In order for an invading microorganism to be pathogenic, it must overcome a variety of obstacles and perform several crucial functions. First, a sufficient number of bacteria must gain entry to the host, adhere to a mucosal surface, compete with the native flora, evade host defenses, and, in some cases, penetrate into the epithelium. The organisms must then multiply and colonize their selected niche, continuing to withstand assault from host defenses. Lastly, they must injure the host in some fashion, often by producing toxins or destructive enzymes, or by inflicting mechanical damage to host cells (Beachey, 1981; Mims, 1982; Ofek and Beachey, 1980; Reed and Williams, 1978).

T. hyodysenteriae preferentially colonizes the large intestine and cecum of swine. During the acute stages of the disease, large numbers of spirochetes can be detected in the mucus, within the pseudomembrane and necrotic debris on the luminal surface, and in the crypts of Lieberkuhn (Hughes, et al., 1975; Joens et al., 1981; Glock and Harris, 1972; Glock and Kluge, 1974). Electron microscopic examination of infected colonic tissue revealed

the presence of T. hyodysenteriae within the cytoplasm of damaged epithelial cells, and aggregated around vessels in the lamina propria (Glock and Harris, 1972; Teige and Nordstoga, 1979; Teige et al., 1981; Taylor and Blakemore, 1971). Although T. hyodysenteriae appears to associate with colonic epithelial cells, there is no definitive proof that spirochetes attach to these cells in vivo. On the other hand, T. hyodysenteriae binds avidly and irreversibly to a variety of cultured cells in vitro. Knoop et al. (1979) studied the attachment of T. hyodysenteriae to isolated swine intestinal epithelial cells and to mouse adrenal cells. Maximum adherence was attained after 60 minutes of incubation, and within a temperature range of 37-42°C. Treponemes that were inactivated at 56°C for 60 minutes, or at 23°C for several days, lost their ability to attach to the cells. This observation was thought to be related to the presense of a heat-sensitive or metabolically active treponemal surface adhesion. Wilcock and Olander (1976) reported a direct correlation between the motility of T. hyodysenteriae cultures and frequency of attachment. They also found that T. hyodysenteriae adhered to the surface of all cell lines tested: swine buffy coat, swine testicle, Hela, pig kidney, and green monkey kidney. In addition, this attachment appeared to be irreversible

since spirochetes remained attached to the cells throughout repeated washings in saline, formalin, alcohol and xylene.

The pathophysiology of SD is very characteristic for the disease and has been described by many researchers. The clinical signs of dysentery appear following a 10-14 day incubation period, and begin with yellowish-to-grey soft feces, partial anorexia and elevated rectal temperature. Within several days copious amounts of mucus and flecks of blood usually appear in the stools; as the disease progresses a watery, mucohemorrhagic diarrhea develops in which shreds of white, mucofibrinous exudate may be seen. Prolonged, severe diarrhea leads to dehydration, cachexia, emaciation, weakness and incoordination. Death is generally attributed to dehydration and the resultant acidosis and hyperkalemia (Harris and Glock, 1981).

Gross lesions are limited to the large intestine and ileocecal region, although occassional hypermia of the small intestine and fundic portion of the gastric mucosa have been noted (Hughes et al., 1979). Typical lesions include hyperemia and edema of the walls and mesentery of the cecum and colon. The mucosa swells, losing its normal rugose appearance, and is often covered with a mucohemorrhagic exudate. The colonic submucosal glands and mesenteric

lymph nodes become swollen and more prominent, and ascites fluid may accumulate in the peritoneal cavity (Harris and Glock, 1981; Hughes et al., 1975). The serosal surface may become reddened, with foci of petechial hemorrhage, and a slight fibrinous exudate may be formed. The edema in the intestinal wall may decrease in the advanced stages of dysentery, and a thick fibrinonecrotic pseudomembrane may form over the mucosal surface. A marked superficial necrosis may also be observed (Harris and Glock, 1981; Hughes et al., 1975; Glock and Harris, 1972; Glock and Kluge, 1974; Teige and Nordstoga, 1979; Teige et al., 1981; Taylor and Blakemore, 1971).

Microscopic lesions are found primarily in the colon, and to a lesser extent in the cecum and rectum. Sloughing of the epithelium occurs, and partial to complete erosion of the luminal surface with separation of the epithelium from the lamina propria can be seen. Damage to the microvilli can range from mild irregularity to complete cellular degeneration. The mitochondria and endoplasmic reticulum of epithelial cells may appear swollen. Both the mucosa and submucosa become edematous; and infiltration of PMN's into the mucosal region and mononuclear leukocytes into the lamina propria and submucosa occurs. The crypts become hyperplastic and dilated, and excessive mucus

production with goblet cell exhaustion occurs. Infected portions of the mucosa undergo coagulative necrosis, and the mucosal surface may become covered with pseudomembranous exudate consisting of mucus, fibrin, erythrocytes, cellular debris and bacteria. Numerous spirochetes can be found within the crypts, invading into and between necrotic epithelial cells, and adhering to the epithelial surface. Spirochetes are not seen invading deeper than the lamina propria. Fibrinoid thrombi may occlude many of the superficial vessels in the lamina propria, and neutrophils may be seen in congested vessels on the luminal surface (Harris and Glock, 1981; Hughes et al., 1975; Glock and Harris, 1972; Glock and Kluge, 1974; Teige and Nordstoga, 1978; Teige et al., 1981; Taylor and Blackemore, 1971).

The presence of microthrombi in the small vessels of the lamina propria indicates stasis, and may be one of the initiating events in the development of the disease (Teige and Nordstoga, 1979). Occlusion of these blood vessels may lead to hypoxia and ischemia and the subsequent necrosis of the overlying tissue. T. hyodysenteriae is most proliferative at sites of coagulative necrosis, and the increased numbers of spirochetes seem to correlate with more extensive tissue damage. The resultant outpouring of blood and fibrin favor the overgrowth of opportunistic

pathogens. Hughes et al. (1975) suggested that these bacteria, in conjunction with T. hyodysenteriae, induce an inflammatory response which can be seen most clearly between the zone of superficial necrosis and the underlying normal mucosa.

The occurance of fibrin and red blood cells in the intestinal lumen, as well as the considerable loss of fluids, indicate severe defects in membrane permeability (Teige and Nordstoga,1979). Colonic transport studies undertaken by Argenzio et al. (1980) demonstrated that the diarrhea produced in SD is caused entirely by colonic absorptive failure, predicated by a sharp decrease in the lumen-to-blood fluxes of Na^+ and Cl^-. A subsequent investigation by Schmall et al. (1983) disproved the hypothesis that these alterations in ion transport occurred in response to increased levels of cAMP and/or cGMP. E. coli, Vibrio cholerae, and Salmonella typhimurium produce enterotoxins that cause cAMP/cGMP-induced diarrhea (Schmall, et al., 1983; LeVine and Cuatrecasas,1981; Mims, 1982). It seems apparent that if T. hyodysenteriae does produce an enterotoxin, its mechanism of action differs from that of these pathogens.

No specific virulence factors have been isolated yet from T. hyodysenteriae that can produce the lesions

found in SD. T. hyodysenteriae is not an invasive organism: although spirochetes are found inside necrotic tissue they are never seen invading healthy tissue. In addition, T. hyodysenteriae never has demonstrated cytopathic effects in experimental assays with various cell lines (Knoop et al., 1979; Wilcock and Olander, 1979). The pathology of the disease seems to indicate the involvement of a toxin; however, no pathological changes have been observed in cell cultures following treatment with culture filtrates, supernatants, and homogenates (Wilcock and Olander, 1979). Only in the presence of viable, pathogenic spirochetes are lesions produced (Teige et al., 1981). Wilcock and Olander (1979) proposed that a vasoactive toxin may be involved in pathogenesis, evoking the formation of microthrombi in the luminal capillaries. Several possible candidates have come to light which may account for the circulatory alterations, but may not directly cause the colonic maladsorption. A heat-labile, oxygen-tolerant hemolysin has been detected in cell-free filtrates of beta-hemolytic T. hyodysenteriae (Saheb et al.,1980). Intact, viable spirochetes are required, and maximum production of the hemolysin occurs during the log-phase of growth (Knoop, 1981; Lemcke and Burrows, 1982). Weakly beta-hemolytic, nonpathogenic spirochetes produce only

small amounts of hemolysin, and only during a limited period of the logarithmic phase (Lemcke and Burrows, 1982). The treponemal hemolysin has a number of characteristics in common with the streptococcal hemolysin, Streptolysin S (Saheb et al, 1980). Both are oxygen-tolerant and heat-labile, and production of hemolysin is dependent on the presence of an RNA-core carrier. T. hyodysenteriae hemolysin does not display any proteolytic or lipolytic activities, and its role as a potential virulence factor has yet to be determined (Saheb et al., 1980).

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria, and elicits a wide range of endotoxic and immunogenic responses in a host. LPS is believed to induce the following responses: fever, leukopenia, shock, disseminated intravascular coagulation, activation of complement, neutropenia and sequestration followed by neutrophilia, direct damage to endothelial tissue, and activation of macrophages (Slauson and Cooper, 1982). T. hyodysenteriae produces LPS which is serotype specific (Baum and Joens, 1979b), and its involvement in the pathogenesis of SD has been investigated. Nuessen et al. (1982) demonstrated that treponemal LPS was toxic to peritoneal macrophages, enhanced C3 and IgG-Fc receptor-mediated internalization by macrophages, was

mitogenic to mouse splenocytes, and was chemotactic for porcine leukocytes in the presence of normal pig serum. The immunogenic role of LPS was demonstrated by its ability to competitively inhibit serotype-specific opsonization of T. hyodysenteriae to mouse peritoneal cells in the presence of the homologous antisera (Nuessen and Joens, 1982). In another study, T. hyodysenteriae colonized the large intestine of both the LPS-sensitive C3HeB/FeJ mice and the LPS-resistant C3H/HeJ mice, but produced gross and microscopic lesions only in the FeJ mice (Nuessen et al., 1983). In the presence of Actinomycin D, 1 mg of treponemal LPS was lethal for the FeJ but not the HeJ mice; and the LPS was chemotactic for macrophages from FeJ mice only. It thus appears that LPS may be a virulence factor of T. hyodysenteriae. The vasoactive properties of LPS may account for the vascular congestion observed in the colon. In addition, the host response to LPS may trigger the inflammatory reaction that occurs in the large intestine.

Inflammation and circulatory stasis are probably amoung the initial events leading to the degenerative processes that occur in the gut during SD. Although these processes take place early on in the course of the disease, the order in which the two events occur is not known. LPS and hemolysin are the only potential virulence factors that

have been isolated to date, and their mechanisms of action have yet to be defined. Colonic maladsorption appears to be responsible for producing the clinical signs of SD, but the factors that induce adsorptive failure have not been determined. These enigmas, and many other issues, need to be resolved before the pathogenesis of SD is fully clarified.

Immunity

Pigs that have recovered from SD are resistant to subsequent challenge with viable, pathogenic T. hyodysenteriae, and have elevated antibody titers for up to 8 weeks post-infection (Nuessen et al., 1982; Joens et al., 1979). Many researchers have attempted to vaccinate pigs in a variety of ways, and have attained varying degrees of success. Hudson et al. (1976) employed a complicated vaccination regimen in which the pigs were first orally inoculated with live, attenuated T. hyodysenteriae on three occassions, and then vaccinated with either live T. hyodysenteriae intraperitoneally, or killed treponemes intramuscularly. This regimen protected 9 of 18 pigs, while 16 of 18 unvaccinated pigs developed SD. Glock et al. (1978) vaccinated pigs with multiple, intravenous inoculations of increasing doses of formalin-treated T. hyodysenteriae. Although the symptoms were less severe for the vaccinated

pigs, only 2 of 8 of the animals were resistant to challenge with T. hyodysenteriae. Jenkins et al. (1979) demonstrated that oral administration of attenuated T. hyodysenteriae offered minimal protection, indicated by a slight delay in the onset of symptoms, and a decrease in the total number of days of diarrhea. In addition, they found that administration of a low dose of virulent T. hyodysenteriae prevented the development of diarrhea in pigs subsequently challenged; but they feared that this procedure could potentially introduce the disease into susceptible herds. Harris and Schuitman (1982) conferred protection to 3 of 4 pigs following parenteral and oral innoculations of inactivated T. hyodysenteriae; however, parenteral or oral inoculations alone were not sufficient to protect pigs from SD.

Fernie et al. (1983) discovered that a single parenteral vaccination with formalin-treated T. hyodysenteriae in an oil adjuvant prevented diarrhea in 3 of 5 pigs, encouraged spontaneous recovery, and reduced the severity of all clinical manifestations of the disease. Levamisole is an antihelminthic which is thought to enhance immunity by augmenting the generation of IgM-producing lymphocytes, and by intensifying the cell-mediated immune response. Jenkins et al. (1982a, 1982b) vaccinated pigs with either

particulate or soluble treponemal antigen, with or without the concurrent administration of levamisole. Fewer clinical signs and less shedding of organisms were observed in the group that received soluble antigen and levamisole than in any other treatment group.

Joens et al. (1983) evaluated swine that had recovered from infection with specific serotypes of T. hyodysenteriae for their resistance to challenge with homologous and heterologous serotypes of the spirochetes. To examine this phenomenon, the large intestine of convalescent pigs was surgically ligated into a series of isolated segments, into which the various treatments were injected. Typical dysenteric lesions developed in those segments that received the heterologous serotype of T. hyodysenteriae, while no lesions were produced in the segments inoculated with the homologous serotype. Immunity appeared to develop in response to treponemal antigens that are serotype-specific. In addition, Joens et al. (1983) noted a positive correlation between lesion development and colonization of the mucosal surface with T. hyodysenteriae, suggesting that protection may be related to preventing the spirochetes from adhering to and/or colonizing the colonic mucosa. The evidence indicates that SD should be preventable by vaccination; however, a practical, effective, polyvalent

system of immunization has yet to be developed.

Bacterial Adherence

Bacteria that are capable of adhering to the surface of host cells gain several advantages by this ability. Organic compounds tend to adsorb to the cell surface, creating a nutritionally rich environment for microorganisms. The bacteria are also in position to utilize host cell secretions, products of extracellular enzyme activity, host cell surface components, and intestinal epithelial mucus (Jones, 1977). The most important function of bacterial adherence, however, is to secure the bacteria against removal from the mucosal surface by the movement of bodily fluids. Microorganisms have evolved a variety of structural appendages or biochemical adhesins with which to adhere to cell surfaces. In many species, the presense of structural adhesins is directly related to the pathogenicity of the microorganism. In other cases, the relationship is an indirect one: adhesins allow the bacteria to remain in proximity to the mucosal surface, wherein bacterial toxins or enzymes may be released, or tissue-invasion may begin.

The aim of many researchers is to determine the specific nature of the adherence of bacteria to mucosal surfaces in order to develop measures to prevent this

adherence before tissue damage occurs. Inhibition of attachment can be achieved by a number of methods. Saturation of the host cell receptors with compounds that are identical to, or structural analogs of, the bacterial adhesins would preclude bacterial attachment. Another method would involve the destruction or alteration of the bacterial adhesins, or saturation of the adhesins with receptor analogs. If the specific biochemical structure of the bacterial adhesin could be determined, isolated, and rendered immunogenic, then perhaps this antigen could be used as a vaccine to stimulate production of antibody against the adhesin. Anti-adhesin antibodies would conceal the ligands from the receptor sites and thus prevent attachment.

One of the most extensively researched micro-organisms is Escherichia coli, and a comprehensive literature exists concerning the surface antigens and attachment properties of this bacteria. The majority of E. coli strains produce type 1, mannose-sensitive pili which enable the bacteria to adhere to a variety of eukaryotic cells (Gaastra and de Graaf, 1982). The involvement of pili in the attachment mechanism is demonstrated by the ability of anti-pili antibody to inhibit the adherence of E. coli to epithelial cells (Weinstein and Silverblatt, 1983). It has

been shown that D-mannose, methyl-D--mannopyranoside, and yeast mannan inhibit the attachment of E. coli to mouse phagocytes (Bar-Shavit et al., 1977) and to human mucosal cells (Ofek et al., 1977), indicating the possibility of a mannose receptor on the host cell. The nature of the host cell receptor site was elucidated by Davis et al. (1981), who reported a decrease in the attachment of E. coli to epithelial cells pretreated with sodium metaperiodate or lipase. In addition, they found that treating the cells with neuraminidase or colominic acid increased adherence, while coincubation of the cells and bacteria with N-acetyl-neuraminic acid inhibited attachment. Based on these results, they concluded that the host-cell receptor may be a glycolipid.

A number of enteropathogenic E. coli strains produce surface adhesins that differ from the type-1 pili, primarily in that they are not mannose-sensitive. The K88 antigen is produced by E. coli that cause neonatal diarrhea in piglets; the K99 pilus is produced by E. coli that infect pigs, calves and lambs; and the 987-P antigen is from isolates that infect pigs. These antigens are proteinacious, filamentous appendages that project from the bacterial cell surface. They are immunogenic, granting passive immunity to the offspring of vaccinated dams; and

they mediate the adherence of E. coli to intestinal cells (Gaastra and de Graaf, 1982; Whipp, 1980). Cell-free pilus antigen adheres to epithelial cells, and antisera against the pili inhibits attachment (Jones and Rutter, 1972). Production of the K88 and K99 pili is plasmid-controlled, and transfer of the plasmid into K88- or K99- negative E. coli confers both the adhesive and hemagglutinating properties of these pili to the recombinant E. coli (Burrows, et al, 1976; Gaastra and de Graaf, 1982; Jones and Rutter, 1974). While K88-positive E. coli colonize primarily the anterior portion of the small intestine, the 987-P- and K99- positive strains of E. coli proliferate in the posterior small intestine (Gaastra and de Graaf, 1982). The host-cell receptors for these adhesins are thought to involve glycolipids and glycoproteins, but no specific receptors have been defined as yet (Gaastra and de Graaf, 1982).

The receptor-adhesin interaction has been clarified in a number of other host-pathogen systems. Vibrio cholerae is a flagellated, motile bacteria that causes the severe diarrhea of cholera. Although a relationship had been noted between motility, adherence, and virulence, the exact role of the flagella was not understood until recently. Attridge and Rowley (1983) demonstrated that

motility was not required for adherence, but that the flagella bore the adhesins required for the attachment and subsequent pathogenicity of Vibro cholerae.

Another organism whose properties of attachment have been investigated extensively is Treponema pallidum, the caustive agent of syphilis. A correlation seems to exist between attachment and pathogenicity, since only virulent strains of T. pallidum adhere to host cells. Actively metabolic host cells and viable treponemes are required for adherence, and T. pallidum multiplies in vitro only when attached to mammalian cells (Wong, et al, 1983a; 1983b; Fitzgerald et al, 1977). These treponemes have been observed to attached to host cells with an end-on orientation, leading to the proposal that the terminal structures represent a specialized attachment organelle (Hayes et al., 1977; Fitzgerald and Johnson, 1979). However, while studying the motility of Leptospira interrogans, Charon et al. (1981) discovered that after the spirochetes had attached to fixed, antibody-coated latex beads, the beads were displaced to the terminus of the leptospira as they attempted to swim away. This indicates that surface antigens are able to move laterally through the lipid bilayer of the outer membrane. It may be that the adhesins on T. pallidum are actually distributed thoughout the outer

membrane sheath, and that once attached to a cell, the treponemes eventually assume an end-on-end configuration.

Syphilis is a generalized infection and T. pallidum is found attached to a variety of tissues within the host. Fitzgerald et al. (1979) proposed that T. pallidum may bind to ground substance, the intercellular "cement" between tissue cells, which is found throughout the body. Ground substance consists of proteins complexed with acidic mucopolysaccharides, the latter of which are primarily chondroitin sulfate and hyaluronic acid. Fitzgerald was able to demonstrate the presence of acidic mucopolysaccharides in the tissues to which T. pallidum attached. In addition, treatment of the host cells with hyaluronidase resulted in a decrease in attachment of the treponemes, indicating that the host receptor may indeed be an acidic mucopolysaccharide. A subsequent study by these researchers demonstrated that T. pallidum possesses hyaluronidase activity, which was indicated by the ability of treponemal cultures to degrade long-chain mucopolysaccharides, and by the presence of antibodies to hyaluronidase in the serum of syphilic rabbits (Fiztgerald and Johnson, 1979). It was proposed that the action of treponemal hyaluronidase on host tissue may facilitate the invasion of T. pallidum into the tissue.

Receptor-binding proteins of T. pallidum were

identified by Baseman and Hayes (1980). They found that three of the proteins detected by SDS gel electrophoresis bound to HEp-2 cell cultures, and that one of the proteins seemed to be a major antigenic stimulus for antibody production. Peterson et al. (1983) investigated the role of a membrane protein, fibronectin, as a host receptor for T. pallidum. Virulent treponemes bound selectively to coverslips coated with fibronectin, and anti-fibronectin antibody inhibited attachment.

It is apparant that attachment of microorganisms to host cells is an important event in the pathogenesis of many diseases. Knowledge of the specific interactions between the host cell and pathogen not only gives a better understanding of the mechanisms of infectious disease, but also may be useful in developing methods of treating or preventing disease. Little is known at the present time concerning the attachment of T. hyodysenteriae to epithelial cells and the role of this event in the pathogenesis of SD. The purpose of the following studies was to investigate a number of parameters pertaining to the attachment of T. hyodysenteriae to intestinal epithelial cells. The requirements for motility and viability of the spirochetes for attachment were established; the ability of colonic secretions and antisera to inhibit adherence was examined;

enzyme probes were used to determine receptor moieties on epithelial cells; and competitive inhibition studies were performed to confirm the receptor moiety, and to define the receptor-binding adhesin on T. hyodysenteriae.

CHAPTER 2

MATERIALS AND METHODS

Tissue Culture

A continuous intestinal epithelial cell line was used as the host model for all adherence tests. Human Embryonic Intestinal (HEI 407) cells were purchased from American Type Culture Collection, and frozen stocks were stored in liquid nitrogen. Cells were cultured in 25 cm^2 tissue culture flasks (Corning Glass Works, New York) and maintained in minimal essential media (MEM; Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; Gibco) and 2% Antibiotic-Antimycotic (Gibco). Cells were subcultured every two to three days, and incubated at 37°C in a humidified atmosphere of 5% CO_2. Hemocytometer counts were performed at the time of assay, with the concentration of cells averaging 10^5 cells/ml. Viability was determined by trypan blue exclusion.

Treponemal Cultures

Pathogenic strains of *T. hyodysenteriae* B234 and B204, representing serotype 1 and 2 respectively, were

grown in 15 ml anaerobe tubes containing 7.0 ml of a reduced medium of trypticase soy broth (TSB; BBL, Baltimore, Md.) supplemented with 7% FBS, as described by Kinyon and Harris (1974); and in 500 ml flask cultures as described by Joens et al. (1978). Motility was assessed by phase microscopy and viability determined by plating serial 10-fold dilutions on trypticase soy agar with 5% bovine blood.

Adherence Test

Log-phase cultures of T. hyodysenteriae (16-20 hour growth) were used for the adherence assay. For each assay, 0.7 ml of treponemal culture was added to MEM to a final volume of 2.0 ml. The pH of the solution was adjusted to approximately 7.0 with 8M NaOH. HEI cells were grown to confluency, rinsed with MEM, and released from the flask with 0.5 ml trypsin-EDTA. The enzyme was then neutralized with 0.5 ml FBS, 7.0 ml MEM added, and the cells centrifuged at 900 rpm for 10 minutes. The cells were resuspended in MEM and centrifuged two additional times, and the final pellet resuspended in 8.0 ml MEM. Cells were seeded into 6-well tissue culture plates (CoStar, Cambridge, Mass.) at a ratio of four wells per flask of cells, and incubated at 37°C in 5% CO_2 for 2 hours. After incubation, the wells were rinsed 6 times with MEM via

pipetting to remove nonadherent cells. 2.0 ml of the MEM/T. hyodysenteriae solution were added to each well of cells, and coincubated for 2 hours at 37°C. The wells were rinsed 8 times with MEM to remove unattached spirochetes. The preparations were then saturated with FBS, air-dried, stained with Wright's, and examined at 1000x magnification for attachment of the spirochetes to cells. The motility and vibility of the spirochetes was assessed following all treatments, with the exception of the antisera and colonic secretion assays.

Nonpathogenic T. hyodysenteriae

The adherence test was performed to compare the attachment capabilities of two nonpathogenic strains of T. hyodysenteriae (ACK 300/8, B3143, and T22) and T. innocens strain B3143, to that of the pathogenic T. hyodysenteriae strain B204. 0.7 ml of each of the four isolates was added to 1.3 ml MEM (pH 7.0) and coincubated with HEI cells for 2 hours at 37°C. The preparations were then rinsed 8 times with MEM, fixed and stained.

Heat, Cold, and Formalin

T. hyodysenteriae was cultured in a 500 ml flask, and divided into four equal portions for the following treatments. One portion was placed in a 56°C water bath

for 30 minutes, cooled to room temperature, then incubated with HEI cells. A second portion was held at 4°C for 24 hours prior to addition to the cells. The third portion was treated with formalin at a final concentration of 0.03% and incubated for 24 hours at 37°C. The formalin-treated spirochetes then were centrifuged at 6500 rmp for 20 minutes and resuspended in phosphate-buffered saline (PBS) three times. The final pellet was resuspended in Hank's balanced salt solution (Gibco) to the original volume. The fourth portion was untreated and served as a control. Each treatment group was assayed for motility and cultured for viability on blood agar prior to coincubation with the HEI cells. The untreated control and the heat-treated spirochetes were assayed on day one; the formalin- and cold-treated spirochetes assayed on day two.

Colonic Secretions

In both normal pigs and pigs recovered from experimental infection with T. hyodysenteriae strain B204, colonic loops were surgically isolated and externalized using silastic cannuli (Joens and De Young, personal communication). Colonic secretions were obtained by flushing the loops with 200 ml PBS. The PBS washings were heat-inactivated at 56°C for 30 minutes, clarified by centrifugation, filtered through a 0.8 um Nalgene filter,

and concentrated 10X by ultrafiltration. The colonic secretions were then assayed for inhibition of attachment of T. hyodysenteriae to HEI cells. 0.2 ml of normal or convalescent washings was added to 0.7 ml T. hyodysenteriae strain B204 in 1.1 ml MEM. After 30 minutes, 2.0 ml of each preparation were added to the HEI cells, and the assay continued as described.

Convalescent and Hyperimmune Antisera

Serum was collected from pigs that had recovered from experimental infection with T. hyodysenteriae strain B204 or B234, and from rabbits hyperimmunized with killed, whole-cell preparations of B204 or B234. The antisera were heat-inactivated at 56°C for 30 minutes, cooled to room temperature, and then added in 0.2 ml aliquots to 0.7 ml of isolate B204 or B234 in 1.1 ml MEM. Each sample was added to the HEI cells within 15 minutes, and coincubated for 90 minutes at 37°C.

Enzyme/Chemical Pretreatment

Protease type IV (1.0 U/mg; Sigma Chemical Co., St. Louis, Mo.), phospholipase C type I (10-20 U/mg; Sigma), and sodium-M-periodate (Sigma) were dissolved in MEM (pH 7.2) and diluted to the following final concentrations: protease = 0.0001, 0.001, 0.005, 0.01, 0.05 mg/ml; phospho-

lipase C = 0.0001, 0.001, 0.01, 0.1, 1.0 mg/ml; and periodate = 0.004, 0.004, 0.04, 1.0, 2.0, 4.0 mg/ml. Neuraminidase type VI (4.9 U/mg; Sigma) was dissolved in MEM (pH 5.0) and diluted to final concentrations of 0.0001, 0.001, 0.01, 0.1, and 1.0 mg/ml. HEI cells were washed with MEM and seeded into 6-well plates as described. After incubating 1 1/2 hours, the cells were rinsed with MEM, and 2.0 ml of the enzyme preparations were added to the cells for 30 minutes at 37°C. The cells were rinsed 8 times with MEM to remove the enzyme, and then coincubated with T. hyodysenteriae for 2 hours at 37°C. Control cells were treated identically, adding MEM to the cells instead of enzymes. The pH of the MEM added to the control cells was adjusted to that of the treatment groups to eliminate differences in adherence due to pH.

Post-adherence Enzyme/Chemical Treatment

Neuraminidase type VI was diluted to 0.01 or 0.1 mg/ml in MEM (pH 5.0), and sodium-M-periodate dissolved in MEM (pH 7.2) to a final concentration of 2.0 mg/ml. T. hyodysenteriae was coincubated with HEI cells for 1 1/2 hours. The cells were rinsed to remove unattached spirochetes, and 1.0 ml of enzyme solution was added to each well for one hour at 37°C. The preparations were then rinsed 8 times with MEM, fixed and stained.

Carbohydrate and Glycoprotein Coincubation

N-acetylneuraminic acid (NANA; Sigma), colominic acid (Sigma), D-glucuronic acid (Sigma), N-acetylglucosamine (GlcNAc; Sigma), N-acetylgalactosamine (GalNAc; Sigma), yeast mannan (Sigma), fetuin type IV (Sigma), and ovalbumin grade VI (Sigma), were dissolved in MEM (pH 7.2). NANA, colominic acid, GlcNAc, GalNAc, and mannan were added to 0.7 ml T. hydodysenteriae in 1.3 ml MEM to a final concentration of 10 or 15 mg/ml, and fetuin and ovalbumin to 5.0 mg/ml. These solutions were coincubated with HEI cells for 2 hours at 37°C. Colominic acid was dissolved in 2.0 ml MEM to a final concentration of 15 mg/ml, and added to the HEI cells for 30 minutes at 37°C. The cells were rinsed 8 times with MEM to remove the sugar, and coincubated with T. hyodysenteriae for 2 hours at 37°C.

Phenol-phase Antigens

Surface antigens were isolated from T. hyodysenteriae strains B204 and B234 using a hot phenol-water extraction method (Baum and Joens, 1979a). The phenol-phase extracts were filter-sterilized through a 0.45 um syringe filter (Nalgene, Rochester, NY), 0.3 ml added to 0.7 ml of B204 or B234 in 1.0 ml MEM, and then coincubated with the HEI cells for 2 hours.

Culture Supernatants and Washings

Supernatant from 48-hour B204 flask cultures (1000 ml) was collected following the removal of spirochetes by centrifuging at 6500 rpm for 20 minutes. Culture washings were collected after resuspension of the T. hyodysenteriae pellet in PBS and a second centrifugation. The supernatant was concentrated 60X, and the washings 75X, in an Amicon YM10 ultrafiltration unit. 60X concentrated cell-free culture media (TSB with 7% FBS) served as a control for the culture supernatant. 0.3 ml of the concentrated supernatant, washing, or culture media in 1.7 ml MEM was added to each well of HEI cells. A second set of controls received 2.0 ml MEM. After incubating 30 minutes at 37°C, the cells were rinsed with MEM, and 0.7 ml T. hyodysenteriae in 1.3 ml MEM was added to the cells for coincubation at 37°C for 2 hours.

Thiobarbituric Acid Assay for Sialic Acid

The thiobarbituric acid (TBA) assay for the determination of sialic acid was described by Warren (1959), and adapted for use here by Dr. William Grimes (Dept. of Biochemistry, University of Arizona; personal communication). Triplicate samples of 0.1 ml of each substrate were treated with 50 ul of 0.2 M $NaIO_4$ in 9 M

H_3PO_4, vortexed briefly, and held at room temperature for 20 minutes. 0.4 ml of 12.5% Na-arsenite in 0.5 ml $NaSO_4$ and 0.1 N H_2SO_4 was added, and samples vortexed, capped, and placed in a boiling water bath for 15 minutes. The samples were then placed in a cold water bath for 5 minutes. 1.0 ml of cyclohexanone was added to each sample, vortexed twice,and centrifuged at 3000 rpm for 3 minutes. The organic phase was read at 549 and 532 nm on a Beckman DV-6 spectrophotometer.

Sialic acid was released from each substrate prior to the TBA assay by one of two methods: treatment of the substrate with 0.1 N H_2SO_4 (final concentration) for one hour in an 80°C water bath; or treatment with 0.01 mg/ml neuraminidase type VI for 20 hours at 37°C.

The following substrates were assayed for their sialic acid content: HEI cells in MEM; T. hyodysenteriae strain B204 in its culture medium (TSB/FBS); B204 washed 3 times in PBS at 6500 rpm for 20 minutes and resuspended in PBS to the original volume; cell-free culture media (TSB/FBS); B204 supernatant collected after the first centrifugation; 75X concentrated B204 culture washing; 60X concentrated B204 culture supernatant, and 60X concentrated cell-free culture media.

The protein content of a T. hyodysenteriae broth culture, and of culture media, were estimated by U.V. absorbtion, using the method of Warburg and Christian (1941).

Fluorescent Lectin/Antibody Examination

FITC-labelled wheat germ agglutinin lectin (WGA; Sigma) was diluted 1:100 in PBS. Mouse anti-B204 monoclonal antibody was filtered through a 0.22 um syringe filter (Nalgene), and used undiluted. Rhodamine-labelled anti-mouse IgG (RITC-Ab; Miles Scientific, Napersville, Ill.) was diluted 1:100 in PBS. HEI cells were washed and plated as for an adherence assay, incubated 2 hours at 37°C, then rinsed with MEM. Half of the preparations were treated first with 0.3 ml WGA for 30 minutes at 4°C, rinsed, and then coincubated with 0.7 ml T. hyodysenteriae in 1.3 ml MEM for 2 hours at 37°C. The other half of the cells were first coincubated with the T. hyodysenteriae in MEM for 2 hours at 37°C, and then treated with 0.3 ml WGA for 30 minutes at 4°C. Both sets of preparations were then rinsed with MEM, treated with 0.3 ml of anti-B204 antibody for 30 minutes at 37°C, rinsed, and then then reacted to 0.3 ml RITC-Ab for 30 minutes at 4°C. The preparations were rinsed a final time with MEM, and then fixed in 2.0 ml paraformaldehyde (1% solution) for 20 hours at 4°C. To

determine if the WGA was binding to the spirochetes, a third trial was performed in which WGA alone was added to the attached spirochetes, avoiding any potential interference from rhodamine. All preparations were examined under fluorescent microscopy, and photographed.

Statistical Analysis of Data

The raw data concerning the number of spirochetes attached to each cell was transformed to $\sqrt{x + 0.5}$ in order to compensate for those cells with zero attachment. Transformed data were subjected to analysis of variance using multi-wave factorial analysis or simple one-way analysis where appropriate. Treatment means were compared by least significant difference to confirm the significance of the results of the analysis of variance (Sokal and Rohlf, 1981). Differences were considered significant if the probability of differences being due to chance was below 5% ($p < 0.05$), as determined by Student's t-test.

CHAPTER THREE

RESULTS

Adherence of T. hyodysenteriae to HEI Cells

T. hyodysenteriae attached readily to HEI cells, with a maximum adherence of up to 20 spirochetes per cell attained within 90 minutes (Figure 1). The optimal pH for adherence appeared to be in the neutral to slightly acidic range: alkaline conditions (pH > 8.0) decreased the incidence of attachment, while a highly acidic environment (pH < 5.0), caused disruption of the HEI cells. A positive correlation was noted between the titer of T. hyodysenteriae and the number of spirochetes that attached per cell (Figure 2). The relationship between titer and adherence applied only to the controls and not to the treated cells. Washing the spirochetes in PBS at 6500 rpm reduced adherence by as much as 50%, despite retention of their motility and viability. Multiple washings further reduced adherence; however, spirochetes washed in PBS with 10% FBS retained their ability to attach to a higher degree than those washed strictly in PBS.

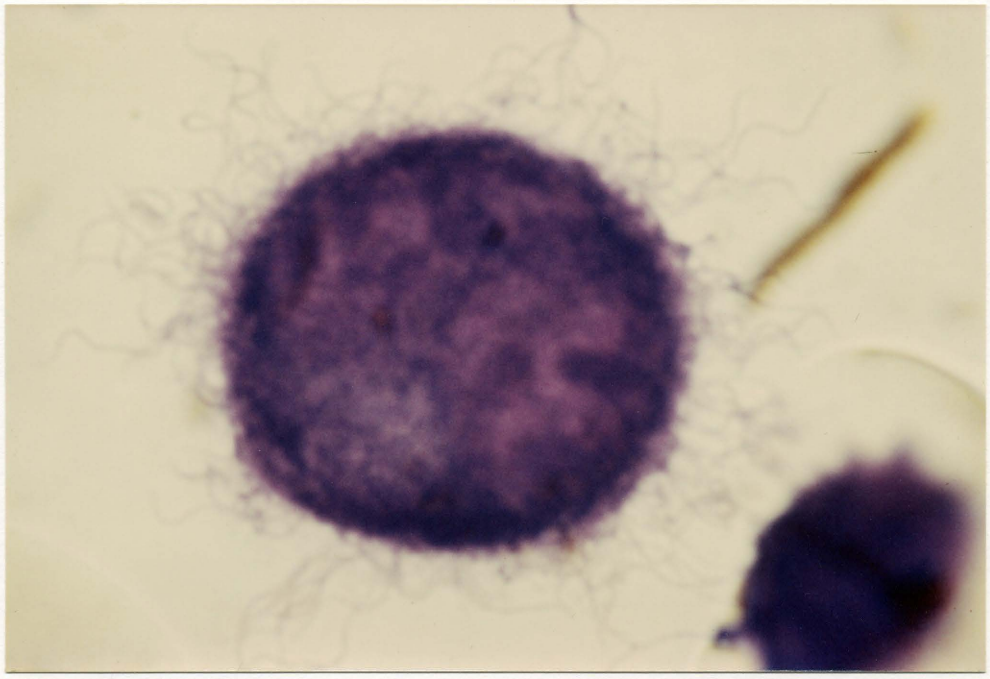

Figure 1. Attachment of T. hyodysenteriae to HEI cell.

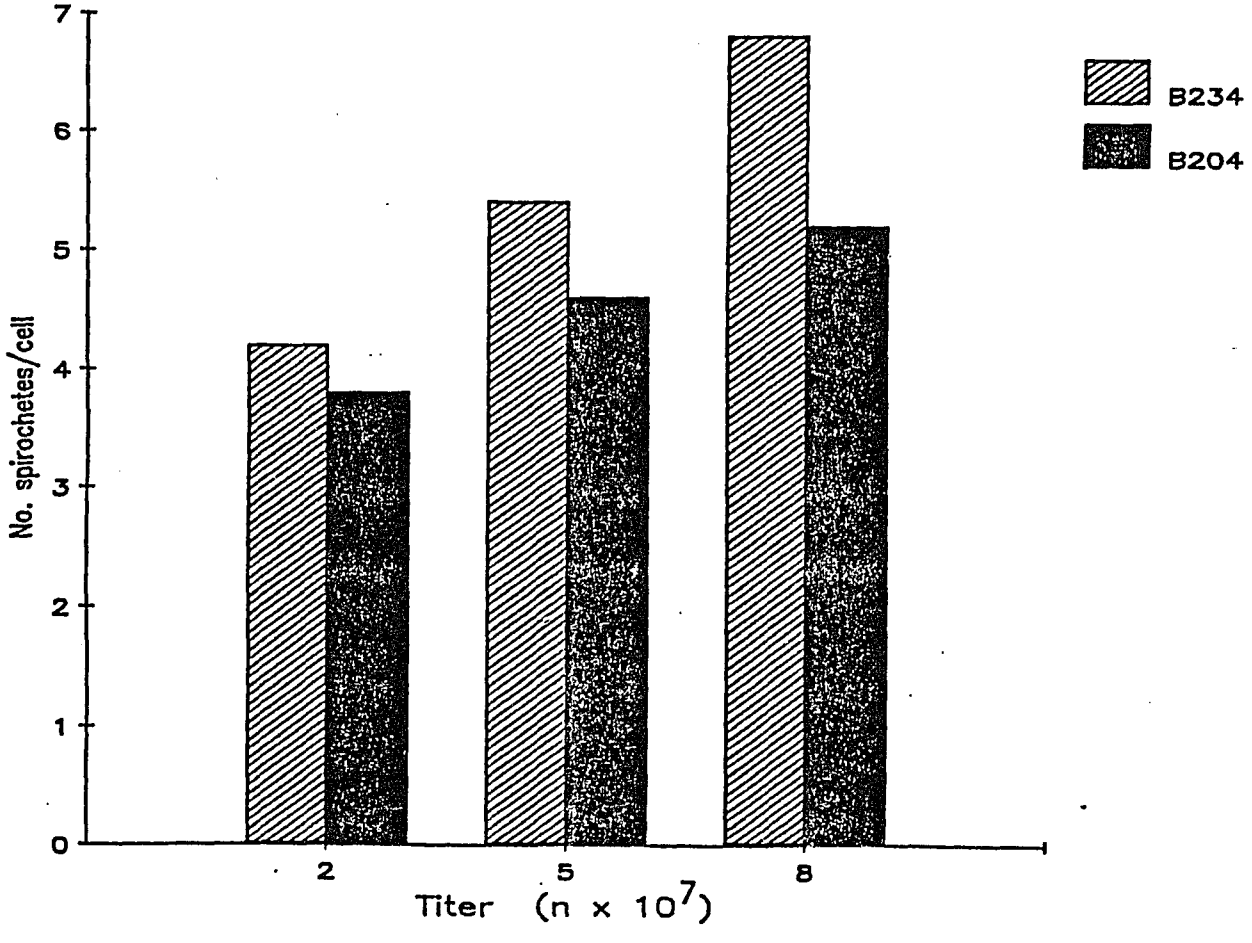

Figure 2. Relationship between concentration of T. hyodysenteriae and frequency of attachment.

Nonpathogenic T. hyodysenteriae

Two nonpathogenic strains of T. hyodysenteriae and one T. innocens isolate were assayed for their ability to adhere to HEI cells. All three strains adhered as readily as the pathogenic strain B204.

Heat, Cold, and Formalin Treatments

T. hyodysenteriae was treated with heat, cold or formalin to determine if motility and/or viability were requirements for attachment. As shown in Table 1, exposure to heat or formalin inactivated the spirochetes and decreased adherence by 65 to 90%. Maintaining the spirochetes at 4°C impaired motility without affecting viability, and adherence was reduced by nearly 50%.

Colonic Secretions and Antisera

Pigs that have recovered from swine dysentery are immune to reinfection with the same strain of T. hyodysenteriae. Colonic secretions from convalescent pigs were assayed to determine if the secretions prevent recurrence of disease by inhibiting the attachment of spirochetes to the mucosal epithelium. These secretions contain a measurable quantity of IgG and IgA antibody (Joens, 1983, personal communication), but at a level much lower than

TABLE 1. EFFECTS OF HEAT, COLD, AND FORMALIN ON MOTILITY, VIABILITY, AND ADHERENCE OF T. HYODYSENTERIAE TO HEI CELLS

Treatment[a]	Isolate	Motility[b]	Viability[c]	% Control[d] Adherence	p Value[e]
Heat	B204	-	-	2.8 (6)	<0.001
	B234	-	-	6.1 (4)	0.05
Cold	B204	+	++	44.5 (6)	0.01
	B234	+	++	41.4 (4)	NS
Formalin	B204	-	-	35.3 (6)	0.001
	B234	-	-	10.1 (4)	0.05

[a] Heat = T. hyodysenteraie were heat inactivated at 56 C for 30 minutes. Cold = T. hyodysenteriae were kept at 4 C for 24 hours. Formalin = T. hyodysenteriae were treated with 0.03% formalin for 24 hours at 37 C. Following treatments, the spirochetes were incubated with HEI cells for 2 hours at 37 C.

[b] Motility of the spirochetes was assessed by phase microscopy prior to and following each treatment; ++ = same level as control; + = 50-75% level of control; - = none detected.

[c] Viability was determined by plating 10-fold dilutions of T. hyodysenteriae on trypticase soy agar supplemented with 5% bovine blood and incubating the plates for 48 hours at 42 C, in an atmosphere of 50:50 H_2:CO_2.

[d] % Control Adherence = $\frac{\#\text{treated spirochetes per cell}}{\#\text{control spirochetes per cell}} \times 100$

Number in parenthesis indicates the number of trials performed; 100 cells counted per trial.

[e] Significant p=.05; NS= not significant.

that found in serum. There was no significant difference in attachment between the controls and cells that had been treated with secretions from either normal or convalescent pigs, nor between the two treatment groups (Table 2).

Antisera was tested against homologous and heterologous serotypes of T. hyodysenteriae for inhibition of attachment. Both hyperimmune and convalescent antisera decreased adherence by as much as 80%. The only exception was that rabbit anti-B234 had no significant effect on the adherence of T. hyodysenteriae strain B204 to cells.

Enzyme and Periodate Modification

The HEI cell surface was altered chemically or enzymatically to determine the biochemical nature of the receptor site for T. hyodysenteriae (Table 3). Pretreatment of the cells with the enzyme protease significantly reduced adherence at concentrations greater than 0.001 mg/ml. Phospholipase C altered attachment in a dose-dependent fashion: there was a slight increase in attachment at 0.0001 and 0.001 mg/ml; adherence was reduced by more than 80% at 0.01 mg/ml; and concentrations of 0.1 and 1.0 mg/ml caused lysis of the HEI cells (Figure 3). Periodate also had a dose-dependent effect on attachment, with the most significant reduction seen at 2.0 mg/ml

TABLE 2. EFFECTS OF COLONIC SECRETIONS AND ANTISERA ON THE ATTACHMENT OF T. HYODYSENTERIAE TO HEI CELLS

Treatment[a]	Isolate	% Control[b] Adherence	p Value[c]
Normal Colonic Secretion	B204	74.6 (5)	NS
Convalescent Colonic Secretion	B204	75.6 (5)	NS
Rabbit anti-B204 hyperimmune serum	B204	16.5 (6)	<0.001
	B234	15.5 (6)	0.001
Rabbit anti-B234 hyperimmune serum	B204	101.7 (6)	NS
	B234	22.0 (4)	0.001
Swine anti-B204 convalescent serum	B204	42.2 (5)	0.01
	B234	62.7 (4)	0.025
Swine anti-B234 convalescent serum	B204	24.1 (5)	0.01
	B234	22.9 (6)	0.01

[a] Colonic secretions were obtained from normal pigs, or pigs convalescent from swine dysentery, by PBS washes of 30cm of spiral colon. Antisera was collected from rabbits hyperimmunized with formalized preparations of T. hyodysenteriae or from pigs recovered from experimental infection with T. hyodysenteriae strains B204 or B234. Spirochetes were coincubated with secretions or antisera and HEI cells for 2 hours at 37 C.

[b] $\text{\% Control Adherence} = \frac{\text{\# treated spirochetes per cell}}{\text{\# control spirochetes per cell}} \times 100$

Number in parenthesis indicates the number of trials performed; 100 cells counted per trial.

[c] Significant. p = .05; NS = not significant.

TABLE 3. EFFECTS OF CHEMICAL AND ENZYMATIC TREATMENT OF HEI CELLS ON ADHERENCE OF T. HYODYSENTERIAE

Treatment[a]	Pretreatment		Post-adherence treatment	
	% Control[b] Adherence	p Value[c]	% Control[b] Adherence	p Value[c]
Protease	45.1 (4)	Ø.Ø25	---	---
Phospho-lipase C	17.2 (2)	<Ø.ØØ1	---	---
Periodate	28.Ø (4)	<Ø.Ø1	88.5 (7)	NS
Neuramini-dase	88.8 (11)	NS	63.5 (8)	<Ø.ØØ1

[a] HEI cells were treated with various concentrations of enzymes or periodate before or after incubation with T. hyodysenteriae isolate B2Ø4 for 2 hours at 37 C. Protease = Ø.ØØ5, Ø.Ø1, Ø.Ø5 and Ø.1 mg/ml; phospholipase C = Ø.Ø1 mg/ml; periodate = 2.Ø mg/ml; neuraminidase = Ø.Ø1 and Ø.1 mg/ml.

[b] % Control Adherence = $\frac{\text{\# spirochetes per treated cell}}{\text{\# spirochetes per control cell}} \times 1ØØ$

Number in parenthesis indicates the number of trials performed; 3Ø or 5Ø cells counted per trial.

[c] Significant p = .Ø5; NS = not significant.

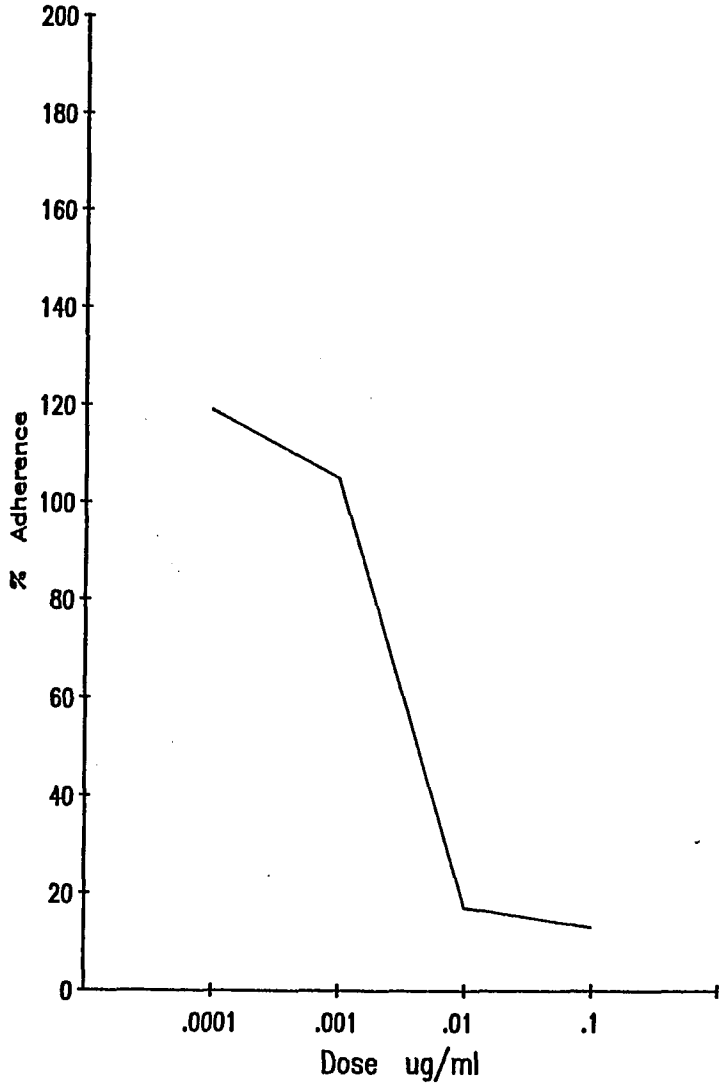

Figure 3. Effect of concentration of phospholipase-C pretreatment on attachment of T. hyodysenteriae to HEI cells.

(Figure 4). Neuraminidase had no significant effect on adherence (Table 3). Lipase and trypsin were also assayed, but treatment of the HEI cells with these enzymes consistently resulted in cell lysis.

Post-adherence Neuraminidase/Periodate Treatment

The decreased attachment observed when cells were pretreated with periodate suggested that the receptor-site might consist of sialic acid and/or a glycoprotein. To clarify the role of these constituents, the cells were treated following attachment of T. hyodysenteriae (Table 3). In this assay, periodate had no significant effect; whereas treatment of the cells and spirochetes with neuraminidase significantly reduced the number of attached spirochetes.

Carbohydrate and Glycoprotein Coincubation

The decreased adherence resulting from treatment with protease, neuraminidase, and periodate suggested glycoprotein as the cell-receptor for T. hyodysenteriae. Several glycoproteins and glycoprotein constituents were assayed for competitive inhibition of the spirochetes to the cells (Table 4).

Both coincubation of NANA with cells and spirochetes, and pretreatment of the HEI cells with colominic

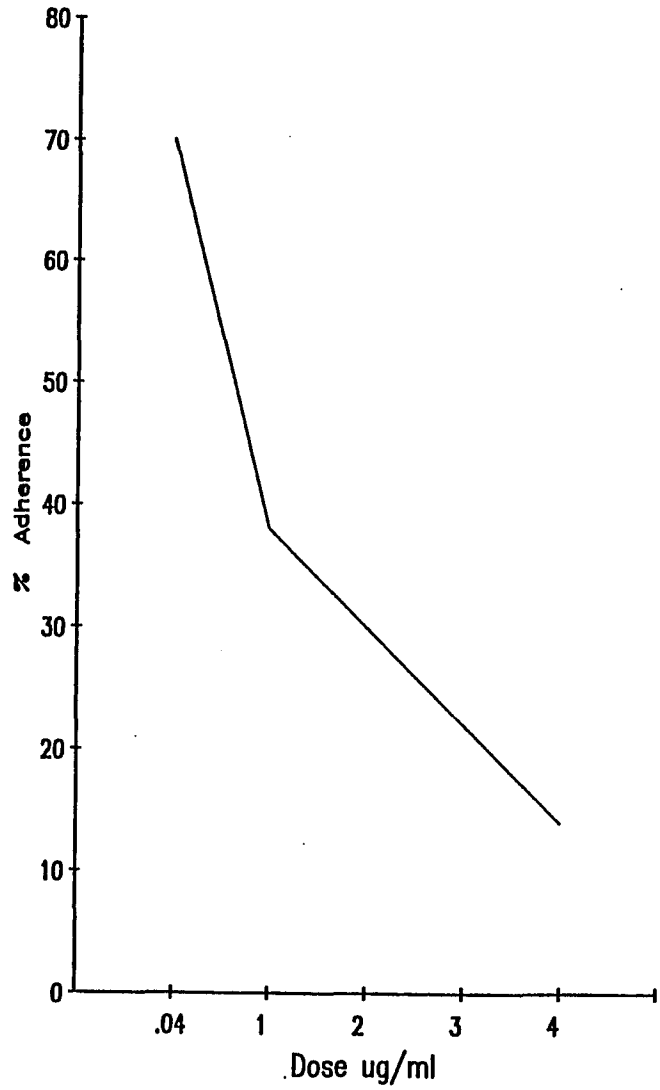

Figure 4. Effect of concentration of periodate pretreatment on attachment of T. hyodysenteriae to HEI cells.

TABLE 4. EFFECTS OF SELECTED SUGARS AND GLYCOPROTEINS ON ADHERENCE OF T. HYODYSENTERIAE TO HEI CELLS

Treatment[a]	Dose (mg/ml)	% Control[b] Adherence	p Value[c]
N-acetylneuraminic acid	15	48.5 (8)	0.001
Colominic acid	15	90.2 (5)	0.025
D-glucuronic acid	10	96.4 (6)	NS
	15	46.8 (6)	0.025
N-acetylglucosamine	15	129.5 (7)	0.025
N-acetylgalactosamine	10	86.7 (6)	NS
	15	72.1 (6)	0.025
Mannan	15	130.3 (8)	0.05
Ovalbumin	5	71.8 (4)	NS
Fetuin	5	62.8 (5)	0.025

[a] Treatments were added to solutions of 1.3 ml MEM and 0.7 ml T. hyodysenteriae isolate B204 at final concentrations indicated; treated cultures were then incubated with HEI cells for 2 hours at 37 C. HEI cells were pretreated with colominic acid dissolved in 2.0 ml MEM for 30 minutes, washed with MEM, and coincubated with spirochetes for 2 hours at 37 C.

[b] % Control Adherence = $\frac{\text{\# spirochetes per treated cell}}{\text{\# spirochetes per control cell}} \times 100$

Numbers in parenthesis indicates the number of trials performed; 30 or 100 cells counted per trial.

[c] Significant p = .05; NS = not significant.

acid (poly-2,8-N-acetylneuraminic acid), significantly inhibited attachment. D-glucuronic acid, while not a glycoprotein constituent, is similar to NANA in that it has a negatively charged carboxyl substituent on the hexose ring. This sugar did not affect attachment at 10 mg/ml, but there was a significant reduction in adherence at 15 mg/ml. GlcNAc and GalNAc are structurally identical except for the orientation of the substituents at C-2; yet their positions in glycoprotein structure are very different. As seen in Table 4, GlcNAc coincubation increased adherence by 30% while GalNAc decreased adherence by nearly 30%.

The glycoproteins examined were mannan, ovalbumin and fetuin. Coincubation of T. hyodysenteriae and cells with mannan resulted in a 30% increase in adherence; ovalbumin had no significant effect; and fetuin inhibited attachment by almost 40%.

Phenol-phase Extracts and Culture Supernatants

Competitive inhibition assays were conducted using phenol-phase extracts and culture supernatants of T. hyodysenteriae. Surface antigens isolated from T. hyodysenteriae by phenol-phase extraction were assayed for their potential involvement in the attachment of spirochetes to HEI cells. Culture supernatants and washings

were assayed to determine if substances involved in the binding of the spirochetes to the cells had become dissociated from the spirochetes during centrifugation. The supernatants and washings were concentrated 60X and 75X respectively, to ensure concentrations sufficient to competitively inhibit attachment. Concentrated cell-free culture media served as a control.

Phenol-phase extracts significantly reduced adherence in both the homologous and heterologous systems (Table 5). 60X concentrated supernatant inhibited attachment by 40%, while neither the 75X washing nor the 60X culture media significantly affected adherence.

Thiobarbituric Acid Assay

Neuraminidase treatments and carbohydrate coincubation assays indicated that sialic acid (NANA) may be involved in the adherence of T. hyodysenteriae to HEI cells. In order to determine the location of NANA in this system, materials present in the adherence assay were examined for their sialic acid content using the thiobarbituric acid assay (Table 6).

Virtually no sialic acid was released from the HEI cells. An average of 11.76 ug/ml NANA was recovered from T. hyodysenteriae in broth culture, compared to 5.36 ug/ml from washed spirochetes. Cell-free culture media (TSB/FBS)

TABLE 5. EFFECTS OF TREPONEMAL EXTRACTS AND CULTURE SUPERNATANTS ON ADHERENCE OF T. HYODYSENTERIAE TO HEI CELLS

Treatment[a]	Isolate	% Control[b] Adherence	p Value[c]
B204 Phenol-phase extract	B204	41.5 (5)	<0.001
	B234	70.4 (4)	0.025
B234 Phenol-phase extract	B204	34.2 (6)	<0.001
	B234	28.6 (6)	0.001
B204 Culture supernatant (60X)	B204	61.5 (7)	0.05
B204 Culture washing (75X)	B204	82.5 (5)	NS
Culture media (60X)	B204	82.2 (6)	NS

[a] Phenol-phase extracts from T. hyodysenteriae; supernatant collected after centrifugation of T. hyodysenteriae isolate B204 (1000 ml flask culture) at 6500rpm for 20 minutes and concentrated 60X on YM10 ultrafiltration unit; washing collected following resuspension of T. hyodysenteriae pellet in PBS and a second centrifugation, and concentrated 75X on YM10 ultrafiltration unit; TSB/FCS cell-free culture media concentrated 60X on YM10 ultrafiltration unit: 0.3 ml of the above solutions were added to 0.7 ml T. hyodysenteriae and 1.0 ml MEM and then coincubated with HEI cells for 2 hours at 37 C.

[b] % Control Adherence = $\frac{\text{\# treated spirochetes per cell}}{\text{\# control spirochetes per cell}} \times 100$

The number in parenthesis indicates the number of trials performed; 100 cells counted per phenol-phase trial; 30 cells counted per supernatant trial.

[c] Significant p = .05; NS = not significant.

TABLE 6. SIALIC ACID CONTENT OF BIOLOGICAL MATERIALS PRESENT IN THE ADHERENCE ASSAYS INVOLVING T. HYODYSENTERIAE AND HEI CELLS

Substrate[a]	Sialic acid[b] g/ml	Preparations (no.)
HEI cells	0.78	5
T. hyodysenteriae broth culture	11.76	5
T. hyodysenteriae (washed cells)	5.36	5
Culture media (TSB/FBS)	6.96	7
T. hyodysenteriae culture supernatant	17.52	2
Culture washing (75X concentrated)	3.40	3
Culture supernatant (60X concentrated)	125.28	6
Culture media (60X concentrated)	27.08	6

[a] HEI cells in growth media of MEM with 10% FBS and 2% antibiotic-antimycotic; T. hyodysenteriae isolate B204 in culture media (TSB with 7% FBS), 24 hour culture; spirochetes washed 3 times in PBS at 6500 rpm for 20 minutes and resuspended in PBS to original volume; trypticase soy broth with 7% fetal bovine serum; supernatant collected following centrifugation at 6500 rpm for 20 minutes to remove spirochetes; washing collected following resuspension of T. hyodysenteriae pellet in PBS and a second centrifugation, and concentrated 75X on YM10 ultrafiltration unit; T. hyodysenteriae culture supernatant concentrated 60X on YM10 ultrafiltraion unit; and TSB/FBS cell-free culture media concentrated 60X.

[b] Sialic acid content determined by the thiobarbituric acid assay. Average values given. ug/ml NANA = 6.48 x A_{549} - 2.35 x A_{532}.

contained 6.96 ug/ml NANA and culture supernatant had 17.52 ug/ml, while the 75X concentrated washings had only 3.40 ug/ml. 125.28 ug/ml NANA was released from the 60X concentrated culture supernatant, compared to 27.08 ug/ml from the 60X concentrated culture media.

The estimated protein content of T. hyodysenteriae was 13.33 mg/ml, and was 12.01 mg/ml for the culture media.

Fluorescent Lectin/Antibody Examination

N-Acetylglucosamine is known to be associated with glycoproteins on the surface of upper villus cells in the intestine (Weiser, 1973). To test for the presence of this carbohydrate on either the HEI cells or T. hyodysenteriae a fluorescent assay was conducted using an FITC-labelled WGA lectin specific for GlcNAc. Since it was not known how WGA would affect adherence of the spirochetes, half of the preparations were treated with WGA prior to coincubation with T. hyodysenteriae, and half were treated after the coincubation. No differences were seen between the two methods. As seen in Figures 5 and 6, GlcNAc is present on the HEI cell, and appears to concentrate in pockets on the cell surface. In Figure 5, RITC-Ab labelled spirochetes are visible, but whether or not they are adhering to WGA/GlcNAc on the cell surface cannot be determined. In Figure 6, the spirochetes were not labelled with RITC-Ab

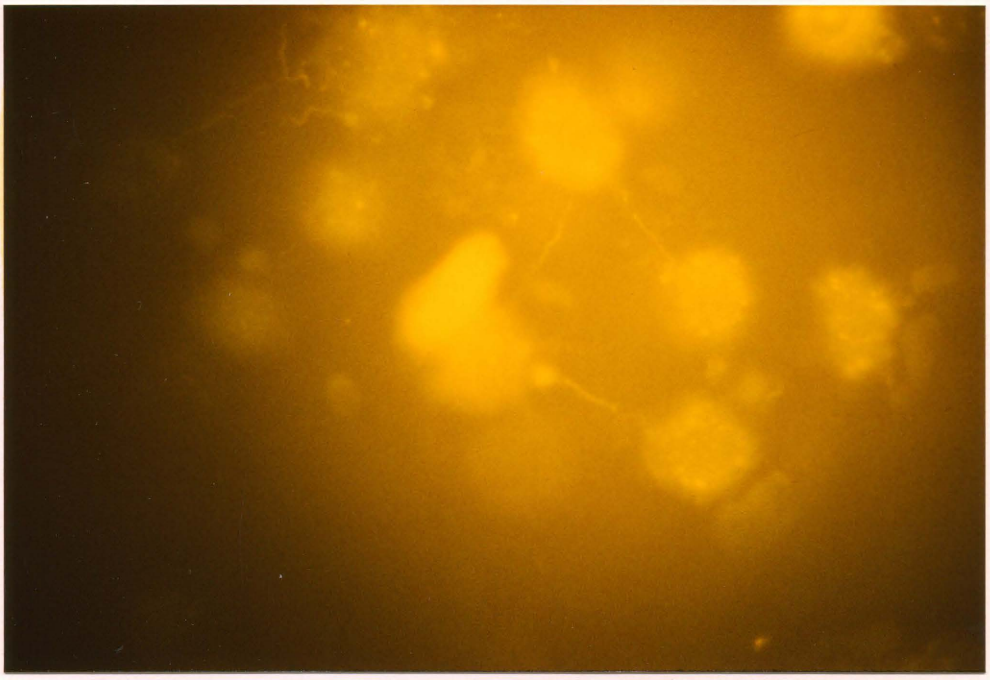

Figure 5. Attachment of RITC-labelled spirochetes to HEI cells labelled with FITC-WGA lectin.

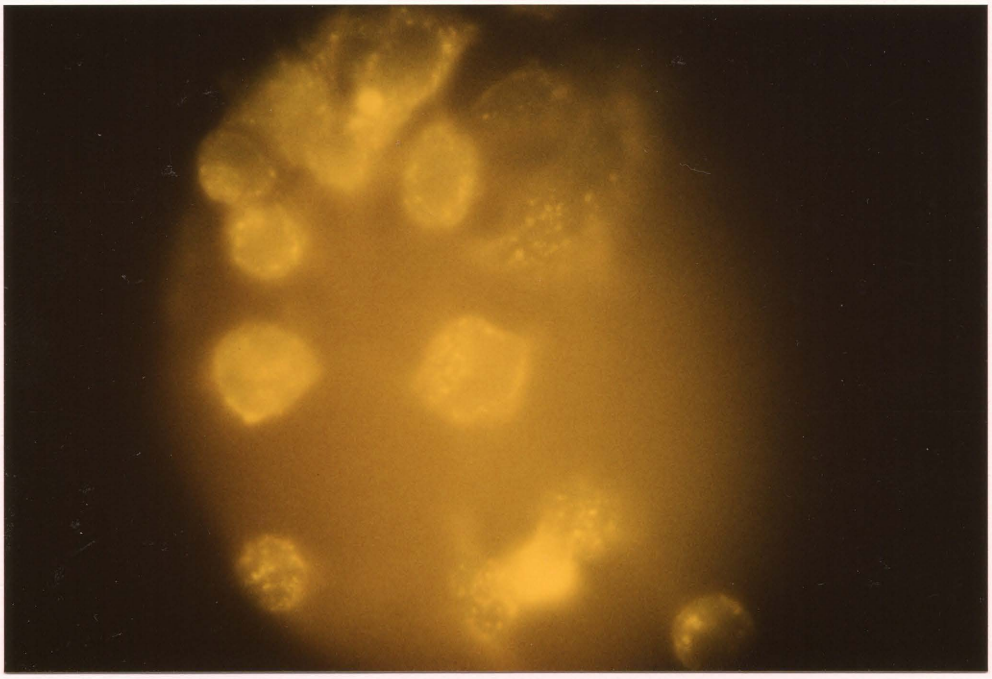

Figure 6. Attachment of unlabelled spirochetes to HEI cells labelled with FITC-WGA lectin.

and cannot be visualized, indicating that WGA did not adsorb to the surface of the spirochetes.

CHAPTER FOUR

DISCUSSION

Characterization of the adherence of bacteria to host epithelial cells can provide information about the pathogenesis of a given micro-organism. Although much attention has been given to the etiology and pathophysiology of swine dysentery, few attempts have been made to define the nature of the adhesion-receptor interaction between T. hyodysenteriae and host cells. Several researchers have laid the groundwork for in vitro studies of the attachment of T. hyodysenteriae to cells. Cultured cell lines were deemed appropriate models for studying attachment: T. hyodysenteriae adhered to cultured mouse adrenal cells at the same rate and frequency as to swine colonic epithelial cells (Knoop et al., 1979); and T. hyodysenteriae readily adhered to a variety of other mammalian cell lines (Wilcock and Olander, 1979). They also have defined the basic parameters of incubation time and temperature, but little else has been studied. We attempted here to

investigate a broader spectrum of interactions between T. hyodysenteriae and a cultured epithelial cell line.

Adherence is directly related to pathogenicity for a number of bacterial species. Only virulent strains of T. pallidum adhere to host cells; nonvirulent isolates do not form attachments (Wong et al., 1983b). K88-positive E. coli adhere to intestinal cells, and a 50% fatality rate is associated with these isolates; K88-negative E. coli do not adhere, and the fatality rate is only 3% (Jones and Rutter, 1972). In contrast with these pathogens, initial studies of nonpathogenic T. hyodysenteriae and T. innocens demonstrated that these adhered to tissue culture cells with the same frequency as pathogenic isolates of T. hyodysenteriae. While we believe that adherence is a prerequisite for colonization and the expression of pathogenicity of T. hyodysenteriae, adherence apparently is not directly responsible for the pathophysiological changes seen in SD.

There are many factors that determine the specific region in which bacteria become established. The native microbial population of a given mucosal niche, available nutrients, pH, and other environmental considerations all determine the eventual locale of a particular pathogen. It was interesting to note in our in vitro assays that adherence was greatest within a pH range 6.0-8.0: the media

in which T. hyodysenteriae is propagated has a pH of about 6.9, and the pH of the colon is generally about 6.7. The only other portions of the gastrointestinal tract where the pH falls within this range are the middle portion of the small intestine, and the cecum (Smith and Jones, 1963).

Spirochetes are particularly well adapted to colonize a niche where mucosal secretions are high, such as in the colon. Their spiral motility aids them in propulsion through the mucus layer to the epithelial cell surface, where they can form specific attachments (Lee, 1980; Mims, 1982; Murray, 1976). T. hyodysenteriae must be viable to attach to cells, and motility appears to augment the rate of adherence. How strong the correlation is between motility, viability, and adherence, however, cannot be determined by our investigations alone. Exposing the spirochetes to high temperatures may have altered their cellular surface as well as killing them. The purpose of treating the spirochetes with formalin was to preserve the integrity of the outer membrane while inactivating the spirochetes. It was necessary to wash the formalin from T. hyodysenteriae before incubation with the HEI cells, however, and it was discovered later that the process of washing and centrifuging the spirochetes disrupted the adhesins and reduced adherence. Although it may be that

the surface adhesins are sensitive to heat, and can be removed by centrifugation, throughout all our experiments we consistently observed that inactivated spirochetes did not adhere, regardless of the source of inactivation. Spirochetes exposed to cold for 24 hours had a lower rate of attachment than controls, and we associated this with the reduced motility that was observed concurrently. Wilcock and Olander (1979) reported a direct relationship between motility and frequency of attachment. It is possible that exposure to cold suppressed the production of metabolically active adhesins rather than having affected adherence by reducing motility. In vivo, motility is an important element in the survival of spirochetes. Here, as well as in the laboratory, motility may serve to increase the probability of random contact with a host cell, or to facilitate an active search of and attachment to host tissue. A definitive assay of the motility of T. hyodysenteriae needs to be explored, one in which spirochetes are treated with chemotactic inhibitors such as valinomycin or nigericin, or with motility inhibitors like potassium cyanide.

One of the mechanisms by which the immune system prevents disease is by inhibiting the attachment of pathogenic bacteria to mucosal surfaces. Secretory IgA

confers local immunity at mucosal surfaces; and while sIgA inactivates viruses, it does not appear to be bactericidal by itself, nor with complement or PMN's. Rather, sIgA seems to prevent the adherence of certain bacteria to epithelial cells (Reed and Williams, 1978; Savage, 1980). Pigs are refractory to reinfection with the same strain of T. hyodysenteriae to which they have been previously exposed. In colonic loop studies, Joens et al. (1983) demonstrated that colonic lesions were not produced in loops rechallanged with the homologous serotype of T. hyodysenteriae. In addition, spirochetes were found only on the luminal surface and not in the crypts, and only 10^3 organisms were isolated per square centimeter. In contrast, colonic loops exposed to heterologous serotypes of T. hyodysenteriae harbored 10^6 organisms/cm^2; and spirochetes were found both on the luminal surface and in the crypts, and lesions were produced. As part of the present study, colonic secretions from pigs recovered from SD were assayed for inhibition of attachment. In our initial survey, these secretions had no significant effect on adherence. Although it is possible that colonic secretions do not protect pigs from reinfection by inhibiting adherence, this may be an erroneous conclusion, for two reasons: first, this would be in contrast with the

ligated-loop studies, which inferred a relationship between immunological protection and inhibition of treponemal adherence; and second, only one concentration of colonic secretions was assayed, and there may not have been sufficient antibody present to effectively inhibit adherence.

Immune sera, on the other hand, significantly inhibited adherence. The function of antibodies produced against surface antigens of pathogens are: to agglutinate and hasten the destruction of micro-organisms; to opsonize pathogens and facilitate their ingestion by macrophages; and/or to inhibit attachment of micro-organisms to host cells. Antisera can prevent adherence in several fashions, e.g. antibodies can arise against specific adhesins on the bacterial surface and prevent adherence by competitive inhibition or stearic hindrance, or antibody can agglutinate the bacteria (Ofek and Beachey, 1980). In our studies, convalescent swine antisera was cross-reactive against strains B204 and B234 in adherence tests. Rabbit anti-B204 was also cross-reactive, but rabbit anti-B234 was specific for strain B234. This interstrain reactivity may indicate that there are immunogenic surface adhesins common to both isolates. These common antigens should be isolated

and assayed for competitive inhibition of adherence, for they may be useful in future vaccine studies.

In both the small and large intestine, a single layer of columnar cells line a basement membrane which overlies the lamina propria. In the small intestine, the epithelium covers villi which protrude finger-like into the lumen. The luminal aspect of epithelial cells in both the small and large intestine are subdivided into tiny hair-like projections called microvilli. The microvilli have a thin coating of glycoproteins and glycolipids, known as the glycocalyx. The glycocalyx is in turn covered by mucus, which is produced by the goblet cells and consists primarily of glycoproteins and acidic mucopolysaccharides (Savage, 1980; Lee, 1980; Weiser, 1973). The individual cells that comprise animal tissue are enclosed within a highly specialized membrane. The inner domain of the membrane harbors the hydrophobic compounds such as lipids and proteins, while the hydrophilic carbohydrates are located externally (Jones, 1977).

Proteins are retained within the hydrophobic domain, and although essential for the integrity and survival of eucaryotic cells, they rarely are directly involved with bacterial interactions (Jones, 1977). In contrast, glycoproteins, which consist of carbohydrate

moieties linked to a polypeptide backbone, are intimately involved with the attachment of bacteria to host cells (Kornfeld and Kornfeld, 1976; Weiser, 1973; Sharon, 1984b). The enzyme protease cleaves nonspecific peptide bonds in proteins and glycoproteins. In our studies, protease pretreatment of HEI cells reduced adherence of the spirochetes. This may reflect a generalized disruption of cell membrane integrity, or may indicate a protein-conjugated substance such as glycoprotein as the cell receptor for T. hyodysenteriae.

The lipid bilayer of eucaryotic cells is largely comprised of phospholipids (Loh and Law, 1980). Phospholipid polar head groups (inositol, serine, ethanolamine, and choline residues) interact with the external, water phase, while the apolar hydrocarbon chains form the internal domain. There is also a particular distribution of phospholipids within the lipid bilayer, wherein phosphatidylcholine tends to be located on the external surface of membranes, and phosphatidylserine and phosphatidylethanolamine on the inner surface. External hydrophilic regions of the lipid bilayer often serve as receptor sites for biochemical interactions involving hormones, toxins, and other cellular signals (Loh and Law, 1980), and as sites for bacterial adherence (Jones, 1977). Phospholipase

C is an enzyme that cleaves the polar head group from phospholipids. The dose-dependent reduction in adherence of T. hyodysenteriae that occurred with phospholipase C pretreatment of HEI cells may signify several things. One implication is that the epithelial cell receptor for T. hyodysenteriae may be a phospholipid. Another possibility that should be considered for this, and other pretreatments, is that the enzyme may not have been completely washed from the cells prior to addition of the spirochetes. In this case, phospholipids may actually be associated with the bacterial, rather than the cellular, ligand. The major phospholipid present in the genus Treponema is phosphatidylcholine (Johnson, 1976). As mentioned earlier, this compound is located externally and thus is susceptible to phospholipase degradation. A third possibility, another which pertains to all assays involving cell surface modification, is that the reagent may destroy not only the receptor, but may alter the entire cell surface (Jones, 1977).

Competitive inhibition assays using phospholipids were not successful. Sphingomyelin, phosphatidylcholine, cerebroside, and ceramide were insoluble in the water-based media used in all assays. The phospholipids were miscible in 1% acetate and in 10% DMSO. To ensure that the

solvents themselves did not interfere with adherence, controls were run in which cells and T. hyodysenteriae were coincubated with 1% acetate or 10% DMSO. Unfortunately, acetate tended to increase adherence, and DMSO decreased adherence. As such, nothing definitive could be stated regarding the role of phospholipids in adherence.

Sodium-M-periodate oxidizes α-glycols on carbohydrates, cleaving the bond between adjacent carbons possessing hydroxyl or carbonyl groups. Examples of sugars susceptible to periodate degradation include NANA, mannose, galactose, GlcNAc, and GalNAc. When a glycoprotein is oxidized by periodate, not only are terminal residues degraded, but internal sugars may be oxidized as well. When susceptible internal sugars are present, the entire sugar chain external to the internally located one will be released by periodate (Neufield and Ginsburg, 1966). In our assays, pretreatment of HEI cells with periodate significantly reduced adherence of T. hyodysenteriae while post-attachment treatment did not alter adherence. One explanation for this disparity is that the structure of the alleged glycoprotein receptor is such that the peripheral, but not internal, sugar moieties are sensitive to periodate oxidation. Another possibility is that once spirochetes become attached to the HEI cells, periodate-sensitive

residues are stereochemically concealed by treponemal adhesins and are no longer accessible for periodate oxidation.

Neuraminidase cleaves terminal sialic acid residues from glycoproteins and other compounds. N-acetylneuraminic acid, N-glycolylneuraminic acid, and colominic acid are the most common sialic acids present in both microorganisms and higher animals. NANA in particular is an important constituent of animal tissues, and is found in red blood cells, mucins, connective tissue, immunoglobulins, and colonic epithelial tissue (Gottschalk, 1960; Oegema and Cooper, 1983; Kornfeld and Kornfeld, 1976). Sialic acid has also been isolated from bacterial products such as hemolysin, LPS, and capsular material; in addition, there are species of bacteria that possess lectins specific for NANA (Sharon, 1984b). Attachment of T. hyodysenteriae to HEI cells was reduced following coincubation of cells and spirochetes with NANA, suggesting the involvement of sialic acid in adherence of spirochetes. Furthermore, the following observations indicate that sialic acid may be associated with the adhesin on T. hyodysenteriae rather than the cell receptor: post-attachment neuraminidase treatment significantly decreased adherence while pretreatment of cells had no effect; pretreatment of cells with

colominic acid decreased adherence; and NANA was isolated from T. hyodysenteriae but not from HEI cells in the TBA assay.

Intestinal epithelial cells, ground-substance (intercellular "cement"), and the glycocalyx coating the microvilli, are rich in polysaccharides and glycoproteins (Lee, 1980; Weiser, 1973). Intestinal cell-surface constituents vary depending on the location in the intestinal tract (Lee, 1980), and along the gradient of differentiation from crypt to villus (Weiser, 1973). These factors contribute to the specific localization of bacteria in the intestinal tract. T. hyodysenteriae preferentially colonizes the large intestine, and migrates into the mucopolysaccharide-abundant crypts. Since T. pallidum was shown to bind to the acidic mucopolysaccharides chondroitin sulfate and hyaluronic acid (Fitzgerald et al., 1979), we attempted to assay hyaluronic acid and porcine stomach mucin for competitive inhibition of attachment. However, we encountered problems similar to those we had had with the phosphlipids. Sodium hydroxide was required to dissolve the mucopolysaccharides, and the resultant alkalinity was sufficient to reduce adherence. In addition, hyaluronic acid and mucin tended to form globules in which the spirochetes agglutinated. Although these

micelles did adsorb to the HEI cell surface, it was not possible to quantify the attachment. Nonetheless, this event may warrant further investigation, for there are intestinal bacteria that colonize the mucus layer without ever forming attachments with the epithelial surface.

Glycoproteins are involved in a variety of important biological activities, not the least of which is the process of intercellular recognition. Included in this category is the function of the glycoprotein as cell receptor for bacteria (Sharon, 1984a; 1984b). The great diversity of glycoprotein capabilities is due to its structure and its location in the cell. The protein portion of the compound remains imbedded in the cell membrane, while the carbohydrate substituents extend outside of the cell membrane and thus are available for interaction with other cells or substances. The extensive branching of polysaccharide side-chains and the 3-dimensional configuration of the carbohydrate moiety allow for great heterogeneity and stereospecificity (Sharon, 1984a; Harper, 1977). The most prevalent hexoses found in glycoproteins are mannose, galactose, fucose, acetylglucosamine, and acetylgalactosamine; sialic acid is also a common constituent. Fucose and sialic acid always occupy peripheral positions, while acetylglucosamine and galactose

usually are located near the protein (Harper, 1977). The linkage of the carbohydrate moiety to the polypeptide chain follows an established pattern: GlcNAc is always linked N-glycosidically to the amide nitrogen of asperagine; GalNAc (or fucose, xylose, mannose, or galactose) forms an O-glycosidic linkage to the hydroxyl group of serine or threonine (Kornfeld and Kornfeld, 1976). In our competitive inhibition studies of sugars and glycoproteins, GlcNAc and GalNAc had opposite effects on adherence: GlcNAc caused an increase in attachment, while GalNAc decreased attachment. The exact role of either of these sugars in adherence cannot be deduced from this work; however, the results do suggest a highly stereospecific recognition of receptor analogs by the spirochetes. Mannose has been implicated as a receptor for certain strains of E. coli and other enterobacteria. Our initial survey of the effect of this sugar on the adherence of T. hyodysenteriae produced no significant results, and was not included in this report. Coincubation with mannan, a yeast-derived glycoprotein comprised of straight-chained polymannose units linked to serine, enhanced attachment of T. hyodysenteriae to cells. Ovalbumin, consisting of repeated units of mannose linked by GlcNAc to asparagine, had no effect on adherence. Ovalbumin is a bulkier compound than mannan in that its

mannose units form three branches rather than a simple linear configuration, and perhaps for this reason was unable to interact with the spirochetes and cells. Fetuin, a component of fetal bovine serum, is a doubly sialated glycoprotein in which GalNAc forms the linkage to serine or threonine. Fetuin inhibited adherence of T. hyodysenteriae to cells, which further supported the involvement of sialic acid in attachment.

There is another consideration that ought to be kept in mind when evaluating the effects of sialic acid on adherence, and that is the involvement of charge interactions. The surfaces of most bacteria and interfaces are negatively charged, and mechanisms have evolved to overcome like-charge repulsion. Bridges of extracellular polymers, and/or lectin-carbohydrate interactions often serve to adsorb bacteria to host cells (Lee, 1980). Sialic acid is a negatively-charged compound. Coincubation of neuraminic acid with spirochetes and cells may result in decreased adherence because of an increase in static repulsion. D-glucuronic acid is also a negatively-charged sugar, and it, too, inhibited adherence. These results may support a charge-interaction theory; on the other hand there is substantial evidence indicating a sialic acid treponemal adhesin. In addition, glucuronic acid is a primary sugar

constituent of mucopolysaccharides and may have competitively inhibited attachment.

The increase in adherence following coincubation of cells and T. hyodysenteriae with GlcNAc or mannan may or may not have been biologically significant. WGA lectin established the presence of GlcNAc on the HEI cell surface; carbohydrate side-chains are synthesized on the membrane itself (Harper, 1977); and villus cells have a high rate of incorporation of D-glucosamine into surface glycoproteins (Weiser, 1973). One hypothesis is that GlcNAc acts as a cell receptor for T. hyodysenteriae: when GlcNAc is added to the system it becomes adsorbed to the HEI cells, providing additional receptor sites for the spirochetes. Another possibility is that the presence of GlcNAc or mannan serves to stabilize the adherence of spirochetes to the cells. Biochemical stabilization would allow firm attachments to occur more frequently, and would result in a net increase in adherence.

Isolating and characterizing cellular components, and investigating the ability of individual constituents to inhibit attachment, is the most efficacious method to determine the exact chemical composition of bacterial adhesins and cell receptors. K88 fimbrial adhesin of certain E. coli strains adheres to host cells, and anti-K88

serum inhibits the adherence of both K88 antigen and K88-positive E. coli (Gaastra and de Graaf, 1982). Antibody to mannose-sensitive pili of E. coli also inhibits adherence (Weinstein and Silverblatt, 1983). T. pallidum has been shown to adhere to fibronectin, and anti-fibronectin serum blocked this attachment. In addition, three proteins isolated from the outer envelope of T. pallidum were implicated as potential ligands, and selectively bound to fibronectin on a fibronectin-Sepharose column (Peterson et al., 1983). Direct relationships between distinct bacterial adhesins and cell receptors, and the action of blocking antibodies, serve not only to elucidate the pathogenesis of diseases, but provide information crucial to developing anti-bacterial vaccines. Lipoprotein surface antigens that are unique to T. hyodysenteriae were extracted by a hot phenol-water method (Baum and Joens, 1979a). Phenol-phase antigens from two serotypes of T. hyodysenteriae inhibited attachment of spirochetes of the same serotype, as well as the heterologous serotype. In immunodiffusion assays, the strongest reactions were between phenol antigens and antisera of the same serotype of T. hyodysenteriae, but weak interactions were observed between heterologous serotypes, too. As with the convalescent swine antisera, these cross-reactive antigens may

contain substances involved as binding ligands. To determine if this is indeed the case, antisera raised against phenol extracts should be assayed for inhibition of adherence, and cell-free phenol-phase antigens should be labelled and examined for attachment to host cells.

During the initial studies with enzymes, T. hyodysenteriae was pretreated as well as the cells. Treated spirochetes were washed and centrifuged to remove enzymes; untreated spirochetes were washed and centrifuged and used as one set of controls; and untreated, unwashed spirochetes served as another control. This experimental model allowed for direct comparison of washed and unwashed T. hyodysenteriae, although this was not our original intention. The washed spirochetes retained their motility, and had titers equal to, and often greater than, that of unwashed spirochetes; however, we discovered that washed spirochetes adhered to cells at about half the frequency of unwashed spirochetes. Adding 10% FBS to the PBS in which T. hyodysenteriae was washed somewhat enhanced attachment, but did not restore it to the level of the unwashed spirochetes. We deduced that treponemal adhesins, or constituents associated with adherence, became disassociated from the outer membrane during centrifugation. Adding supernatant to washed spirochetes (0.3 ml to 0.7 ml

T. hyodysenteriae in 1.0 ml MEM) did not restore adherence capabilities, nor inhibit the attachment of unwashed spirochetes. 60X concentrated supernatant, on the other hand, did significantly inhibit adherence of intact spirochetes. Spirochete-free culture media was concentrated 60X and assayed concurrently to eliminate the chance that concentrated media constituents were inhibiting attachment. Since T. hyodysenteriae was usually washed and centrifuged 2 or 3 times to remove enzymes, the supernatant was decanted and assayed after the second centrifugation (termed "PBS washing" here). Concentrated 75X, these PBS washings had no effect on adherence, leading us to the conclusion that the adhesins were displaced primarily in the first centrifugation.

Having postulated that sialic acid was involved as the ligand on T. hyodysenteriae we measured the sialic acid content of HEI cells, T. hyodysenteriae, media, supernatants, and washings. In addition, the protein content of the culture media and of T. hyodysenteriae in broth culture was ascertained. Culture media contained 12.01 mg/ml protein, and T. hyodysenteriae 13.33 mg/ml; the corresponding sialic acid contents were 6.96 and 11.76 ug/ml, respectively. Thus the spirochetes contained 51% more NANA per milligram of protein than the culture media. Washed

spirochetes "lost" nearly 50% of their detectable NANA, which coincides with the reduction in attachment seen after washing the spirochetes. As much sialic acid was detected in the culture supernatant as was in both T. hyodysenteriae and the culture media combined. Supernatant may have a higher concentration of free NANA because sialic acid adhesins are released into the broth during centrifugation. 75X concentrated washings had relatively little sialic acid, which may correspond to the lack of competitive inhibition seen with this substance. At 125 ug/ml, the 60X concentrated supernatant had the largest amount of NANA detected: four times that of the 60X concentrated media, and eight times that of T. hyodysenteriae. The parallels observed between sialic acid content and adherence of the spirochetes are noteworthy, and further support the conjecture that sialic acid is the adhesin on the spirochete.

A number of parameters have been established here, and many new questions raised concerning the adherence of T. hyodysenteriae to HEI cells. Based on the information gleaned from our experiments, several avenues of investigation have appeared that may lead to expedient methods of preventing or treating SD. Administration of antibiotics or other substances that would interfere with motility of

the spirochetes, or that would inhibit production of surface adhesins, may prevent adherence and thus disease. Application of large doses of receptor analogs have been shown to inhibit attachment (Beachey, 1981). Caution must be exercised when using this method, however, because attachment of the pathogen to phagocytic cells may also be inhibited, having deleterious consequences for the host. Isolation of binding ligands on the spirochete that are both immunogenic and unique (i.e. not found in host tissue) could be applied in a vaccination regime. Given that phenol-phase antigens and antisera inhibited attachment of T. hyodysenteriae, and were effective across serotypes, there is promise for the development of an efficacious vaccine program.

Ultimately, the significance and relevance of any in vitro experimentation cannot be assessed until it is evaluated in the living host. No matter how profound laboratory results may be, the laboratory serves only as a simplified, controlled model of the living system. Once applied to the living system, "significant" results may no longer have meaning. In order to thoroughly investigate a vastly complicated interaction such as that between a pathogen and host, one must, of necessity, simplify, and isolate, and examine individual components. One often

loses sight of the whole picture, however, after concentrating for long periods on a single component, and herein lies the problem so often encountered in research of any nature. Once the scientist has discerned the underlying patterns and functions on an elementary level, he must refrain from proclaiming at once that he has discovered the modus operandi of that which he has explored. Instead, he must first "reconstruct" the living organism, carefully observing the interrelationships between each of the pieces he has examined, and their relation to the organism as a whole. Only by tempering the knowledge gained in the laboratory with the interactions observed in vivo, can true progress be made toward attaining a beneficial end.

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