Immunobiology of Treponema hyodysenteriae

outer membrane components

by

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GENERAL INTRODUCTION

Swine dysentery is a disease of swine characterized by mucohemorrhagic diarrhea, that can result in dehydration and death (10). Most countries with modern swine production report the occurrence of swine dysentery (60). The etiologic agent of the disease was shown to be an anaerobic spirochete given the name Treponema hyodysenteriae (27,96). The organism has been extensively studied and yet swine dysentery remains a serious health problem for the pork industry. Affected, untreated herds may experience up to 90% morbidity and 25% mortality (24).

Resistance to swine dysentery has been shown to develop in pigs experimentally infected with T. hyodysenteriae and allowed to fully recover (23,39,72). However, the mechanism of acquired resistance was not determined. A specific IgG response to the organism has been detected in ligated colonic loops from convalescent pigs (41) however, the mechanism of resistance to swine dysentery in the intact large intestine of the pig has not been examined.

A non-pathogenic anaerobic spirochete is often present in the large intestine of swine and has been named Treponema innocens (49). The presence of this organism in the normal swine colon can complicate the

identification of T. hyodysenteriae. A simple method of differentiating the two species is needed.

The present investigation focused on three areas. These were 1.) an examination of the outer membrane proteins of T. hyodysenteriae, 2.) investigations of the mechanism of resistance to infection by T. hyodysenteriae in convalescent pigs and 3.) evaluation of monoclonal antibodies to protect mice from infection with T. hyodysenteriae.

Explanation of dissertation format The alternative format has been used for this dissertation, which includes three manuscripts. Two of the manuscripts were written in the style of Infection and Immunity. The third manuscript was written in the style of The American Journal of Veterinary Research. The first manuscript is entitled "Analysis of Outer Membrane Components of Treponema hyodysenteriae and Treponema innocens". The second manuscript is entitled "The Immune Response of Swine to Antigens of Treponema hyodysenteriae". The third manuscript is entitled "Serotype Specific Protection of Mice from Infection with Treponema hyodysenteriae with Monoclonal Antibody". This dissertation also contains three appendices. The first appendix is entitled "Monoclonal Antibodies Reactive to Treponema hyodysenteriae and Treponema innocens". The

second appendix is entitled "APIZYM Reactions of Treponema hyodysenteriae and Treponema innocens Isolates". The third appendix is entitled "Investigation of Age-related Response of Pigs to Antigens of Treponema hyodysenteriae."

A literature review precedes the first manuscript and a general summary follows the last manuscript. Literature cited in the general introduction, literature review and general summary are cited in a literature section following the general summary. Literature cited in a manuscript follows the manuscript.

LITERATURE REVIEW

Background characteristics of Treponema

hyodysenteriae The disease swine dysentery was first described by Whiting et al. in 1921 (103). In 1944 Doyle (9) suggested that the causative agent was Vibrio coli, however, as reviewed by Harris and Glock (25), subsequent investigators failed to reproduce the disease with this organism. Research focused on anaerobic spirochetes as the causative agent of the disease after Terpstra et al. (98), described spirochetes in the intestines of pigs suffering from swine dysentery. Taylor and Alexander (96) and Harris et al. (27) isolated the organism which was named Treponema hyodysenteriae. This spirochete was described to be 6 - 8.5 um long, 0.32 - 0.38 um wide, with 7 - 9 axial fibrils attached at each end of the organism which overlap near the middle of the protoplasmic cylinder (50). When incubated anaerobically on blood agar media, these spirochetes produced zones of strong beta-hemolysis. Similar organisms, which were not pathogenic for pigs were reported by Taylor and Alexander (96). These organisms also produced less complete beta hemolysis on blood agar than the pathogenic isolate. Subsequently Kinyon et al. (51) found weakly

beta-hemolytic spirochetes in 334 pigs from five herds, representing 20-40% of the total number of pigs tested. Thirteen of these weakly beta-hemolytic isolates were administered to 2-week old specific pathogen free (SPF) pigs. All 13 isolates were found to be non-pathogenic (50). In 1979, Kinyon and Harris described these non-pathogenic spirochetes as a new species, Treponema innocens (49). This species was found to be morphologically similar to and serologically cross-reactive with T. hyodysenteriae.

The main criterion for differentiating between the two species has been the hemolytic properties of the organisms on blood agar. Other criteria have been described including: indole production, enteropathogenicity in mice, enteropathogenicity in ligated colonic loops of swine or rabbits, and reactions in the APIZYM system (described in this work). T. hyodysenteriae strains were found to produce indole while T. innocens strains did not (48). Pathogenicity tests to differentiate these two species requires identification of gross or microscopic evidence of cecal lesions at postmortem (45). Enteropathogenicity in ligated colonic loops of the rabbit (52) or swine (101) caused an accumulation of fluid, gross hemorrhagic lesions in the intestinal mucosa and the presence of spirochetes in the

lesions which could be identified by darkfield microscopy.

The APIZYM system is a procedure which may be used to differentiate T. hyodysenteriae from T. innocens on the basis of enzymes possessed by the organisms. Hunter and Wood (36) were the first to use this system and found a number of differences between pathogenic and non-pathogenic spirochetes isolated in Great Britain. Recently the APIZYM system was used to evaluate enzyme differences between a number of strains of T. hyodysenteriae and T. innocens (see Appendix 2). Pathogenic strains consistently lacked an alpha galactosidase which was present in T. innocens.

Serotypes of T. hyodysenteriae Gram-negative bacteria, particularly Salmonella sp., have been serotyped on the basis of the antigenic diversity of their lipopolysaccharide (LPS). Lipopolysaccharide is found only in the outer membrane of Gram-negative bacteria. With Salmonella sp. the serotype specific antigens were found in the O-polysaccharide portion of the LPS molecule (69).

This method of serotyping was used to evaluate serologic differences between T. hyodysenteriae strains (4). Hot phenol/water extraction was used to isolate a

water-soluble antigen from pathogenic T. hyodysenteriae strains. These antigens were used in agar gel diffusion tests with antisera produced in rabbits against whole cells. Four serotypes of T. hyodysenteriae were described. In 1985, Mapother and Joens (64) proposed three new serotypes of T. hyodysenteriae. Although the authors noted some cross-reactivity with hyperimmune rabbit sera, specific precipitation reactions could be observed. Lemcke and Bew (56) have also described three new serotypes of T. hyodysenteriae based on agglutination studies with hyperimmune rabbit serum. It is unknown if these new serotypes described by Lemcke and Bew (56) are the same as those described by Mapother and Joens (64). There is a need for a reliable test that would allow for serotyping T. hyodysenteriae isolates from around the world.

Different colony types of T. hyodysenteriae have been described. The five colony types observed were: (1) Growth beneath the surface of the agar, (2) colorless, transparent, circular, flat colonies with strong swarming activity, and with a diameter of 1 - 2 mm, (3) colorless, transparent, circular, convex colonies with entire edges and some swarming activity and a diameter of 1 mm, (4) colorless, transparent, circular convex pin-point colonies with entire edges and a diameter of 0.2 - 0.5

mm, (5) colorless, transparent, irregular colonies with slightly raised margins, entire edges and a diameter of 1-3 mm. Colony types were evaluated after incubation for four days at 41.5 C in an atmosphere of 50% CO₂ and 50% hydrogen on trypticase soy agar with 5% calf blood, 400 ug/ml spectinomycin and 5 ug/ml polymyxin (30). Others have described two biotypes of *T. hyodysenteriae*. Both biotypes are pathogenic. Biotype 1 organisms are beta-hemolytic, produce indole, do not ferment fructose and cause a mucohemorrhagic diarrhea. The biotype 2 organisms are weakly beta-hemolytic, have variable indole production, and fructose fermentation and cause a grey-green diarrhea (6).

Resistance to swine dysentery Resistance to swine dysentery in convalescent pigs after several exposures to infected feces was first reported by Olson in 1974 (72). Joens et al. (39) reported immunity to challenge with pure cultures of *T. hyodysenteriae* in pigs that had recovered from swine dysentery. These had been previously infected with the same isolate 4 to 17 weeks earlier. Of 29 convalescent pigs in this study, 13 developed diarrhea but only 2 developed dysentery. However, 23 of 28 controls developed dysentery, 10 of which died. Using a microagglutination test with whole

cells of T. hyodysenteriae as the antigen, these authors showed that the convalescent pigs had seroconverted after initial exposure and maintained a high serum antibody titer for up to 8 weeks. Only 6 of 29 convalescent pigs were found to shed the organism but shedding was detected for up to 25 days after initial exposure. The authors concluded that a low level of T. hyodysenteriae in the large intestine of recovered pigs stimulates the immune system and provides the pig with adequate protection against rechallenge with T. hyodysenteriae. An anamnestic serum antibody response was demonstrated in previously challenged, convalescent pigs upon rechallenge.

It has been reported that treatment of acutely ill animals with antibiotics decreases the amount of antigen available to induce protective immunity (23,37). However, repeated treatment of pigs, exhibiting clinical signs of swine dysentery, with the antibiotic tiamulin for 2 to 10 days followed by drug withdrawal resulted in the swine becoming immune to a subsequent challenge (73). It was hypothesized that drug withdrawal permitted the reoccurrence of diarrhea that was necessary to stimulate immunity. If the pigs recover naturally without antibiotics, a protective immune response can be maintained. However, if the animals are stressed by poor

management, disease, or transportation, clinical symptoms may reappear.

In 1983, Joens et al. (46) reported that immunity was serotype specific in ligated colonic loops of pigs convalescent for 35-50 days after experimental infection with T. hyodysenteriae. By inoculating colonic loops of these pigs with the homologous or heterologous T. hyodysenteriae serotype, less pathology was demonstrated in loops which received the homologous serotype. Upon necropsy no organisms were found in the crypts of these loops and fewer T. hyodysenteriae were observed on the mucosal surface of these loops than in loops challenged with a heterologous serotype of T. hyodysenteriae. Serum from these pigs did not protect mice or ligated colonic loops of swine when they were challenged with heterologous T. hyodysenteriae serotypes. In conjunction with complement, serotype specific serum antibody inhibited in vitro growth of T. hyodysenteriae, which was noted as a decrease in the colony forming units/ml, by several orders of magnitude (46). Nuessen and Joens (71) reported that antisera from convalescent pigs was opsonic for treponemes of the homologous serotype. In addition, Taylor and Stevenson (94) have shown that serotype specific serum antibody will prevent the

colonization of mucosal explants by the homologous organism.

In contrast, it has been reported that pigs that had recovered from swine dysentery caused by one serotype exhibited some immunity to infection after challenge with a heterologous serotype (11). This observation was substantiated by Joens et al. (42) who showed passive protection in swine colonic loops with heterologous antibody and complement. An immune response to treponemal lipopolysaccharide, which is the serotype antigen in T. hyodysenteriae strains, is therefore important in protection against swine dysentery. However, LPS may not be the only bacterial product inducing a protective immune response.

Vaccination to establish immunity A common method of immunization of animals against bacteria induced diseases is by the administration of formalized bacterins. These killed, whole cell vaccines are often mixed with adjuvants and given intramuscularly or subcutaneously. Unique antigen preparations and routes of administration have been used in the effort to protect pigs from swine dysentery. Hudson et al. (32) attempted to protect 8 pigs by administering an oral vaccine on 3 successive days using an attenuated strain of T.

hyodysenteriae (strain A1). However, no protection was demonstrated in the vaccinates following challenge with a virulent strain. However, Glock et al. (20) reported acquired resistance to swine dysentery after parenteral vaccination of pigs with a formalin-killed suspension of pathogenic T. hyodysenteriae 6 times, every 6 days, over a period of 36 days. Dysentery was observed in only one of eight vaccinated pigs and none died. Three of the eight control pigs died of complications associated with swine dysentery. These studies demonstrated that immunization of swine could eliminate mortality caused by T. hyodysenteriae and greatly reduce the associated morbidity.

Lysons et al. (62) attempted to improve the protection to T. hyodysenteriae induced through vaccination by combining oral and parenteral vaccinations. Pigs were primed with an intra-muscular (i.m.) injection of formalin killed T. hyodysenteriae strain P18A suspended in adjuvant (Marcol 52/Arlacel A). This injection was followed by 3 oral doses of live avirulent T. hyodysenteriae strain VS1. A second i.m. injection of killed P18A was given 3 weeks after the first. This regime resulted in protection of 83% of the pigs from clinical swine dysentery after challenge with P18A as compared with 41% protection provided by two i.m.

injections alone. Their hypothesis was that the colonization of the colon/cecum was necessary to induce sufficient stimulation of the immune response to produce protection. Therefore, colonization with the live avirulent T. hyodysenteriae may mimic the natural course of the infection and induce protection similar to that observed in convalescent pigs.

A subunit vaccine has been used experimentally in an attempt to protect pigs from swine dysentery. Peterson (77) immunized pigs with subcellular material derived from the outer envelope of T. hyodysenteriae. These outer membrane fractions failed to protect pigs against subsequent challenge with T. hyodysenteriae. However, one group of pigs immunized intraperitoneally (i.p.) with an immunoprecipitate of T. hyodysenteriae cell wall proteins and rabbit anti-T. hyodysenteriae antibodies had a greater weight gain than controls. There is no effective vaccine for the prevention of swine dysentery even though there has been much effort devoted to develop one. A better understanding of the immune response of pigs to T. hyodysenteriae infection would aid in the incorporation of important virulence attributes of the organism into an effective vaccine.

Passive immunity to swine dysentery has been reported in pigs. For example, Genho et al. (15)

reported limited immunity to swine dysentery in pigs injected i.m. with precipitated globulins from convalescent swine. Schwartz and Glock (89) also reported passive resistance in swine which were given injections of serum from convalescent, hyperimmunized pigs. Swine receiving immune serum were observed to have a delay in the onset of clinical signs (14.6 days) following experimental infection in comparison to the appearance of signs in swine receiving non-immune serum (3.4 days). The usual incubation period for the disease, before the development of clinical signs, is 10 to 14 days (26) which indicates the immune serum did not have a clearing effect on the organism. These authors also demonstrated that immune serum to a heterologous strain of T. hyodysenteriae failed to protect pigs from challenge.

Peterson (77) reported that 3 of 5 sows convalescent after swine dysentery, provided lactogenic protection to nursing piglets. The sows with protected piglets had higher titers of IgA in their milk to various outer envelope fractions of T. hyodysenteriae than did the milk from sows whose piglets developed the disease. It was not established what antibodies were important in this passive protection. In addition, the piglets became susceptible to T. hyodysenteriae infection after weaning

upon challenge. This indicated that the piglets had not developed protective immunity even though they had been previously challenged with the organism which was apparently cleared by the lactogenic antibody. This would support the hypothesis of Lysons et al. (62), that sufficient replication of the organism must take place in the colon in order to induce protective immunity to antigens of T. hyodysenteriae.

Diagnostic procedures for detecting T.

hyodysenteriae The diagnosis of swine dysentery requires isolation and identification of T. hyodysenteriae from feces, colonic lesions, or colonic mucosa, along with an evaluation of clinical signs. The clinical signs associated with swine dysentery include mucohemorrhagic diarrhea, depression and dehydration with chronically affected pigs becoming emaciated (23). T. hyodysenteriae is identified by strong beta-hemolysis on blood agar. Isolation is difficult due to overgrowth of other colonic bacteria and the presence of T. innocens which produces weak beta-hemolysis on blood agar.

Songer et al. (93) reported the use of a selective blood agar media which contained 400 ug/ml of spectinomycin. This method has been changed by the addition of other antibiotics, to reduce the growth of

other intestinal organisms, or yeast derived RNA, to enhance growth and hemolysis (57). Recently, Kunkle and Kinyon (53) added five antibiotics to blood agar medium for the primary isolation of T. hyodysenteriae. This medium enhanced the recovery of T. hyodysenteriae from intestinal contents by greatly inhibiting the growth of the other organisms while not affecting the growth of T. hyodysenteriae (93).

Numerous tests have been developed to detect T. hyodysenteriae in tissue or rectal swabs, or to detect serum antibody to T. hyodysenteriae antigens. In 1968, Terpstra et al. (98) used fluorescein tagged antiserum from convalescent swine to detect spirochetes in colonic lesions. In 1977, Hunter and Saunders (35) showed that the fluorescent antibody test was false 10% of the time if the serum was not absorbed with non-pathogenic treponemes.

Lemcke and Burrows (58) developed a growth inhibition test for differentiating T. hyodysenteriae strains from other intestinal spirochetes. Filter paper discs impregnated with specific antiserum to T. hyodysenteriae were placed onto agar plates containing various isolates of intestinal spirochetes. A zone of no growth around the disc indicated inhibition of T. hyodysenteriae growth and therefore indicated that the

original isolate streaked on these plates were T. hyodysenteriae. The mechanism of this growth inhibition is independent of complement but must involve surface antigens.

Finally, Taylor and Stevenson (95) have developed an enzyme-linked immunosorbant assay (ELISA) for detection of T. hyodysenteriae in pig feces and lagoon sewage using rabbit anti-T. hyodysenteriae IgG absorbed with T. innocens cells. This IgG was coated onto 96 well microtiter plates to which samples of feces or lagoon slurry were added and incubated. They reported this test was accurate in the detection of low numbers of the organism in feces and slurry (100 colony forming units/gm wet weight) and was more sensitive than culturing the organism on blood agar plates.

A reliable serological test to detect T. hyodysenteriae from suspected fecal samples would be beneficial in the routine diagnosis of the disease as well as an improvement over the current method of streaking rectal swabs on blood agar plates.

Tests to detect serum antibody to T. hyodysenteriae have also been developed. In 1973, Hunter and Saunders (34) developed a serum agglutination test for the diagnosis of swine dysentery. Lee et al. (55) used an indirect fluorescent-antibody test for detecting T.

hyodysenteriae antibody in swine sera. In 1978, Joens et al. (40) reported a microagglutination test (MAT) for detection of antibody to T. hyodysenteriae using whole washed cells.

In 1982, Joens et al. (43) reported an ELISA test for detection of antibody to T. hyodysenteriae using extracted lipopolysaccharide as the antigen. This test was reported to be serotype specific and more sensitive than the microagglutination test. Lysons et al. (62) developed an ELISA test which incorporates sonicated T. hyodysenteriae cells as the antigen in the detection of asymptomatic carrier pigs. Since this test was not based on serotype specificity, the authors reported a higher incidence of positive samples than that detected by the LPS antigen ELISA test, although an increase in false positive results is likely.

Pathogenesis of swine dysentery The lesions associated with swine dysentery are described as a severe catarrhal enteritis with hemorrhage. The organism infects the cecum and large intestine. The highest incidence of the disease is in 8-12 week old pigs. The disease has an incubation period ranging from 2 days to 3 months (26). Clinical signs are first noted with the appearance of grey, soft or watery feces. Within several

days, a watery diarrhea containing blood and mucus develops (1,17,78,104). Intestinal lesions are further described as a superficial coagulative necrosis of the mucosa and of the crypts of Lieberkuhn with an associated edema of the lamina propria and mucosa. Varying amounts of blood are present, along with strands of fibrin and areas of denuded epithelium (1).

Chronic lesions are characterized by the presence of a fibrin cast and a fibrinonecrotic layer of material which may cover the hemorrhagic mucosa. Necrosis, which is usually superficial, involves only the mucosa although edema and congestion of blood vessels in the lamina propria are seen (1,2,3,25,87).

Several investigators have studied the ultrastructure of lesions at various times after infection and have hypothesized mechanisms of pathogenesis (1,78,104). Albassam et al. (1) conducted a detailed ultrastructural and sequential study of the lesions produced by T. hyodysenteriae infection in pigs. The initial lesions detected in pigs consisted of marked edema at the crypt shoulders with both intercellular, and to a lesser degree, intracellular edema. The tissue fluid was shown to have the same density as plasma indicating to the authors that the interstitial fluid in the lamina propria was plasma.

Necrotic epithelial cells, in more advanced lesions, had irregular, sparse, short or disrupted microvilli at the openings of the crypts. The cell surface was distended, the cells were vacuolated, the mitochondria were disrupted and the endoplasmic reticulum was dilated (1). There were increased numbers of lysosomes and the density of the cytoplasm was increased, an indication that the cells were degenerating. In the more severe lesions, red blood cells were observed passing between necrotic cells. This is possibly the route of plasma leakage into the intestinal lumen. Spirochetes were also observed within necrotic cells and were seen between the partially sloughed epithelial cells and the intact lamina propria. Neutrophils were also observed and could be seen to contain intracytoplasmic spirochetes. There was also evidence of epithelial regeneration at the margins of the necrotic zones (1).

Animals having diarrhea for several days had severe lesions with large areas of sloughing epithelium. The necrotic epithelium was covered with cellular debris, spirochetes and many bacilli. Many neutrophils in the necrotic areas had degranulated. Other neutrophils and macrophages contained bacteria undergoing degradation. Spirochetes were seen all around necrotic cells whether the cells were in place or sloughing. Spirochetes were

also seen within normal enterocytes and goblet cells (1). Cells with intracellular spirochetes had cytoplasmic degenerative changes (i.e., cells were vacuolated, the endoplasmic reticulum was distended, and the mitochondria were swollen). Spirochetes were often found between normal epithelial cells with minimal damage to the plasma membrane. Treponema were found invading the epithelial cells, from the lateral borders, in areas where adjacent cells had extruded or through adjacent empty goblet cells. Spirochetes were never seen attached to or penetrating normal colonic enterocytes at the apical borders. Spirochetes were found attached to the microvilli of goblet and epithelial cells in the crypts. In these advanced lesions, the subepithelial lamina propria was less edematous and more infiltrated by macrophages, neutrophils and a few lymphocytes (1). Ultrastructural changes in the endothelium of subepithelial blood vessels were also seen. These included folded cell membrane of the luminal surface, folded nuclear membrane, rounded nucleus and distension into the blood vessel lumen (1).

Pohlenz et al. (78) infected gnotobiotic pigs with Treponema hyodysenteriae and reported spirochetes within colonic goblet cells by day 2 post infection. By day 4, spirochetes had invaded enterocytes apparently from

adjacent goblet cells. The colonic mucus from infected pigs was more homogenous, less electron dense and less highly sulfated than that of control, non-infected pigs (78). By day 7 post infection, hypersecretion of mucus, mild erosion of the mucosal surface, marked crypt hyperplasia, with many immature cells in the deep crypts, was present. However, no significant inflammatory response was noted (78).

In the same study, conventional pigs infected with T. hyodysenteriae had a marked inflammatory reaction in the colonic mucosa. This reaction was primarily neutrophilic. Small mucosal blood vessels contained fibrin, platelets and numerous leukocytes. This difference in the inflammatory response between gnotobiotic and conventional pigs may be due to the intestinal microflora which may contribute to lesion development in swine dysentery (78). This work also indicated that T. hyodysenteriae has a tropism for mucus and is attracted to goblet cells. When the spirochete is within goblet cells, a hypersecretion of mucus, different in composition, is stimulated. The presence of the organism in the crypts also stimulates a hyperplasia of immature cells at the base of crypts (78).

Wilcock and Olander (104) also examined lesion development in dysenteric swine. Early morphological

changes consisted of congestion and margination of leukocytes in mucosal capillaries and depletion of mucus from goblet cells lining the crypts, which were followed by superficial mucosal necrosis and crypt cell hyperplasia. Greater than 10^5 colony forming units (CFU) per gram of mucosa were necessary for lesion development. They also suggested that the outpouring of mucus contributes to the critical electrolyte loss in swine dysentery and the persistent mucosal necrosis contributes to the malabsorption syndrome.

Mechanisms of pathogenesis based on experimental findings have been proposed. Albassam et al. (1) suggested that penetration of the superficial colonic enterocytes, although not essential for lesion development, occurs through the lateral borders of cells from adjacent infected goblet cells. Another possibility is the penetration of the spirochetes between enterocytes followed by invasion through the lateral borders of the enterocytes. The spirochetes then multiply within the cytoplasm and eventually destroy the enterocytes. The multiplication of the spirochete within the epithelial cell enables the organism to evade host defense mechanisms. This may also explain how spirochetes infect adjacent enterocytes and develop the carrier state described for swine dysentery (46). The lack of evidence

for penetration of epithelial cells at the luminal surface suggests that the normal brush border serves as a physical barrier against T. hyodysenteriae penetration (18,97).

Albassam et al. (1) suggested that the mucosal necrosis was not caused by invasion of the mucosal surface by the spirochetes but to a bacterial toxin produced by the spirochetes or secondary invading bacteria. They suggest a similarity between the circulatory disturbances in the superficial colonic mucosa in swine dysentery, to those of shigella dysentery in primates (3,82). Shigella dysenteriae produces a toxin which causes disturbances in circulation, cellular maladjustment and hypoxia. Similar events could be due to histamine-type mediators possibly produced by local mast cells activated by the postulated spirochete toxin (1,3).

Others have postulated that the restriction of necrosis to the superficial epithelium is due to direct or indirect action of a poorly diffusible toxin which reaches effective concentrations only at the mucosal surface (33). However, a cytotoxin could not be detected in T. hyodysenteriae broth culture or in colon contents from infected pigs (104). The late lesions, epithelial necrosis, and vascular leakage, favor the growth of

opportunistic anaerobic organisms close to the epithelial surface which may exacerbate lesion development by causing cell damage (78).

Harris et al. (28) and Whipp et al. (102) reproduced swine dysentery in gnotobiotic pigs by oral inoculation of T. hyodysenteriae in combination with either Bacteroides vulgatus, and Fusobacterium necrophorum, or with Listeria denitrificans and Clostridium spp. In both studies, a synergistic bacterial species was needed in order for T. hyodysenteriae to express its pathogenicity in the colon of the gnotobiotic pigs.

In another study, Pohlenz et al. (78) found that T. hyodysenteriae, inoculated into gnotobiotic pigs, invaded goblet cells and adjacent enterocytes. They noted a hypersecretion of mucus, mild erosion of the mucosal surface, and marked crypt hyperplasia. The infection did not produce an inflammatory response. The absence of an inflammatory response in the gnotobiotic pigs indicates a role for secondary infection by normal microflora in lesion development in conventional pigs or a more developed immune response.

Schmall et al. (87) and Argenzio et al. (3) studied the nature of electrolyte loss in dysenteric pigs. there has been no evidence to indicate that there is an active secretion of fluids from the small intestine during swine

dysentery. They attempted to define the mechanism of malabsorption by examining water transportation and sodium influxes in ligated colonic segments. They also measured concentrations of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in colonic mucosa of normal and infected pigs (3,87). In salmonellosis and shigellosis, the intestinal tissue becomes inflammed and there is active secretion of fluid and electrolytes. During salmonellosis, this increase in secretion is due to prostaglandin - induced modulation of cAMP concentrations (3). This same mechanism is implicated in shigellosis and it is well established as the pathogenic mechanism of cholera toxin and the heat-labile toxin of E. coli (8).

Argenzio et al. (3) determined that the severe loss of sodium, chloride and bicarbonate ions during swine dysentery was the result of colonic malabsorption. There was no evidence of active or passive colonic secretion due to increased mucosal permeability. In the study by Schmall et al. (87), the authors found that cAMP or cGMP levels were not increased in colonic mucosa of T. hyodysenteriae infected pigs. The authors concluded that activation of cyclic nucleotide mediated events were not responsible for the change in ion transport. This indicates that the role of inflammation and/or

prostaglandins in the pathogenesis of swine dysentery is not mediated through changes in cyclic nucleotide levels. The authors concluded that net ion and water absorption in the colon is abolished, and the loss of sodium, chloride ion or bicarbonate ion is not a consequence of increased mucosal permeability. An amount of water approximately 50% of the extracellular fluid volume of the pig is presented daily to the colon for reabsorption. This amounts to 1.5 moles of sodium and 8.6 L of water/day. Any impairment of this absorptive process by T. hyodysenteriae would be deleterious for the pig (87).

One possible mediator of the malabsorptive syndrome induced by T. hyodysenteriae is the strong beta-hemolysin (47,52,59,83,84,85,86). The hemolysin can be removed from broth filtrates supplemented with fetal calf serum and yeast RNA (84). Lemcke and Bew (56) determined that T. hyodysenteriae hemolysin is produced maximally at the end of the logarithmic growth phase, produced aerobically as well as anaerobically, and requires a carrier, sodium RNA or bovine serum albumin (BSA) for stability.

Depending on the method of purification, the hemolysin has been reported to have a molecular weight (m.w.) of 74 kilodaltons (kDa) by gel filtration (84), or 19 kDa by electrophoresis (47). The 19 kDa determination would be consistent with the m.w. of streptolysin S (84).

The T. hyodysenteriae hemolysin characterized by Saheb et al. (84) was oxygen resistant (like Streptolysin S), heat labile at 60 C, and stable at a pH range of 2 - 10. The hemolysin does not bind carbohydrate specific stains, and is not inactivated by amylase, suggesting that the hemolysin is not composed of carbohydrate (84). Saheb et al. (84) also determined that the hemolysin was a protein associated with lipids and nucleotides, which may contribute to the greater molecular weight they reported (74 kDa). Pronase, and to a lesser extent lipase, inactivated the hemolysin indicating that it was a polypeptide or a protein associated with lipids. RNase, trypsin, and chymotrypsin did not inactivate the hemolysin. The hemolysin did not stain well in polyacrylamide gels with coomassie brilliant blue or with sudan black which is characteristic of lipoproteins (84). A lipoprotein associated hemolysin, similar to the hemolysin from T. hyodysenteriae, has been described for Vibrio cholerae (El Tor) (81).

Lysis of rabbit erythrocytes by the T. hyodysenteriae hemolysin did not require divalent cation and was not lipolytic or proteolytic (84). It was shown that the interaction of the hemolysin with the erythrocyte surface was temperature independent, unlike Streptolysin S, while erythrocyte swelling and lysis was

temperature dependent (83). These authors suggested that the hemolysin mechanism of action was disorganization of membrane components. They also demonstrated that the hemolytic activity could be partially blocked by the presence of sucrose and inhibited by trypan blue.

Inhibited lysis by trypan blue is similar to lysis induced by saponin, although saponin erythrocyte lysis is temperature independent and not blocked by sucrose (83).

The hemolysins of T. hyodysenteriae (THH) and T. innocens (TIH) have been compared and share many characteristics (85). Both hemolysins had similar molecular weights, oxygen and pH stabilities, were inactivated by pronase and lipase and inhibited by trypan blue. However, TIH differed from THH in that TIH was inhibited by cardiolipin and had less activity on red blood cells from different species than THH (85). It has been shown that THH had no lytic activity on prokaryotic protoplasts or spheroplasts, and had no activity against chinese hamster ovary cells or mouse spleen cells (86). However, it was found that THH decreased the response of mouse spleen cells to concanavalin A and Escherichia coli lipopolysaccharide by 35% or more (86). The authors suggested that this decrease in the mitogenic response may have been due to inactivation of macrophages or inhibition of the mitogens. It was also shown that when

THH was injected into rat ligated ileal loops, it induced fluid accumulation, desquamation of epithelial cells, hemorrhage into the intestinal lumen and neutrophil accumulation suggesting that THH may contribute to the pathogenicity of T. hyodysenteriae (86).

Kent and Lemcke (47) found that the THH was more cytotoxic for fibroblasts than it was for epithelial cells, based on the release of chromium-51 from labeled cells. Porcine lymphoblasts were most sensitive to cytotoxic effects of THH while swine macrophages and neutrophils were much more resistant (47). Kent and Lemcke (47) also reported that T. innocens produced much less hemolysin than T. hyodysenteriae, and that THH was less cytotoxic for in vitro culture of tissue cells. The marked sensitivity of porcine lymphocytes to THH is similar to the pattern of the sensitivity of murine and human lymphocytes to streptolysin S (31).

Antibiotic therapy and epidemiology Many antibiotics have been used for the prevention and treatment of swine dysentery. These include organic arsenicals, bacitracin, tylosin, sulfonamides, carbadox, lincomycin, virginiamycin, gentamicin, neomycin and tiamulin (73). Other compounds that have been found to be efficacious are olaquinox (105), sedacamicin (106) and treponemycin (90) and three nitroimidazoles;

dimetronidazole, ipronidazole and ronidazole (73,74). However many of these compounds have not been approved for the treatment of swine dysentery in United States.

It has been shown that T. hyodysenteriae has developed resistance to several of these compounds. Resistance to sodium arsanilate has been documented. Swine dysentery in the presence of resistant strains and arsanilate is much more severe (74). Tylosin was also reported to be ineffective, apparently due to acquired resistance (73).

Antibiotic therapy affects the development of immunity by suppressing the antibody response to T. hyodysenteriae (23,37). Withdrawal of dimetridazole or tiamulin was necessary to stimulate immunity (73). The stimulation of the immune response by the withdrawal of antibiotics indicates that the organism is probably not eliminated by antibiotic therapy but the growth of the organism is effectively suppressed.

The survival of T. hyodysenteriae outside the host requires a cool (4-6 C) and moist environment. The organism can survive for 2 months in refrigerated feces or anaerobically in a lagoon. However, the organism dies at temperatures higher than 10 C, surviving less than 1 week on grass or in warm, dry soil (23).

Reservoirs of T. hyodysenteriae include carrier pigs and mice. After pigs recover from swine dysentery and become asymptomatic they continue to pass T. hyodysenteriae in their feces for up to 70 days (23). Infected mice have been shown to shed the organism for as long as 200 days. Dogs, flies, birds and rats apparently do not become infected with the organism but could serve as mechanical vectors (23,92).

The most common method of introducing T. hyodysenteriae into a herd is by asymptomatic carrier pigs. Mechanical vectors (i.e., feces, contaminated boots, coveralls or vehicle tires) may also be responsible for the transmission of T. hyodysenteriae from herd to herd (23,92). The natural spread of swine dysentery from pig to pig is by the ingestion of feces or contaminated feed. Floor feeding favors disease dissemination. Gutter flush systems recycling lagoon water may also result in further spread of swine dysentery (19).

Gram-negative outer membranes The outer membrane of Gram-negative bacteria has been intensively studied (67,69,75,88,91,100). Much of the work has been done on members of the family Enterobacteriaceae, particularly Escherichia coli and Salmonella typhimurium. Electron

microscopic examination of Gram-negative bacteria demonstrated that these organisms are covered by an outer membrane layer outside of the peptidoglycan layer (16). Separation of outer and inner membranes by equilibrium density centrifugation in sucrose, which is based on the higher buoyant density of outer membranes than the inner cytoplasmic membrane (1.22 and 1.15 respectively), revealed the presence of proteins and lipids in the membranous structure (75). To study protein composition of the outer membrane, extraction with mild detergents such as sodium dodecyl sarcosinate (Sarkosyl) can be used to extract insoluble proteins from the outer membrane due to the preferential solubilization of cytoplasmic membrane proteins (13).

The three major components of the outer membrane are phospholipids, similar to those of the cytoplasmic membrane, LPS and a characteristic set of proteins. The LPS of Gram-negative outer membranes is a unique molecule consisting of three parts. The proximal part is the hydrophobic lipid A region consisting of a glucosamine disaccharide backbone substituted with six or seven saturated fatty acid residues which anchor the LPS to the outer membrane. The lipid A is responsible for most of the toxic properties of LPS and is essential for the structure of the outer membrane (67). The next region of

the LPS is the core polysaccharide composed of 3-deoxy-D-manno-octulosonic acid (KDO), L-glycero-D-mannoheptose phosphate and variable oligosaccharide units that may vary within a species. The core is important in maintaining the barrier property of the outer membrane. The core is also a connection between the glucosamine back bone of the lipid A and the distal hydrophilic O polysaccharide region, important in host parasite interaction (69).

The outer membrane has a characteristic protein composition, consisting of a few major proteins and a number of minor proteins which account for 9-12% of the total cellular proteins (91). These proteins are generally examined and characterized by the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The major types of proteins in the outer membrane of the Enterobacteriaceae are murein lipoproteins, porins, diffusion specific proteins and a few enzymes. Murein lipoprotein is a small protein covalently linked to the peptidoglycan layer. This protein has a fatty acid residue linked to the N-terminal cysteine. The protein is apparently not surface exposed but is important in maintaining the stability of the outer membrane-peptidoglycan complex (29).

The porins are proteins that form nonspecific diffusion channels or pores to allow for the passage of small molecular weight, hydrophilic molecules across the outer membrane. Several of the porins of E. coli and S. typhimurium have been thoroughly studied, their genes cloned and their gene regulation elucidated. One of these porin proteins, PhoE, is unusual in that it is produced only under conditions of phosphate starvation (100). Other porins, OmpF and OmpC are usually present in the outer membrane in large amounts. Porins make up approximately 2% of the total cellular protein of E. coli (approximately 10^5 copies per cell (69). The structure of these porins consists of transmembrane, beta-sheets associated in trimers forming three channels on the surface. These channels may merge toward the inner surface or remain separate (5).

There are also proteins of the outer membrane involved in specific diffusion processes in E. coli. When induced these proteins exist in large numbers on the bacterial surface. The solutes transported by these proteins include maltose, nucleosides, vitamin B₁₂, ferric iron, ferric iron linked to enterobactin or aerobactin, ferrichrome, ferric citrate and amino acids. All of the proteins involved in the passage of iron chelates are induced by iron starvation and can become

major components of the outer membrane. Most of these diffusion-associated proteins require a functional Ton B gene product. The product is a protein thought to supply the energy required for the diffusion process (79).

Also, most of these diffusion proteins and the porins act as receptors for numerous phages and colicins (69). One interesting difference between porins and OmpA, a protein probably involved in the transport of amino acids, is their migration during SDS-PAGE. Porins are converted to monomers when heated at 100 C in the presence of SDS, and thereby increasing their mobility in an acrylamide gel. However, motility of the OmpA protein is reduced after heating in SDS (88). The final two types of outer membrane proteins are enzymes, such as phospholipase A1 and proteases (69), and LPS-binding protein which has a molecular weight of 15 kDa (91) which may be a common surface antigen within the Enterobacteriaceae. Porins, diffusion proteins, enzymes and LPS-binding proteins, with apparently analogous functions as those described above, have been found in Pseudomonas aeruginosa, Neisseria gonorrhoeae and Neisseria meningitidis (91).

The outer membrane proteins (OMPs) of Neisseria spp. seem to be conserved. The three classes of Neisseria OMPs are designated as reduction - modifiable protein, heat-modifiable protein and the major outer membrane

protein porin. The reduction-modifiable protein is so called because it migrates at 31 kDa when reduced but at 30 kDa when unreduced. The heat-modifiable protein, protein III, acts to block bactericidal antibody by binding non-cytocidal antibody and shields the outer membrane from antibodies that would be lethal to the organism (7).

Outer membrane proteins, as a source of immunogenic proteins, have been examined extensively in recent years. OMP vaccines would lack the lipid A toxicity of whole cell vaccines, would bypass the nonimmunogenic nature of polysaccharide capsules of some Gram-negative pathogens and induce a protective immune response to surface antigens of a pathogen. Two organisms which have received particular attention in this regard are Neisseria meningitidis and Hemophilus influenzae type b (91).

The SDS-PAGE technique has been used for the separation and identification of OMPs in a number of organisms (i.e., Hemophilus influenzae type b, E. coli and Salmonella spp., 21). The profiles obtained with these organisms have been shown to be stable, useful for epidemiology and useful for subtyping strains of pathogens (21). Differences in the expression of OMPs have been shown to reflect differences in virulence of

pathogenic strains of Bordetella spp. (12) and Neisseria spp. (54). The outer membrane of Gram-negative bacteria acts as the physical interface between the organism and its environment or host. Therefore, the morphologic and immunologic aspects of the organism, as well as virulence attributes, are often correlated with outer membrane structures. It is these structures which can confront and influence the immune system and which can determine the outcome of the infection in the presence of host defense mechanisms (69).

Treponemal outer membranes Spirochetes are unique helical shaped organisms. Unlike other motile bacteria, the spirochetes are covered with an outer sheath which surrounds the protoplasmic cylinder and periplasmic flagella. These periplasmic flagella, also called axial filaments, are inserted at the ends of the protoplasmic cylinder. Most of the work on the outer membrane sheath (OM) of treponemes has been on Treponema pallidum. The reported protein composition of the OM isolated from T. pallidum varies widely. For example, Penn et al. (76) were unable to detect protein in the OM preparations while Moskophidis and Muller (68) demonstrated the presence of at least 13 OM proteins. These differences may be due to the procedures used to radiolabel the surface proteins. The presence of

individual surface exposed proteins, with variations in m.w. from 19 to 47 kDa has been described (65,70,80). Outer membrane proteins of other Treponema spp. have also been identified. T. phagedenis biotype Reiter has at least one major protein species of 69 kDa on its surface (66). T. pertenue was shown to have at least six surface proteins (99). T. denticola was shown to have at least two OM proteins of 31 kDa and 58 kDa (38). In addition, the nature of surface proteins can be difficult to determine since many of these organisms require complex growth media in vitro or only grow in vivo. For instance, T. pallidum binds host proteins on its surface, including immunoglobulin, albumin, fibronectin and other serum proteins which must be differentiated from native OMPs (14,76).

The OMPs of pathogenic spirochetes are believed to be important in the pathogenesis as well as in the immune response. For instance, the 39 kDa surface polypeptide of T. pallidum is recognized by serum from infected rabbits (70). Another report indicated an antibody response in infected rabbits to proteins of 20, 25, 30, 45, and 59 kDa (99). Eight to 22 proteins of T. pallidum were recognized by western blot analysis using sera from syphilitic humans (22). However, these antigens were not identified as surface exposed proteins.

The OMPs of T. hyodysenteriae has not been characterized, however, Joens and Marquez (44) described the antigenic profile of treponemal antigens using convalescent porcine serum. The location of these antigens or their chemical nature (i.e., protein or carbohydrate) was not addressed. The following work includes the elucidation of the protein composition of the T. hyodysenteriae outer membrane and the immune response directed at these antigens.

SECTION I. ANALYSIS OF OUTER MEMBRANE COMPONENTS OF
Treponema hyodysenteriae AND Treponema innocens

ABSTRACT

Outer membrane protein (OMP) profiles of Treponema hyodysenteriae and Treponema innocens were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cells were treated by six different extraction methods for the preparation of outer membrane fractions. These extraction procedures were performed with Zwittergent, Triton-X 100, lithium chloride or N-lauryl sarcosinate (sarcosyl), sucrose gradient sedimentation or N, N'-dicyclohexylcarbodiimide (DCCD). Sarcosyl extracts were found to be the most useful for differentiating T. hyodysenteriae strains from T. innocens strains. All T. hyodysenteriae strains examined had similar SDS-PAGE profiles. T. innocens strains were grouped into three subgroups on the basis of their OMP profiles. T. hyodysenteriae produced an additional 70 kilodalton (kDa) OMP when grown under iron limiting conditions.

INTRODUCTION

Treponema hyodysenteriae, an anaerobic to microaerophilic (27) spirochete, is the etiological agent of swine dysentery (SD) (11). The disease is characterized by a mucohemorrhagic diarrhea that can result in dehydration and death. Affected herds may experience 90% morbidity and 25% mortality (10). Most countries with modern swine production report the occurrence of SD, which is a serious economic problem for the pork industry (15).

Isolates of T. hyodysenteriae have been grouped into seven serotypes based on a phenol-water extracted lipopolysaccharide antigen (1,14,16). Other groupings have been made based on clinical signs, biochemical tests and colony morphology (2,12). Little is known about the OMPs of the organism. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of OMPs from other Gram-negative pathogens has resulted in the identification of potentially useful differentiation, virulence, or protective antigens (25).

Expression of OMPs has been shown to be controlled by both genetic and environmental mechanisms. Stability of OMP profiles has been shown to vary as a function of in vitro passage and often these profiles are affected by in vivo passage as well (25). In many Gram-negative

organisms, iron regulates OMP expression (9). These proteins are often associated with the organism's ability to compete for and transport iron across the outer membrane (9,19), and thus the organisms are able to survive in the iron deficient milieu of the host's tissues.

The purpose of this study was to examine the SDS-PAGE profiles of outer membrane enriched fractions of T. hyodysenteriae and T. innocens strains. Patterns obtained were used to differentiate T. hyodysenteriae from T. innocens.

MATERIALS AND METHODS

Bacterial strains All of the T. hyodysenteriae and T. innocens strains used were from the culture collection of the Veterinary Medical Research Institute, Iowa State Univ., Ames, Iowa. Cultures were frozen and stored in growth medium at -70C. Cultures were grown in anaerobically prepared trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) supplemented with 20 ml/l of VPI salt solutions A and B, 0.5% yeast extract (BBL), 0.05% cysteine-HCl (Sigma Chemical Co., St. Louis, MO) and 5.0% horse serum (HyClone Inc., Logan, Utah), (TSBYS) at 37C for 18-24 hours. VPI salt solution A consisted of 0.4 g/l CaCl_2 and 0.4 g/l MgSO_4 . Solution B contained 2 g/l K_2HPO_4 , 2 g/l KH_2PO_4 , 20 g/l NaHCO_3 and 4 g/l NaCl .

T. hyodysenteriae B204 (serotype 2) was subcultured over 100 times in TSBYS before OMP preparation. To evaluate the effect of in vivo passage on OMP profiles, specific pathogen free pigs were infected with strain B204. After the development of clinical signs in the infected animals, a serotype 2 organism was isolated as previously described (26) and subcultured in TSBYS. OMP preparations of this T. hyodysenteriae isolate (passage 2) were obtained and evaluated by SDS-PAGE and compared to strain B204 (passage 12).

Outer membrane protein preparation Outer

membrane protein enriched fractions were prepared by six methods. Detergents or chaotropic agents used in the procedures described in this paper were obtained from commercial sources and used without further purification. The detergents used were Triton X-100 (Sigma), Zwittergent 3-14 (Calbiochem-Behring, LaJolla, CA), and sodium-N lauryl sarcosinate (Sarcosine) (Sigma). The chaotropic agents used were lithium chloride (Fisher Scientific Co., Fairlawn, N.J.) and N,N'-dicyclohexylcarbodiimide (DCCD) (Sigma). A ratio of 12.5 mg (dry weight of cells)/1 ml detergent was used for each extraction procedure. Cells were extracted with Zwittergent according to a modification of the method of Blake and Gotschlich (4). Briefly, cells were washed with phosphate buffered saline, (pH 7.2, 0.05M) (PBS) and then resuspended in a solution of 20mM Zwittergent in 10mM tris hydroxymethyl aminomethane-hydrochloride (tris), pH 7.4, and stirred for 2 hrs at room temperature. The mixture was then centrifuged at 15,000 x g for 30 min at 20 C. The supernatant was collected and centrifuged at 100,000 x g for 2 hrs at 4 C. The pellet was collected and washed 3 times with .15M NaCl at 100,000 x g for 2 hrs at 4 C and resuspended in .15M NaCl.

Cells were extracted with Triton X-100 according to a modification of the methods of Penn et al. (22). Washed cells were resuspended with 2.0% Triton X-100 in 10mM Tris containing 5mM $MgCl_2$. The mixture was incubated at 37 C for 30 min and then centrifuged at 5000 x g for 30 min. The supernatant was centrifuged at 100,000 x g for 2 hrs at 4 C. The pellet was then washed 3 times with 0.15M NaCl and resuspended in 0.15M NaCl.

Cells were extracted with lithium chloride by the addition of 1M LiCl in 10mM Tris (18). Glass beads were added to the mixture and shaken for 2 hrs at 37 C. The supernatant was collected and the beads were rinsed with 10mM Tris. The supernatant and wash were then centrifuged at 12,000 x g for 20 min at 4 C. The supernatant was collected and centrifuged at 40,000 x g for 45 min at 4 C. The supernatant was then centrifuged at 100,000 x g for 2 hrs at 4 C. The pellet was washed 3 times with 0.15M NaCl and resuspended in 0.15M NaCl.

Cells were extracted with sarcosine according to a modification of the methods of Cooper et al. (7). In brief, washed cells were spheroplasted using 0.2% lysozyme (Sigma) in cold 10mM Tris (pH 7.4) with 8% sucrose. The mixture was incubated for 5 min on ice followed by addition of 3 volumes of cold water. The spheroplasts were disrupted by sonification (Model W225R,

Heat Systems-Ultrasonics Inc., Farmingdale, NY) at 50% maximum output. Cells were sonicated with four bursts of 15 sec each, while cooling in an ice bath. Whole cells and debris were removed by centrifugation at $1500 \times g$ for 20 min at 20 C. The supernatant was centrifuged at $40,000 \times g$ for 45 min at 4 C and the pellet was resuspended in 10mM Tris with 1% sarcosine. The mixture was incubated, stirred at room temperature for 30 min and then centrifuged at $100,000 \times g$ for 2 hrs at 4 C. The pellet was collected and washed 3 times in 0.15M NaCl and resuspended in 0.15M NaCl.

Cells were extracted with DCCD according to the methods of Masuda and Kawata (17). Briefly, washed cells were resuspended in 10mM Tris - HCl buffer (pH 7.4) with 50um DCCD and incubated for 30 min at 37 C. This suspension was then centrifuged at $6000 \times g$ for 10 min and the pellet discarded. The supernatant was recentrifuged at $25,000 \times g$ for 30 min. The pellet was resuspended in 0.1M sodium acetate - HCl buffer, pH 3, and incubated for 2 hrs at 4 C, then recentrifuged at $25,000 \times g$ for 30 min. The pellet was then washed two more times in 10mM Tris - HCl, pH 7.4.

Cells were also extracted by cell fractionation and outer membrane fractions collected by sucrose gradient centrifugation by a modification of the methods of Osburn

and Munson (21). Washed cells were resuspended in 10mM Tris - HCl buffer, pH 7.4, with 0.75M sucrose. Lysozyme (Sigma) was added to give a final concentration of 0.1 mg/ml and the suspension incubated on ice for 2 min. Two volumes of cold 0.15M EDTA were slowly added over the next 10 min. This suspension was then centrifuged at 6000 x g for 10 min. and the pellet discarded. The supernatant fluid was recentrifuged and washed 2 times at 25,000 x g for 30 min.

All OMP extracts were resuspended to a concentration 0.1 to 0.8 mg/ml protein and stored at -70°C. Protein concentrations were determined by a Coomassie G-250 dye binding protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (BSA) as a standard.

SDS-PAGE Discontinuous SDS-PAGE was performed with a 4% stacking gel and 10% separating gel and the two buffer system of Laemmli (13). A Hoefer SE 600 vertical slab electrophoresis chamber was used (Hoefer Scientific Instruments, San Francisco, CA). The OMP fractions were solubilized by boiling at 100°C for 5 min in 0.0625M Tris sample buffer containing 2% sodium dodecyl sulfate (SDS, Sigma), 5% 2-mercaptoethanol and 10% glycerol, pH 6.8.

Samples were loaded into wells at a concentration of 15-20 µg protein per lane and run at a constant current of 30mA per gel until the tracking dye (bromophenol blue,

Fisher) was approximately 1.5 cm from the bottom of the gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R250. Carbohydrate or lipid bands were visualized by the method of Tsai and Frasch (28). Apparent molecular weights were calculated by comparison to standards of known molecular weight (Dalton Mark 7-L, Sigma) containing: bovine albumin (66 kilodaltons, kDa), egg albumin (45kDa), glyceraldehyde-3-phosphate dehydrogenase (36kDa), carbonic anhydrase (29kDa), trypsinogen (24kDa), trypsin inhibitor (20.1kDa) and alpha-lactalbumin (14.2kDa).

Surface labeling with Iodine-125 Iodine-125
labeling of surface exposed proteins was accomplished using the Enzymobead lactoperoxidase-glucose oxidase system (Biorad). Briefly, a fresh 18 hr 10 ml broth culture of T. hyodysenteriae was harvested by centrifugation at 1500 x g for 15 min in 250 ml of PBS. The cells were gently resuspended in PBS and diluted until a 1:10 dilution of the cells gave an optical density (OD) of 1.0 at 550 nm using a Spectronic 20 spectrophotometer (Bausch and Lomb Inc., Rochester, NY). The concentrated suspension (100 ul) was added to 490 ul of PBS containing 10^{-5} M KI. Twenty-five microliters of 2% D-glucose in water was then added followed by 100 ul of Enzymobeads and 0.5 mCi of Na (125 I) iodide (Amersham

Corp., Arlington Heights, Ill.) and allowed to incubate at room temperature for 30 min. After incubation, 10 ml of PBS containing 10^{-4} M KI was added and the sample was centrifuged at 600 x g for 10 min to recover the Enzymobeads and stop the reaction. The supernatant fluid was collected and centrifuged (10,000 x g for 20 min) at 4 C with PBS containing 10^{-4} M KI. The resulting pellet of labeled cells was washed three times by centrifugation as above. The bacterial cell pellet was then sonicated and extracted with sarcosine. Samples were subjected to SDS-PAGE and the resulting gel was autoradiographed using Kodak XAR-5 film.

Growth of *T. hyodysenteriae* under reduced iron

Broth cultures were used to determine the growth curve of *T. hyodysenteriae* in the presence of desferal mesylate (DF) (CIBA-GEIGY, Summit, N.J.). Tubes were prepared in triplicate with 10 ml of TSBYS and 1% horse serum and pre-treated with increasing concentrations of DF. An 18 hr culture of *T. hyodysenteriae* grown in TSBYS with 1% horse serum was standardized to an OD of 0.2 at 600 nm. This stock culture was used to inoculate test tubes with 0.2 ml of inoculum /10 ml broth. Tubes were then incubated with stirring at 37 C. The OD was read every four hrs at 600 nm. Organisms grown in similar media were collected by centrifugation after 18 hr growth and

their OMPs were extracted with sarcosine, as described above.

RESULTS

OMP profiles of T. hyodysenteriae and T. innocens

T. hyodysenteriae was grown in vitro in several concentrations of DF and with or without added ferric nitrate. The growth of the organism decreased with increasing concentrations of DF (Fig. 1). The addition of iron to the media increased the growth of the organism in the presence of DF (Fig. 1). Outer membrane protein-enriched fractions prepared from T. hyodysenteriae by six different procedures showed some differences. Outer membrane proteins extracted by the detergent Zwittergent resulted in more than 10 bands resolved by SDS-PAGE. The other procedures, Triton X-100, 1% sarcosine, or 1M lithium chloride resulted in SDS-PAGE profiles exhibiting 6 major bands and several minor bands (Fig. 2).

Supernatant fluid from sonicated T. hyodysenteriae cells gave many bands, several of which did not align with detergent extracted outer membrane proteins. The profile obtained with the low passage (2P) (Fig. 2, lane 2), reisolated T. hyodysenteriae was identical to that obtained with organisms that had been passed in vitro 12 times (Fig. 2, lane 5). Organisms that had been passed in vitro over 100 times (Fig. 2, lane 6) had a profile similar to the 12 passage organisms. The high passage

profile contains more lightly staining bands but lacked a minor band between 45 and 36 kDa. Novobiocin treated cells (Fig. 2, lane 8) gave a similar profile to the 12 passage organisms as did cells grown in the presence of 10% fetal calf serum (Fig. 2, lane 10) instead of 5% horse serum (Fig. 2, lane 5). Cells grown in the presence of the iron chelator, DF, displayed a new OMP at 70 kDa (Fig. 2, lane 9). No change in OMP profile was observed in the presence of increasing concentrations of novobiocin which has been used to inhibit the expression of plasmid coded proteins (Fig. 2, lane 8).

The sarcosine and lithium chloride extracted material produced SDS-PAGE profiles which could be used to resolve differences between T. *hyodysenteriae* and T. *innocens*. Although the profiles obtained by the sarcosine and LiCl methods were similar, greater yields of OMP were obtained with the sarcosine procedure. Thus, the sarcosine procedure was used for all subsequent extractions.

All strains of T. *hyodysenteriae* analyzed, representing five serotypes, had similar sarcosine OMP profiles on SDS-PAGE (Fig. 3, lanes 1,2,5,6,9,10). The major protein bands observed were a doublet at 46.5-45.5 kDa and singlet bands at 26, 29, 31, 36.5, 39, and 41 kDa (Table 1). An additional band at 61 kDa was observed in

strain B169, serotype 3. Minor bands were observed at 36, 37.5, and 40 kDa.

Strains of T. innocens isolated from swine were also analyzed by SDS-PAGE of sarcosine extracted OMPs. These six strains had different profiles than the T. hyodysenteriae strains. The T. innocens profiles also varied between strains. The strains were divided into three groups on the basis of their OMP patterns. Group one contained strains B256 and 4/71 (lanes 3,4), group 2 contained the Taylor strain and B359 (lanes 7,8), and B1555a and B6571 (lanes 11,12) were placed in group 3 (Figure 3).

The unique mobility of major OMPs was confirmed by mixing sarcosine OMP extracts from T. hyodysenteriae strain B204 and T. innocens strain B256. The resulting profile contained the 49.5 kDa and 30 kDa of T. innocens which was unique to that species, while the 46.5-45.5 kDa doublet of T. hyodysenteriae was unique to that species (Fig. 4, lane 4).

To determine that the sarcosine OMP profiles are indeed outer membrane proteins, surface exposed proteins of the organism were labeled with iodine-125 (24). After radioisotope labeling the cells, the OMPs were extracted by treatment with sarcosine sonication and the supernatant collected after centrifugation. The SDS-PAGE

patterns of the samples were analyzed by autoradiography revealing which sarcosine extracted OMPs were surface exposed (Fig. 5). The heaviest labeled band was at 45.5 kDa. Other heavy bands were 31, 36.5, 39 and 41 kDa which corresponded to bands in the sarcosine OMP profile. A labeled band also appeared at 33 kDa although no corresponding band was present in the sarcosine OMP profile. Faint bands also appeared at 12, 14, 19, 46.5, 55, 60, 62.5, 68 kDa for which corresponding bands were absent in the sarcosine OMP profile. Electron microscopic examination of the sarcosine extracted outer membrane revealed the material to have a bilaminar appearance (Fig. 6, arrow) indicating the extraction of intact membrane material.

DISCUSSION

Several extraction methods were evaluated for their ability to solubilize all but the intrinsic membrane proteins in the outer envelope (22), the OMPs from T. hyodysenteriae and T. innocens. Outer membrane extraction, followed by SDS-PAGE can also be used to differentiate strains of T. hyodysenteriae from strains of T. innocens (Fig. 3). Sarcosine extracted outer membrane from T. innocens (Fig. 3) demonstrated variations in protein mobility indicating heterogeneity within the species. T. hyodysenteriae strains, however, had similar profiles regardless of serotype (Fig. 3). Antibodies to these T. hyodysenteriae OMPs were removed by absorption when antiserum from convalescent swine was incubated with washed whole cells of T. hyodysenteriae, indicating that these OMPs were surface exposed (18) (Data not shown). Radiolabeling of surface protein with iodine-125 followed by sonification and detergent extraction, SDS-PAGE and autoradiography also showed these OMPs to be surface exposed (Fig. 5). This confirmed the usefulness of the sarcosine extraction procedure to study surface exposed OMPs. However, several minor bands and one major band were seen by surface labeling that were lost by detergent extraction. Interestingly, the upper band of the T. hyodysenteriae

45.5-46.5 kDa doublet was less radiolabeled, indicating that the protein apparently had less available tryrosine residues for radiolabeling by lactoperoxidase (3).

The sarcosine extracted OMP pattern was also similar to that obtained by spheroplasting T. hyodysenteriae cells with lysozyme and sonification followed by sucrose gradient separation (Fig. 5). It was also similar to the pattern obtained by extraction with DCCD, which had been used to remove the outer sheath of another treponeme (17). The OMP pattern of T. hyodysenteriae remains stable after 2 or over 100 in vitro passages, an important consideration for classification. The OMP pattern is altered after treatment with the iron chelator desferal mesylate. To successfully grow in the intestinal tract, organisms must have a mechanism to acquire iron from the environment. Many enteric pathogens in an iron deficient environment synthesize high-molecular weight proteins which act as receptors for iron-siderophore complexes (9). These iron-regulated OMPs can be detected when cells are grown under conditions of iron limitation (5,19,20,23,24). The 70 kDa peptide is similar in size to iron-regulated 74 and 76 kDa OMPs from Campylobacter jejuni (8) and those of 79 and 81 kDa from Yersinia species (6). The ability to elicit a similar protein in T. innocens is currently

under investigation and may be related to differences in virulence of the two organisms (2).

Finally, the organism apparently does not lose an OMP that may be coded for by extra chromosomal DNA. Novobiocin has been used to cure plasmids from bacteria by interference with initiation of plasmid DNA replication (29). However, since the organism was not clone purified the effect of novobiocin could be present but not detected. There are no reports in the literature that T. hyodysenteriae harbors extrachromosomal DNA.

The major objective of this study was to differentiate between treponemal swine isolates by comparing their OMP SDS-PAGE profiles. Sarcosine extracted profiles of T. hyodysenteriae and T. innocens can be used to distinguish these different species. This method can be added to the previously used procedures to differentiate these species on the basis of hemolysis on blood agar, biochemical tests, and mouse enteropathogenicity. Similar growth conditions and extraction procedures are necessary to view consistent OMP differences between these two species. Further study on the regulation of the iron induced T. hyodysenteriae OMP is on going. There is a need to investigate whether T. hyodysenteriae does harbor extrachromosomal plasmid DNA and whether this DNA encodes for OMP expression or

other virulence attributes. Finally, the role of I. hyodysenteriae OMPs in the pathogenesis of the organism and on the immune response of infected swine also should be addressed.

REFERENCES

1. Baum, D. H., and L. A. Joens. 1979. Partial purification of a specific antigen of Treponema hyodysenteriae. Infect. Immun. 26:1211-1213.
2. Binek, M., and Z. Szykiewicz. 1984. Susceptibility of Treponema hyodysenteriae, Treponema hyodysenteriae intermediate type and Treponema innocens to different chemotherapeutics. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 194.
3. Bio-Rad Bulletin 1071. 1986. BioRad Laboratories, Richmond, CA.
4. Blake, M. S., and E. C. Gotschlich. 1982. Purification and partial characterization of the major outer membrane protein of Neisseria gonorrhoeae. Infect. Immun. 36:277-283.
5. Calver, G. A., C. P. Kenny, and K. J. Kushner. 1979. Inhibition of the growth of Neisseria meningitidis by reduced ferritin and other iron-binding agents. Infect. Immun. 25:880-890.
6. Carniel, E., D. Mazigh, and H. H. Mollaret. 1982. Expression of iron - regulated proteins in Yersinia species and their relation to virulence. Infect. Immun. 55:277-280.
7. Cooper, M. D., M. J. Wannemuehler, R. D. Miller, and M. F. Fedyk. 1981. Role of outer envelope contamination in protection elicited by ribosomal preparations against Neisseria gonorrhoeae infection. Infect. Immun. 32:173-179.
8. Field, L. H., V. L. Headley, S. M. Payne and L. J. Berry. 1986. Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in Campylobacter jejuni. Infect. Immun. 54:126-132.
9. Finkelstein, R. A., C. V. Sciortino, and M. A. McIntosh. 1983. Role of iron in microbe-host interactions. Rev. Infect. Dis. 5:S759-S777.
10. Harris, D. L., and R. D. Glock. 1973. Swine Dysentery. Vet. Scope 17:2-7.

11. Harris, D. L., R. D. Glock, C. R. Christensen, and J. M. Kinyon. 1972. Swine Dysentery. Inoculation of pigs with Treponema hyodysenteriae (new species) and reproduction of the disease. Vet. Med. Small Anim. Clin. 67:61-64.
12. Hovind-Hougen, K., and P. Høgh. 1984. Morphological and serological heterogeneity among strongly hemolytic spirochetes isolated from pigs with swine dysentery. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 181.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
14. Lemcke, R. M., and J. Bew. 1984. Antigenic differences among isolates of Treponema hyodysenteriae. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 183.
15. Liven, R. 1979. Prevalence of Treponema hyodysenteriae in healthy pigs. ACTA Vet. Scand. 20:387-395.
16. Mapother, M. E., and L. A. Joens. 1985. New Serotypes of Treponema hyodysenteriae. J. Clin. Microbiol. 22:161-164.
17. Masuda, K., and T. Kawata. 1987. Cleavage and isolation of the outer sheath of Treponema phagedenis by treatment with N,N'-dicyclohexylcarbodiimide. FEMS Microbiology Letters 41:73-78.
18. McDade, R. L. Jr., and K. H. Johnston. 1980. Characterization of serologically dominant outer membrane proteins of Neisseria gonorrhoeae infection. J. of Bacteriol. 141:1183-1191.
19. Mietzner, J. A., G. H. Luginbuhl, E. Sandstrom, and S. A. Morse. 1984. Identification of an iron-regulated 37,000-dalton protein in the cell envelope of Neisseria gonorrhoeae. Infect. Immun. 45:410-416.
20. Neilands, J. B. 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. 36:285-309.

21. Osburn, J. F., and R. Munson. 1974. Separation of the inner (cytoplasmic) and outer membranes of gram negative bacteria. *Methods Enzymol.* 31A:642-653.
22. Penn, C. W., A. Cockayne, and M. J. Bailey. 1985. The outer membrane of Treponema pallidum: Biological significance and biochemical properties. *J. Gen. Microbiol.* 131:2349-2357.
23. Raventos-Saurez, C., S. Pollack, and R. L. Nagel. 1982. Plasmodium falciparum: Inhibition of in vitro growth by desferrioxamine. *Am. J. Trop. Med. Hyg.* 31:919-922.
24. Sigel, S. P., and S. M. Payne. 1982. Effect of iron limitation on growth, siderophore production, and expression of outer membrane proteins of Vibrio cholerae. *J. Bacteriol.* 150:148-155.
25. Smyth, C. J. 1985. Immunology of outer membrane proteins of gram-negative bacteria. Pp. 177-201. In D. E. S. Stewart-Tull and M. Davies, eds. Immunology of the Bacterial Cell Envelope. John Wiley and Sons, Ltd., N.Y.
26. Songer, J. G., J. M. Kinyon, and D. L. Harris. 1976. Selective medium for isolation of Treponema hyodysenteriae. *J. Clin. Microbiol.* 4:57-60.
27. Stanton, T. B. 1987. Erythrocytes as a source of essential lipids for Treponema hyodysenteriae. *Infect. Immun.* 55:304-308.
28. Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119:115-119.
29. Wolfson, J. S., D. C. Hooper, M. N. Swartz, M. D. Swartz, and G. L. McHugh. 1983.. Novoblocin-induced elimination of F'lac and mini-F plasmids from Escherichia coli. *J. Bacteriol.* 156:1165-1170.

Fig. 2. Comparison of T. hyodysenteriae OMP extraction methods and growth conditions. Lanes: (1) Sonicated cell supernatant, (2) Zwittergent extract, (3) Triton - X 100 extract, (4) LiCl extract, (5) sarcosine extract, 12th in vitro passage (12P), (6) sarcosine extract, 135P, (7) swine reisolate sarcosine extracted, 2P, (8) novobloclin treated cells, sarcosine extract, (9) desferal mesylate treated cells, sarcosine extract (10) cells grown with fetal calf serum, not horse serum, sarcosine extract, (11) m.w. standard.

Fig. 3. Sarcosine extracts of T. innocens (Ti) and T. hyodysenteriae (Th) isolates. Lanes: (1) B234 serotype 1 (Th), (2) B204 serotype 2 (Th), (3) B256 (Ti), (4) 4/71 (Ti), (5) B169 serotype 3 (Th), (6) A1 serotype 4 (Th), (7) Taylor (Ti), (8) B359 (Ti), (9) Ack 300/8 serotype 7 (Th), (10) GS serotype 2 (Th), (11) B1555a (Ti), (12) B6571 (Ti), (13) m.w. standard.

Fig. 4. Lanes: (1) m.w. standard, (2) B256 sarcosine extract (T1), (3) B204 sarcosine extract (Th), (4) mixture of B256 and B204 sarcosine extracts, (5) sucrose gradient OMP of B204, (6) DCCD extract of B204.

1 2 3 4 5 6

66 kDa -

45 kDa -

29 kDa -

24 kDa -

20 kDa -

Fig. 5. Autoradiography of surface exposed OMPs of T. hyodysenteriae. Lane 1 - Autoradiography of log phase B204 cells. Lane 2 - B204 sarcosine extract for comparison (CBB stain).

1

2

Fig. 6. Transmission electron micrograph of sarcosine
extracted outer membrane from T. hyodysenteriae.
Magnification, x26,000

Table 1. OMPs detected by coomassie blue staining
of SDS-PAGE treponemal sarcosine extracts

<u>T. hyodysenteriae</u> B204	<u>T. innocens</u> B256
unique bands	unique bands
61 kDa - B169 only	49.5 kDa Ti only
46.5 Th only	47
45.5 Th only	45 (minor)
41	42
40 (minor)	39
39	37.5
36.5	36.5
36 (minor)	31.5
31	30
29	
26	

SECTION II. THE IMMUNE RESPONSE OF SWINE TO ANTIGENS
OF
Treponema hyodysenteriae

ABSTRACT

Outer membrane protein (OMP) extracts of Treponema hyodysenteriae were used to evaluate the protective immune response in pigs immune to swine dysentery. Western blot analysis was used to identify antigenic bands between 14 and 19 kilodalton (kDa) reactive with antibody in immunized and convalescent pigs. Colonic secretions also contained antibody which reacted with these antigens. These antigens were found to be located on the surface of the bacterium as assessed by absorption of serum with whole cells of T. hyodysenteriae. Treatment of the outer membrane extracts with proteinase K abolished reactivity to the 14 kDa and 19 kDa antigens. Lipase and sodium metaperiodate treatment of OMP antigens altered reactivity to the 14 kDa band. These results indicate that protection is related to the pig's immune response against a low molecular weight macromolecular complex of proteins and lipids.

INTRODUCTION

The etiologic agent of swine dysentery (SD) has been shown to be Treponema hyodysenteriae, an anaerobic to microaerophilic spirochete (4,16). The disease is characterized by a mucohemorrhagic diarrhea that can result in dehydration and death. Affected herds may experience up to 90% morbidity and 25% mortality (3). Most countries with modern swine production report the occurrence of SD, which remains as a serious economic problem for the pork industry (10).

Little is known about the OMP of the organism and their role in the development of the host's immune response. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of OMPs of other Gram-negative pathogens has resulted in the identification of potentially useful virulence or protective antigens (6). SDS-PAGE OMP preparations from T. hyodysenteriae were electroblotted onto nitrocellulose and examined with immune or convalescent sera to determine the cell surface moieties important to the immune response of pigs toward T. hyodysenteriae. These studies indicate that outer membrane components of T. hyodysenteriae are the antigens to which antibodies are produced which form the basis of protective immunity in swine.

MATERIALS AND METHODS

Bacterial strains All *T. hyodysenteriae* strains were from the culture collection of the Veterinary Medical Research Institute, Iowa State Univ., Ames, Iowa. Cultures were stored in growth medium at -70 C. Cultures were grown in anaerobically prepared trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) supplemented with VPI salt solutions A (0.2 g CaCl_2 and 0.2 g $\text{MgSO}_4/500\text{ml}$) and B (1g K_2HPO_4 , KH_2PO_4 , 10 g NaHCO_3 , and 2 g $\text{NaCl}/500\text{ml}$), 0.5% yeast extract (BBL), 0.05% cysteine-HCl (Sigma Chemical Co., St. Louis, MO) and 5.0% horse serum (HyClone Inc., Logan, Utah), (TSBYS), at 37C for 18-24 hrs.

Outer membrane protein preparation Cells were extracted with sodium-N-lauryl sarcosinate (sarcosine) (Sigma) according to a modification of the methods of Cooper et al. (2). In brief, washed cells were spheroplasted using 0.2% lysozyme (Sigma) in cold 10mM Tris (pH 7.4) with 8% sucrose. The mixture was incubated for 5 min on ice after which 3 volumes of cold water were added. The spheroplasts were disrupted by sonification (Model W225R, Heat Systems-Ultrasonics Inc., Farmingdale, NY) at 50% maximum output with four bursts of 15 sec each, while cooling in an ice bath. Whole cells and

debris were removed by centrifugation at 1500 x g for 20 min at 20C. The supernatant fluid was centrifuged at 40,000 x g for 45 min at 4 C and the pellet was resuspended in 10mM Tris with 1% sarcosine. The mixture was incubated, stirring at room temperature for 30 min and then centrifuged at 100,000 x g for 2 hrs at 4 C. The pellet was collected and washed by centrifugation at 100,000 x g 3 more times in 0.15M NaCl and resuspended in 0.15M NaCl.

Lithium chloride extracts of T. hyodysenteriae strains B234 (serotype 1), B204 (serotype 2) and A1 (serotype 4), were prepared by the addition of 1M LiCl in 10mM Tris-HCl buffer, pH 7.4 according to the method of McDade and Johnston (14). Glass beads were added to the mixture which was then shaken for 2 hrs at 37 C. The supernatant was collected and the beads were rinsed with 10mM Tris. The supernatant fluid and rinse were then centrifuged at 12,000 x g for 20 min at 4 C. The supernatant fluid was collected and centrifuged at 40,000 x g for 45 min at 4 C. The supernatant fluid was then centrifuged at 100,000 x g for 2 hrs at 4 C. The pellet was washed three times with 0.15 M NaCl and resuspended in 0.15 M NaCl. Lipopolysaccharide from T. hyodysenteriae was prepared by the method of Baum and Joens (1).

OMP extracts were suspended to a concentration of 0.1 to 0.8 mg/ml protein and stored at -70 C. Protein concentrations were determined by a Coomassie G-250 dye binding protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (BSA) as a standard.

Hemolysin preparation Hemolysin was extracted from T. hyodysenteriae by the method of Kent and Lemcke (7). Briefly, an 18 hr motile culture was pelleted by centrifugation (10,000 x g) and then resuspended with 0.15M phosphate buffered saline (PBS) containing 0.05% RNA core Type 2-C (Sigma). This suspension was incubated for 30 min at 37 C, then the mixture was centrifuged at 10,000 x g for 20 min and the supernatant fluid containing the hemolysin was collected. The hemolysin was lyophilized and stored at -20 C. A 1 mg/ml solution of the hemolysin in PBS was prepared for vaccination or electrophoresis.

Animals All swine used in these experiments were crossbred, specific pathogen free, weighed approximately 30 lbs. at the beginning of the experiments and were acquired from H & K Enterprises, Ames, IA. Animals were fed a commercial feed containing 16% protein without added antibiotics. Experiment 1 contained 6 pigs, 3

vaccinates and 3 controls. Experiment 2 consisted of 15 pigs, 10 vaccinates and 5 controls.

Infection protocol Pigs were challenged with fresh 18 hr cultures of T. hyodysenteriae grown in TSBYS. A sample of the culture was counted using a Petroff-Hauser counting chamber to determine the approximate number of bacteria per ml of suspension. The suspension was diluted, if necessary, with anaerobic, sterile TSBYS to yield approximately 10^8 organisms/ml. Pigs were infected on two consecutive days with 100 ml of TSBYS containing 10^8 T. hyodysenteriae organisms given through a stomach tube. Rectal swabs were taken periodically to monitor infection. These were examined by darkfield microscopy and cultured on blood agar. Pigs were examined daily after infection for clinical signs of swine dysentery evidenced by fecal consistency. A score from 0 (normal stool) to 3 (watery diarrhea) was noted for each pig.

Vaccination and absorption of sera In the first experiment three pigs were vaccinated with 1 mg of protein of the sarcosine preparation intramuscularly in the neck (day 0) and three control pigs were not immunized. Secondary vaccinations were given to the immunized pigs 14 days later with the same preparation.

All pigs were challenged 7 days after the second vaccination (day 21) as described above. Sera were also collected 2 weeks after infection (day 35).

Absorption of swine sera was accomplished by incubation of the sera with whole cells of the homologous strain of *T. hyodysenteriae* used for infection. Log phase *T. hyodysenteriae* grown in TSBYS were centrifuged for 20 min at 12,000 x g and resuspended to a concentration of approximately 10^{10} /ml in 10mM Tris pH 7.4. One ml amounts were centrifuged (Eppendorf Model 5412, Brinkman Instruments, Inc., Westburg, NY) for 10 min. The cell pellet was resuspended in 0.5 ml 10mM Tris pH 7.4 and 0.5 ml of heat inactivated serum (56 C for 30 min) and incubated for 90 min at 37 C. The serum samples were clarified by centrifuged and stored at -20 C (11).

SDS-PAGE Discontinuous SDS-PAGE was performed with a 4% stacking gel and 10% separating gel and the two buffer system of Laemmli (9). A Hoefer SE 600 vertical slab electrophoresis chamber was used (Hoefer Scientific Instruments, San Francisco, CA). The OMP fractions were solubilized by boiling at 100 C for 5 min in 0.0625M Tris sample buffer containing 2% sodium dodecyl sulfate (SDS, Sigma), 5% 2-mercaptoethanol and 10% glycerol, pH 6.8.

Samples were loaded into wells at a concentration of 15-20 ug protein per lane and electrophoresed at a

constant current of 30mA per gel until the tracking dye (bromphenol blue, Fisher Scientific Inc., Fairlawn, N.J.) was approximately 1.5 cm from the bottom of the gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R250 (CBB, Kodak Chemical Co., Rochester, N.Y.). Carbohydrate or lipid bands were visualized by the method of Tsai and Frasch (18). Apparent molecular weights were calculated by comparison to standards of known molecular mass (Dalton Mark 7-L, Sigma) containing: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and alpha-lactalbumin (14.2 kDa).

Immunoblotting After separation by SDS-PAGE, OMP extracts were electrophoretically transferred to nitrocellulose sheets (0.45 um pore size, Schleicher and Schuell, Inc., Keene, NH) using a modification of the procedures of Towbin et al. (17). The transfer buffer was 25M sodium phosphate, with 20% methanol, pH 6.8. Gels were washed in 3 changes of transfer buffer for 15 min each and then transferred for 18 hrs in a transblot cell (Hoefer Inc., San Francisco, CA) at a constant amperage of 0.75 amps.

After the transfer, the nitrocellulose was washed in 0.15M NaCl, pH 6.8 (wash buffer) 3 times, for 15 min. The nitrocellulose was then blocked with 0.5% BSA in wash buffer for 30 min at room temperature and then washed 3 times as before. The nitrocellulose was then cut into strips and incubated with diluted sera, milk or colonic contents in wash buffer for 2 hr at 37 C. Strips were then washed 3 times as before and then incubated for 1 hr with horseradish peroxidase conjugated rabbit anti-swine IgG, heavy (H) and light (L) chain specific (Cappel Laboratories, Cochranville, PA) diluted 1:1000 in wash buffer.

For the determination of the isotypic response in colonic material and immune sows milk, a monoclonal antibody, developed and provided by Dr. P. Paul, Iowa State Univ., was used. Ascites fluid, containing monoclonal antibodies to swine IgA was diluted in wash buffer and incubated with the blot strips for 2 hr at 37 C. Strips were washed 3 times and then incubated for 1 hr with horseradish peroxidase conjugated anti-mouse IgG, H and L chain specific (Southern Biotechnology Associates, Fisher Scientific Co., Fairlawn, N.J.) diluted 1:2000 in wash buffer. Strips were then washed 3 times.

Specific antigens were visualized by incubation with substrate (25 ug/ml of o-dianisidine (Sigma), 0.01% H₂O₂ in 10mM Tris pH 7.4) for 10-20 min. The reaction was terminated by washing with several changes of water. The blots were dried between filter paper and stored in the dark. The efficiency of transfer was determined by reacting one nitrocellulose strip for 2 hr with Pelikan Fount India Ink (Pelikon AG, Hanover, Germany) at a concentration of 1 ul/ml in wash buffer.

Antibody samples Antibody samples were collected from pigs as pre-immunization, pre-infection, post-immunization, post-infection serum and convalescent sera. Colonic washes from protected swine were collected as described below. Milk samples from convalescent sows were from the antisera library of the Veterinary Medical Research Institute, Iowa State Univ. All serum samples were removed from clotted blood, aliquoted and frozen at -20 C. Milk samples were collected from nursing sows and frozen in aliquots at -20 C.

Colonic washes were collected at necropsy from the contents of the spiral colon and cecum of pigs. The contents were diluted with equal volumes of cold PBS with 0.02M phenyl-methyl-sulfonyl fluoride (PMSF) and 50 ug/ml dithiothreitol (DTT, 0.324mM, Sigma) and stirred for 1 hr in the cold. The mixture was then centrifuged

at 10,000 x g for 30 min. The supernatant was collected and placed in dialysis tubing (Fisher) with 12 kDa to 14 kDa cut off and dialyzed against PBS. After 48 hrs, the dialysed material was concentrated 10-fold by covering the dialysis tubing with polyethylene glycol-6000 (Sigma). The concentrated supernatant was then aliquoted and stored at -20 C.

Enzyme and periodate treatment of outer membranes

The sarcosine extract was treated with proteinase K (5) for 30 min at 60 C. The OMP was also treated with 0.01M sodium meta periodate (Fisher) for 18-20 hr at 4 C prior to solubilization in the SDS-PAGE treatment buffer (13). Bovine serum albumin (BSA) was also subjected to proteinase K and periodate treatment to see the effects of these treatments on antibody binding to this antigen. Treated BSA was subjected to SDS-PAGE and transferred to nitrocellulose for western blot analysis with rabbit anti-serum prepared to fetal calf serum. Nitrocellulose membrane strips containing the components of the sarcosine extract of *T. hyodysenteriae* were incubated with various enzymes immediately after electrophoretic transfer and then incubated with porcine convalescent serum. Strips with absorbed antigens were incubated with 0.5 mg/ml trypsin, 1 mg/ml lipase, 1 mg/ml

beta-glucuronidase or 1 unit/ml neuraminidase for 30 min at 37C (Sigma) (19).

RESULTS

Vaccination and challenge Non-immunized, T. hyodysenteriae infected pigs developed clinical signs of disease within 14 days of infection. One of the control pigs eventually died of complications associated with swine dysentery (Table 1). OMP vaccinated swine did not develop clinical signs of disease. This experiment was repeated with more pigs and similar protective responses to vaccination were noted (Table 1). Clinical signs of swine dysentery in immunized and non-immunized swine were recorded daily, based on fecal consistency. Vaccinated pigs had consistently lower clinical sign scores and fewer detectable T. hyodysenteriae (Table 1).

Western blot analysis Serum from OMP immunized swine, which were protected against subsequent challenge, and serum from pigs convalescent from swine dysentery, produced very similar western blot profiles (Fig. 1). This indicates that immunization with OMP provoked a response to the same antigens recognized by natural infection. Of particular interest were the bands appearing between 14-19 kDa (Fig. 1) that did not stain with Coomassie brilliant blue R250 (CBB) (data not shown). The vaccinated swine had developed serum antibodies to these antigens by day 14. Non-vaccinated, challenged

swine did not produce antibody to these bands by day 35 (Fig. 1). Serum samples from both immunized and convalescent swine had antibodies to these antigens.

The nature of these 14-19 kDa bands was investigated by treating the OMP with proteinase K or with 0.01M sodium meta periodate prior to solubilization in the SDS-PAGE treatment buffer (Fig. 2). The proteinase K treatment destroyed reactivity to the antigens while oxidation of carbohydrate molecules by periodate treatment had little effect. SDS-PAGE of the proteinase K or periodate treated OMPs followed by Coomassie staining showed that the overall OMP profile was affected by periodate and was destroyed by proteinase K and silver staining of the gel revealed that lipid and carbohydrate remained intact (data not shown). When bovine serum albumin (BSA, Sigma) was treated with proteinase K and periodate and probed with rabbit antiserum, the antigenicity of the BSA was lost with both treatments (data not shown). To better define the nature of the 14-19 kDa antigens, nitrocellulose strips containing OM antigens, were treated with trypsin, the mollusk saccharidase beta-glucuronidase, neuraminidase and lipase, before reacting with convalescent sera. Strips were also incubated with milk from sows that provided a protective lactogenic immunity to piglets challenged with

I. hyodysenteriae, and milk that did not provide protective immunity to piglets (Fig. 3).

The blot profile was not affected by treatment with beta-glucuronidase, indicating that the antigens do not contain carbohydrates sensitive to this enzyme. The blot was also unaffected by treatment with neuraminidase, used to test whether transferred antigens were glycoprotein containing sialic acid. Trypsin treatment of the blot prevented binding of antibody to all antigens except those at 14, 19, 39, 40 and 45.5 kDa. Intensity of the reaction with the trypsin treated 14 kDa band was decreased, however. This indicates that many of the OM antigens are proteins sensitive to the action of trypsin. Lipase treatment of the nitrocellulose strips reduced binding of antibody to all antigens except those at 19, 39, 40 and 45.5 kDa. This indicates the lipid nature of many of the outer membrane antigens. Of particular interest is the effect of lipase on the 14 kDa antigen which is changed so as to abrogate most of the binding of antibody.

Absorption of the convalescent serum with whole I. hyodysenteriae cells removed antibodies reactive with all antigens except those at 29, 31, 37, 39 and 45.5 kDa (Fig. 3), however, the antigens at 37, 39 and 45.5 kDa are often stained by o-dianisidine non-specifically.

This indicates that only antigens at 29 and 31 kDa are not surface exposed. Radiolabeling of surface proteins with iodine-125 substantiates this finding (Hubbard, unpublished data). Strips were also reacted with protective or non-protective sow milk. Antigenic reactivity was identical except that milk from non-protective sows lacked antibody to the 14 and 19 kDa antigens, which was present in the protective sow milk (Fig. 3).

Western blot tests were also performed using colonic antibody collected from convalescent swine and developed with anti-swine IgA specific monoclonal antibodies. These blot tests revealed bands of specific anti-T. hyodysenteriae IgA directed at the 14-19 kDa antigens (Fig. 4). Milk antibody from convalescent sows was also used to probe transferred T. hyodysenteriae OMPs for specific IgA antibody. These procedures revealed a specific IgA response to antigens at 31 kDa (Fig. 4). Normal sows milk or normal colonic wash from pigs that were not exposed to T. hyodysenteriae did not contain antibody to T. hyodysenteriae OMP antigens in western blot tests (data not shown).

T. hyodysenteriae hemolysin, LPS or LiCl outer membrane extracts of two other serotypes were run on SDS-PAGE and transferred to nitrocellulose. The

nitrocellulose was then reacted with convalescent serum. The resulting reaction indicated the presence of the 14 and 19 kDa antigen in three serotypes of *I.*

hyodysenteriae (Fig. 5). The reaction also indicated the presence of a 19 kDa antigen in the LPS preparation and a 14 kDa antigen in the hemolysin preparation. The hemolysin preparation also had antigens at 55, 61 and 66 kDa.

DISCUSSION

The sarcosine extracted OMP's from T. hyodysenteriae, used to vaccinate swine, induced immunity to T. hyodysenteriae. In a limited experiment, 3 pigs vaccinated with this preparation were protected from infection while 3 control pigs in the experiment developed clinical signs of swine dysentery. Western blot analyses of the serum samples from these pigs revealed qualitative differences in the antibody responses between the two groups of pigs. Further experiments have substantiated these findings (data not shown).

All 6 pigs in this experiment had preimmunization antibodies cross-reactive with antigens of T. hyodysenteriae. These antibodies may have been induced by other bacteria that cross react with T. hyodysenteriae or by previous undetected exposure to the specific organism. This latter possibility is unlikely since all pigs were culturally negative for T. hyodysenteriae prior to experimentation. Another possible explanation would be the appearance of hidden protein epitopes that cross-react with other organisms exposed following denaturation of the OMPs during SDS-PAGE. Detection of antibodies which react with several T. hyodysenteriae OMPs in sera from noninfected pigs was not unusual.

Similar findings have been reported when normal pre-exposure or acute phase human sera have been tested for antibodies to OMPs of H. influenzae type b (12), Campylobacter jejuni (15) and N. meningitidis (18).

Only the immunized pigs in both experiments developed antibodies to low molecular weight (14 and 19 kDa) T. hyodysenteriae antigens. The control pigs failed to develop antibodies to these antigens but the pigs did respond to many of the other T. hyodysenteriae antigens.

These results indicate these low molecular weight antigens are important in the protective immune response to T. hyodysenteriae. Sodium meta-periodate or proteinase K treatment of the OMP preparation before western blot analysis indicated that these epitopes are at least partially proteinaceous, and may contain limited carbohydrate affected by oxidation with periodate. It appears however, that periodate can affect the antigenicity of protein antigens, as evidenced by the abrogation of antibody reactive with BSA after periodate treatment (data not shown). Lipase treatment of the OMP on nitrocellulose strips indicated that these antigens are probably lipid associated (Fig. 3, lane 5). Wu and Heath (21) reported that the lipid A associated protein (LAP), from the outer membrane of Gram-negative organisms, is sensitive to pronase digestion. Wober and

Alaupovic (20) have shown that the further the proteinaceous antigenic sites are from the lipid A the more they are destroyed by pronase treatment. If the low molecular weight antigens of T. hyodysenteriae were composed of LAP it could explain why they were lost by proteinase K and lipase treatment. The reason this material does not stain with CBB may be due to its low concentration, or that it lacks enough aromatic amino acids to be adequately stained by CBB.

Comparison of the OMP profiles with partially purified T. hyodysenteriae antigen revealed that some portions of the LPS as well as the hemolysin of T. hyodysenteriae migrate in the same region as the protective antigens. Western blot tests of these antigens indicate the presence of antibodies to both of these antigens in convalescent serum (Fig. 5).

In an effort to examine whether pigs respond to T. hyodysenteriae infection with a specific anti-T. hyodysenteriae secretory IgA immune response in the colon, colonic samples were collected at necropsy from infected pigs and analyzed for T. hyodysenteriae specific IgA. In swine protected by previous immunization, IgA directed against T. hyodysenteriae antigens were observed. These antibodies were directed against the same low molecular weight antigens as the serum

antibodies (Fig. 4). This is further evidence that antibody to these antigens is important in protection. This also supports the role of secretory IgA in the protective immune response against T. hyodysenteriae. Protective sows milk also contained antibody to a 31 kDa antigen. Antibody to this antigen was also detected in non-protective sows milk which indicates this antigen does not play a role in protection.

It had been previously shown that porcine colonic washings contained IgG to multiple antigens of T. hyodysenteriae (6). The presence of anti-T. hyodysenteriae IgA in colonic washings would be expected to inhibit the organisms ability to colonize the mucosa, however, plasma leakage into the intestinal lumen during early dysentery may allow anti-T. hyodysenteriae serum antibody to exert a protective effect as well.

It is apparent from current work that a specific immune response is elicited to various antigens of T. hyodysenteriae after infection or immunization. Of particular interest are the antigens at 14 and 19 kDa, which may be protective antigens. These antigens may also be virulence factors that elicit protective responses in pigs measured by the presence of antibodies in the serum, milk and colon. It appears necessary to stimulate an immune response to these antigens in

attempts to protect swine from swine dysentery by immunization. Whether the individual antigens detected by convalescent or immunized pig sera are protective against disease needs to be determined. This may be possible by immunization with purified or cloned antigens or by passive protection with monoclonal antibodies specific for these antigens.

REFERENCES

1. Baum D. H., and L. A. Joens. 1979. Serotypes of beta - hemolytic Treponema hyodysenteriae. Infect. Immun. 25:792-796.
2. Cooper, M. D., M. J. Wannemuehler, R. D. Miller, and M. F. Fedyk. 1981. Role of outer envelope contamination in protection elicited by ribosomal preparation against Neisseria gonorrhoeae infection. Infect. Immun. 32:173-179.
3. Harris, D. L., and R. D. Glock. 1973. Swine Dysentery. Vet. Scope 17:2-7.
4. Harris, D. L., R. D. Glock, C. R. Christensen, and J. M. Kinyon. 1972. Swine Dysentery: Inoculation of pigs with Treponema hyodysenteriae (new species) and reproduction of the disease. Vet. Med. Small Anim. Clin. 67:61-64.
5. Hitchcock, P. J., and T. M. Brown. 1983. Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154:269-277.
6. Joens, L. A., and R. B. Marquez. 1986. Molecular characterization of proteins from porcine spirochetes. Infect. Immun. 54:893-896.
7. Kent, K. A., and R. M. Lemcke. 1984. Purification and cytotoxic activity of a haemolysin produced by Treponema hyodysenteriae. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. 8:185.
8. Kunkle, R. A., and J. M. Kinyon. 1986. BJ; selective medium for isolation of Treponema hyodysenteriae. Proc. 67th Conf. Res. Workers in Anim. Dis., Chicago, Ill.
9. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
10. Liven, R. 1979. Prevalence of Treponema hyodysenteriae in healthy pigs. ACTA Vet. Scand. 20:387-395.
11. Loeb, M. R. 1984. Immunoblot method for identifying surface components, determining their

- cross-reactivity, and investigating all topology: results with Haemophilus influenzae type b. Infect. Immun. 43:1032-1036.
12. Loeb, M. R., and D. H. Smith. 1982. Human antibody response to individual outer membrane proteins of Haemophilus influenzae type b. Infect. Immun. 37:1032-1036.
 13. Maeland, J. A. 1968. Antigenic properties of various preparations of Neisseria gonorrhoeae endotoxin. ACTA Pathol. Microbiol. Scand. 73:413-422.
 14. McDade, R. L., Jr., and K. H. Johnston. 1980. Characterization of serologically dominant outer membrane proteins of Neisseria gonorrhoeae infection. J. of Bacteriol. 141:1183-1191.
 15. Mills, S. D., and W. C. Bradbury. 1984. Human antibody response to outer membrane proteins of Campylobacter jejuni during infection. Infect. Immun. 43:739-743.
 16. Stanton, T. B. 1987. Erythrocytes as a source of essential lipids for Treponema hyodysenteriae. Infect. Immun. 55:304-308.
 17. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76:4350-4353.
 18. Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119:115-119.
 19. Wedge, E., and L. O. Froholm. 1986. Human antibody response to a group B serotype 2a meningococcal vaccine determined by immunoblotting. Infect. Immun. 51:571-578.
 20. Wise, K. S., and R. K. Watson. 1983. Mycoplasma hyorhinis GDL surface protein antigen P120 defined by monoclonal antibody. Infect. Immun. 41:1332-1339.

21. Wober, W., and P. Alaupovic. 1971. Studies on the protein moiety of endotoxin from gram negative bacteria. Eur. J. Biochem. 19:340-367.
22. Wu, M. C., and E. C. Heath. 1973. Isolation and characterization of lipopolysaccharide protein from Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 70:2572-2576.

Fig. 1. Western blot analysis of serum antibody against OM antigens of T. hyodysenteriae strain B204:
Lanes 1-6: Pre-immunization serums,
Lanes 7-9: Immunized pig serum two wks after immunization, before challenge,
Lanes 10-12: Serum from non-vaccinated pigs,
Lanes 13-15: Serum from vaccinated, immune pigs 2 wks after challenge,
Lanes 16-17: Serum from pigs with acute swine dysentery 2 wks after challenge.

Fig. 2. Effect of proteinase K and periodate treatment of T. hyodysenteriae strain B204 sarcosine OMP extract and T. innocens strain B256 sarcosine OMP extract vs. convalescent sera:

- Lane 1: Untreated B204 OMP
- Lane 2: Periodate treated B204 OMP
- Lane 3: Proteinase K treated B204 OMP
- Lane 4: Untreated B256 OMP
- Lane 5: Periodate treated B256 OMP
- Lane 6: Proteinase K treated B256 OMP

1 2 3 4 5 6

66kDa

45kDa

20kDa

14kDa

Fig. 3. Western blot of OMP from T. hyodysenteriae vs. convalescent swine sera (Lanes 1-6) or vs. sows milk (Lanes 7-8)

Lane 1: Untreated OMP

Lane 2: OMP vs. CSS absorbed with whole cells of T. hyodysenteriae

Lane 3: OMP blot treated with glucuronidase

Lane 4: OMP blot treated with trypsin

Lane 5: OMP blot treated with lipase

Lane 6: OMP blot treated with neuraminidase

Lane 7: OMP vs. protective sow milk

Lane 8: OMP vs. non-protective sow milk

Fig. 4. Western blot of OMP developed with specific anti
- swine IgA:
Lane 1: OMP vs protective sow milk
Lane 2: OMP vs colonic antibody from immunized
and challenged pig

Fig. 5. Western blots of T. hyodysenteriae antigens vs
convalescent swine serum:
Lane 1: B204 LiCl extracted OMP
Lane 2: B204 LPS
Lane 3: B204 hemolysin
Lane 4: A1 LiCl extracted OMP (serotype 4)
Lane 5: B234 LiCl extracted OMP (serotype 1)

1 2 3 4 5

19kDa-
14kDa-

Table 1. Clinical signs (ave.) and % culture positive OMP vaccinated and control pigs^a

Experiment 1:				
	Day 7		Day 14	
	Culture	CS	Culture	CS
V (n=3)	66%	0.33	66%	0.33
C (n=3)	100%	2.0	100%	3.0
				(1 dead)
Experiment 2:				
	Day 7		Day 14	
	Culture	CS	Culture	CS
V (n=10)	50%	1.3	60%	1.6
C (n=5)	80%	2.2	80%	2.25
				(1 dead)

^aClinical signs and culture detectable *T. hyodysenteriae* in OMP immunized (V) and non-immunized (C) swine, listed as days after challenge. Clinical signs (CS) was an average fecal score based on a scale from 0 (normal stool) to 3 (watery diarrhea).

SECTION III. SEROTYPE SPECIFIC PROTECTION OF MICE
FROM INFECTION WITH Treponema hyodysenteriae
WITH MONOCLONAL ANTIBODY

ABSTRACT

Resistance to Treponema hyodysenteriae infection was studied using a murine model. Serotype specific monoclonal antibody (18.2) was used to passively protect mice (C3H/HeN) from infection with homologous serotype organisms (T. hyodysenteriae, B78, serotype 1). The monoclonal antibody did not protect mice from infection with a heterologous serotype (B204, serotype 2) organism. The protective response was also dose dependent. In vitro cultivation of T. hyodysenteriae in the presence of this monoclonal antibody caused a decrease in motility and viability of the organism. These results indicate that animals can be protected from infection with T. hyodysenteriae by passive administration of monoclonal antibodies.

INTRODUCTION

Treponema hyodysenteriae causes a mucohemorrhagic diarrheal disease of swine known as swine dysentery (6,18). Several serotypes of the organism have been reported (2,13,15), and serotype specific protection of pigs to challenge has been shown (7). The mouse has been proposed as a model system for infectivity and pathogenicity of T. hyodysenteriae (11,16).

This study was conducted to examine the effect of a T. hyodysenteriae specific monoclonal antibody on the development of the disease in an experimental mouse model. The effect of the antibody on organisms grown in vitro was also studied.

MATERIALS AND METHODS

Bacteria T. hyodysenteriae strains B78 (serotype 1) and B204 (serotype 2) were from the culture collection of Dr. M. Wannemuehler, Iowa State University, Ames, Iowa. Organisms were cultivated in trypticase soy broth (BBL Microbiology System, Cockeysville, Md.) supplemented with 5% heat-inactivated horse serum (HyClone Inc., Logan, Utah), 0.5% yeast extract (BBL), 0.05% L-cysteine (Sigma Chemical Co., St. Louis, Mo.) (TSBYS) anaerobically, at 37 C. Cultures were grown to log phase (approximately 1.0×10^8 motile cells per ml). In vitro growth was measured spectrophotometrically at 600 nm (Spec 20, Bausch & Lomb, Rochester, N.Y.).

Mice and infection protocol Mice, C3H/HeN, were produced and maintained in the animal care facilities of the College of Veterinary Med., Iowa State Univ., were housed in plastic cages, bedded in ground corn cobs at four mice per cage, and fed a standard mouse ration. Four to 8 weeks old mice were challenged with 10^8 T. hyodysenteriae on two consecutive days. Mice were fasted and then inoculated intragastrically using a stomach tube with 1 ml of culture on two consecutive days (approximately 10^8 organisms). Food was returned to the mice 1 day after the second inoculation.

Mice were killed by cervical dislocation ten days after they were infected. The cecum was examined for gross lesions, consisting primarily of a profuse mucus exudate and cecal atrophy, and the contents were examined by darkfield microscopy and cultured to detect the number of T. hyodysenteriae colonizing the cecum.

Pour plate counts of T. hyodysenteriae colonizing the cecum were accomplished by the following procedure. The cecum were removed from the mice and individually weighed in sterile plastic bags. A 1:100 dilution (wt/vol) was made in the bags by the addition of 0.01 M phosphate buffered saline, pH 7.4 (PBS). The cecums was disrupted in a stomacher tissue homogenizer (A. J. Steward Co., London) for 1 min. Serial dilutions of this suspension were made with PBS. Agar tubes, consisting of 5 ml of TSBY and 0.6% Bactoagar (Difco, Detroit, Mich.), were melted and equilibrated to 45 C in a water bath. Sterile antibiotics and sheep red blood cells (SRBC) were added to the agar tubes in appropriate concentrations along with 0.1 ml of the diluted cecal contents in duplicate. This suspension was then poured into 60 x 15 mm sterile petri dishes and allowed to cool and harden. Plates were incubated for 4 to 6 days at 37 C in anaerobic jars in an atmosphere of 10% CO₂, 10% hydrogen

and 80% nitrogen with palladium catalysts. Colonies were then counted.

The concentrations of antibiotics (Sigma) used in the blood agar were spectinomycin (200 ug/ml), colistin methanesulfonate (250 ug/ml), vancomycin (250 ug/ml), rifampicin (500 ug/ml) and spiramycin (100 ug/ml), (Sigma) (13). The final concentration of sheep red blood cells was 5%.

Monoclonal antibody Monoclonal antibodies (MAB) reactive to strains of T. hyodysenteriae were developed by the method of Colwell et al. (3). Briefly, BALB/c mice were immunized with lipopolysaccharide from T. hyodysenteriae and then revaccinated two weeks later, 24 hrs before the fusion. Spleen cells from the mice were fused with a non-immunoglobulin secreting myeloma cell line (X63-Ag8.653) in the presence of polyethylene glycol 1500 (Boehringer Mannheim, West Germany). The fused cells were incubated in the presence of hypoxanthine, aminopterin, thymidine (HAT) supplement (Sigma). Culture supernatant from wells displaying hybrids was tested for the presence of antigen-specific antibody by the enzyme-labeled immunosorbent assay (ELISA) (5).

ELISA Whole cells of T. hyodysenteriae strains or T. innocens strains were coated on 96 well microtiter

plates (Dynatech Laboratories Inc., Alexandria, VA) at a concentration of 10 ug dry wt. per well in 100 ul volume. Undiluted hybridoma culture supernatants were added to wells of the ELISA plate in duplicate and incubated for 4 hrs at 37 C or overnight at 4 C. Plates were washed 3 times and blotted dry. Affinity purified goat anti-mouse Ig labeled with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, Ala., SBA) was added to appropriate wells. Plates were incubated for 1 hr at 37 C and then washed as before. Substrate was prepared fresh by dissolving p-nitrophenyl phosphate (1 mg/ml; Sigma 104 phosphatase substrate) in 0.05M sodium carbonate buffer with 0.001M $MgCl_2$ (pH 9.3) and added to each well in 100 ul volumes and incubated for 100 min at 37 C. The resulting absorbance was read at 405 nm using a Biotek EL310 photometer (Biotek Instruments, Winooski, Vt.).

Passive immunization Monoclonal antibodies, 18.2 and 41B.7, were used to passively immunize mice. Both antibodies were of the IgM isotype, however, MAB 18.2 is reactive only to serotype 1 *T. hyodysenteriae* while MAB 41B.7 reacts with all serotypes of the organism. Monoclonal antibody was precipitated from culture supernatant by the addition of saturated ammonium sulfate to 40%, followed by exhaustive dialysis against 0.5M

phosphate buffered saline, pH 7.4. Mice received various doses of MAB in 0.1 ml volumes 2 days prior to challenge. Mice were passively immunized by various routes with 0.1 ml of various concentrations of antibody (Ab) from 0 to 200 ug protein.

Transmission electron microscopy A mouse cecum with typical gross lesions was prepared for transmission electron microscopy by fixation in equal parts of 3.6% glutaraldehyde in distilled water, 0.2M Na cacodylate buffer, pH 6.5, and 1.5 mg/ml ruthenium red in distilled water for 2 hr. After fixation the tissue was stained with OsO₄, dehydrated and examined.

Cyclophosphamide treatment of mice and fluorescent antibody test Two groups of mice were given 100 ug/kg of cyclophosphamide (Cytoxan- Bristol Meyers, Syracuse, N.Y.) intravenously (i.v.). One group was left untreated. Two days later all mice were inoculated with T. hyodysenteriae strain B78 on two consecutive days. On the next day half of the treated mice were passively immunized i.v. with either 5 or 50 ug of monoclonal antibody. Ten days later the mice were killed and their ceca were removed. Cecal contents were diluted 1:100 (wt/vol) in PBS and then spread on glass microscope slides with cotton applicators. After the slides air

dried they were fixed in ethanol, chloroform, and formalin (6:3:1) for 5 min followed by three washes of 5 min in methanol. Slides were stored at -70 C until used. Slides were stained with FITC labeled goat anti-mouse Ig (SBA) and examined with a fluorescent microscope. T. hyodysenteriae cells were identified by their characteristic spiral morphology.

The in vitro effect of monoclonal antibody on T. hyodysenteriae The effect of monoclonal antibody on T. hyodysenteriae cultivated in vitro was examined by incorporating 5 or 50 ug/ml protein of 18.2 MAB, or 50 ug/ml protein of 41B.7 MAB, in 10 ml of TSBYS with or without the addition of guinea pig complement (C'). After the addition of 1×10^6 log phase T. hyodysenteriae strain B78, plate counts of remaining viable bacteria were taken at 3 hr and the optical density (O.D.) measured hourly at 600 nm. The motility of the organisms was also monitored hourly for the first three hours of incubation by darkfield examination of a wet mount from each tube and counting the percent motile organisms in 200 spirochetes (Table 3).

RESULTS

Passive protection by various routes Mice were passively immunized by various routes. All routes showed decreases in the percentage of mice with gross cecitis, percent culture positive and percent darkfield positive (Table 1). Cecitis was present in 77.8% of the control mice. Examination of the cecum of a control mouse by electron microscopy revealed the presence of spirochetes in the crypts of the cecum, apparently moving through the profuse mucous exudate (Fig. 1), and under necrotic cells in the crypt wall (Fig. 2). Diarrhea was not produced in any of the mice and no gross signs were evident in the small or large intestine.

T. hyodysenteriae was detected by culture and by direct examination with darkfield microscopy in 55.6% and 66.7% of control mice respectively. Fewer of the antibody treated mice had cecitis, ranging from 25% for the i.v. treatment group, 28.5 % for the intragastric (i.g.) group and 37.5% for the intraperitoneally (i.p.) treated group. The antibody treated groups also had fewer mice with detectable T. hyodysenteriae (Table 1). The group treated with antibody by the i.v. route had the lowest percent cecitis and detectable T. hyodysenteriae so this route was chosen for further experiments.

The amount of antibody given i.v. which is necessary to provide protection from T. hyodysenteriae was examined (Table 2). The serotype specificity of the protective response was also examined by infecting one group of mice with a heterologous serotype T. hyodysenteriae, strain B204. Infection with the heterologous serotype organism was not hindered by 100 ug of MAB 18.2.

The 18.2 MAB had no protective effect below 100 ug protein. At this level of antibody all mice were protected from cecitis and only 1 of 8 had T. hyodysenteriae detectable by culture. Monoclonal antibody, 41B.7, reactive against both T. hyodysenteriae serotypes 1 and 2, did not protect mice from colonization with serotype 1 T. hyodysenteriae.

Motility of the organisms incubated for three hours in the presence of 50 ug/ml of the MAB 18.2 decreased to 60% in the presence of guinea pig complement and 67% without complement (Table 3). Motility of the organisms decreased steadily during incubation. Organisms incubated in the same media without antibody and with or without complement were 87% motile after three hours while organisms grown in the presence of 5 ug/ml MAB had fewer motile organisms than controls but more motile organisms than the 50 ug/ml group. Tubes that contained guinea pig complement had lower viable cell counts after

three hours incubation and tubes that contained both antibody and complement also had lower viable cell counts than controls (Table 3). The tubes containing the serotype specific antibody 18.2 had higher viable cell counts when also incubated with complement than did the 41B.7 MAB (Table 3).

Cecal smears of cyclophosphamide treated or control mice infected with T. hyodysenteriae followed by i.v. injection of MAB 18.2 were stained with FITC - labeled anti-mouse antibody. Examination of the slides revealed the presence of mouse antibody on bacteria with the characteristic spirochete morphology. Mice that were treated with cyclophosphamide but not passively immunized with MAB did not have detectable antibody labeled spirochetes, although these mice had T. hyodysenteriae present in their ceca as detected by blood agar plate culture.

DISCUSSION

When mice were infected with T. hyodysenteriae, strain B78 (serotype 1), and examined by electron microscopy, spirochetes were seen in intestinal crypts and under necrotic, sloughing epithelial cells. Albassam et al. (1) noted similar findings by electron microscopic examination of intestines from experimentally infected pigs. Mice which were passively immunized with the serotype specific MAB 18.2, had fewer indications of colitis and fewer incidents of detectable T. hyodysenteriae in their ceca than non-antibody treated mice. It was found that the best route of antibody administration, to provide protection of the mice from infection with a subsequent challenge of T. hyodysenteriae, was by i.v. injection. The amount of antibody needed to prevent colonization with the organism was at least 100 ug of protein of the ammonium sulfate precipitated and concentrated antibody. A heterologous serotype (serotype 2, strain B204) T. hyodysenteriae was not eliminated from the ceca of infected mice by administration of 100 ug protein of MAB 18.2. When a T. hyodysenteriae specific MAB was used that was not serotype specific, it did not protect or lead to elimination of the organism from the ceca of infected mice.

In vitro growth studies indicated that the serotype specific antibody, 18.2, did have a killing effect on the bacteria after three hours of growth in the presence of guinea pig complement, as evidenced by lower plate counts. The optical density of the media containing the growing organisms did not vary greatly between groups. However, the percent of motile bacteria did vary with treatments, with a drastic reduction when organisms were incubated with antibody and complement. Motility was less in the presence of 50 ug/ml MAB than 5 ug/ml MAB. Viable cell counts and motility were also lower in the presence of MAB 41B.7 and complement. It was apparent that both MABs fixed complement in vitro, which had a deleterious effect on the organisms. However, in vivo only the serotype specific MAB protected mice, which argues against a prominent role for complement in in vivo protection, at least with the murine disease model.

Fluorescent antibody staining of cecal smears of infected mice treated with cyclophosphamide and then treated with MAB 18.2 revealed antibody on the surface of spirochetes present in the mouse ceca. Since cyclophosphamide inhibits antibody production, the antibody detected on the surface of the spirochetes should be passively administered MAB that entered the cecum after i.v. inoculation. This experiment provides

evidence that serum antibody may enter the cecum during T. hyodysenteriae induced cecitis in mice. In the cecum this antibody can bind to the spirochete and may inhibit motility of the organism. The antibody may also act to kill T. hyodysenteriae cells in the presence of complement. Others have used MAB, passively administered intraperitoneally (i.p.), to protect mice from a previous i.p. challenge by K1 - encapsulated Escherichia coli with some success (12). Also i.p. administered MAB was used to protect mice from an intramuscular challenge with Clostridium chauvoei (17). The present work, showing protection in the intestinal tract to a non-invasive pathogen, is an indication that i.v. administered MAB can have an effect in the intestinal tract.

It has been reported that normal swine serum as a source of complement, plus specific heat-inactivated rabbit antiserum to formalinized whole cells of T. hyodysenteriae, or serum from hyperimmunized or convalescent swine, were lytic for T. hyodysenteriae in vitro (9). The monoclonal antibodies investigated here do have a killing effect in the presence of complement and also a inhibitory effect on motility of the organism. The inhibition of motility may result from death, inhibition of energy production by the organism, or

Inhibition of the fluidity of the undulating outer membrane covering the flagella of the organism.

Serotype specific protection from invasion of the lamina propria in swine ligated colonic loops has been shown in convalescent swine (10) or in susceptible loops inoculated with homologous serotype antisera (8). In the present study similar results were found in the mouse model using a serotype specific monoclonal antibody. This is consistent with earlier reports which showed that pigs which had recovered from swine dysentery were also immune to rechallenge with the homologous serotype (7) and possible heterologous serotypes (4). The lipopolysaccharide of the organism is the basis for serotype specificity in this species (2,14). These results indicate that the LPS is important in the pathogenesis of lesions in the mouse model.

REFERENCES

1. Albassam, M. A., H. J. Olander, H. L. Thacker, and J. J. Turek. 1985. Ultrastructural characterization of colonic lesions in pigs inoculated with Treponema hyodysenteriae. Can. J. Comp. Med. 49:384-390.
2. Baum, D. H., and L. A. Joens. 1979. Serotypes of beta-hemolytic Treponema hyodysenteriae. Infect. Immun. 25:792-796.
3. Colwell, D. E., K. A. Gollahon, J. R. McGhee, and S. M. Michalek. 1982. IgA hybridomas: A method for generation in high numbers. J. Immunol. Meth. 54:259-266.
4. Egan, I. T. 1981. Epidemiological aspects of swine dysentery in the midwestern United States. Ph.D. Dissertation. Iowa State Univ., Ames, Ia.
5. Engvall, E., and P. Perlmann. 1971. Enzyme-linked immunosorbant assay (ELISA): quantitative assay of immunoglobulin G. Immunochemistry 8:871-874.
6. Harris, D. L., R. D. Glock, C. R. Christensen, and J. M. Kinyon. 1972. Swine dysentery. I. Inoculation of pigs with Treponema hyodysenteriae (new species) and reproduction of the disease. Vet. Med. Small Anim. Clin. 67:61-64.
7. Joens, L. A., D. L. Harris, and D. H. Baum. 1979. Immunity to swine dysentery in recovered pigs. Am. J. Vet. Res. 40:1352-1354.
8. Joens, L. A., D. W. DeYoung, R. D. Glock, M. E. Mapother, J. D. Cramer, and A. E. Wilcox. 1985. Passive protection of segmented swine colonic loops against swine dysentery. Am. J. Vet. Res. 46:2369-2371.
9. Joens, L. A., and M. E. Nuessen. 1986. Bactericidal effect of normal swine sera on Treponema hyodysenteriae. Infect. Immun. 51:282-285.
10. Joens, L. A., S. C. Whipp, R. D. Glock, and M. E. Nuessen. 1983. Serotype-specific protection against Treponema hyodysenteriae infection in

- ligated colonic loops of pigs recovered from swine dysentery. Infect. Immun. 39:460-462.
11. Joens, L. A., R. D. Glock, and J. M. Kinyon. 1980. Differentiation of Treponema hyodysenteriae from Treponema innocens by enteropathogenicity testing in the CF1 mouse. Vet. Rec. 107:527-529.
 12. Kaufman, B. M., A. S. Cross, S. I. Futrovsky, H. F. Sldberry, and J. C. Sadoff. 1986. Monoclonal antibodies reactive with K1-encapsulated Escherichia coli lipopolysaccharide are opsonic and protect mice against lethal challenge. Infect. Immun. 52:617-619.
 13. Kunkle, B., and J. Kinyon. 1986. BJ: Selective media for the primary isolation of Treponema hyodysenteriae. Proc. Conf. Res. Workers in Anim. Dis., Abstr. 166.
 14. Lemcke, R. M., and J. Bew. 1984. Antigenic differences among isolates of Treponema hyodysenteriae. Proc. Int. Pig Vet. Soc. Cong., Abstr. 183.
 15. Mapother, M. E., and L. A. Joens. 1985. New serotypes of Treponema hyodysenteriae. J. Clin. Microbiol. 22:161-164.
 16. Suenaga, I., and T. Yamazaki. 1983. Experimental infection with Treponema hyodysenteriae in nude mice. Zbl. Bakt. Hyg., I. Abt. Orig. A. 254:528-533.
 17. Tanaka, M., N. Hirayama, and Y. Tamura. 1987. Production, characterization and protective effect of monoclonal antibodies to Clostridium chauvoei flagella. Infect. Immun. 55:1779-1783.
 18. Taylor, D. J., and T. J. L. Alexander. 1971. The production of dysentery in swine by feeding cultures containing a spirochete. Br. Vet. J. 127:58-61.

Fig. 1. Spirochete (see arrow) within mucous strands in crypt of T. hyodysenteriae infected mouse cecum. Magnification 34,000.

Fig. 2 Spirochetes (see arrow) under necrotic,
sloughing epithelial cell in cecal crypt of T.
hyodysenteriae infected mouse. Magnification
16,200.

Table 1. Passive Protection of mice with MAB
given by various routes

Treatment	% with gross lesions	% culture positive	% darkfield positive
Control n=9	77.8	55.6	66.7
Intravascular antibody n=8	25*	25*	25*
Intraperitoneal antibody n=8	37.5**	25*	25*
Intragastric antibody n=7	28.5**	28.5**	28.5**

*Significant at $P < .05$, Chi-square test.

**Significant at $P < .1$, Chi-square test.

Table 2. Concentration of MAB necessary for protection

Treatment ^a	A	B	C	D
Experiment 1				
Control n=6	33%	1.85 $\times 10^6$	100%	B78, serotype 1
5 ug Ab 18.2 n=6	33%	1.71 $\times 10^6$	100%	"
25ug Ab 18.2 n=6	33%	3.73 $\times 10^6$	100%	"
100ug Ab 18.2 n=6	0*	0	0	"
250ug Ab 18.2 n=6	0*	0	0	"
100ug Ab 18.2 n=6	66.6%	77.8 $\times 10^6$	100%	B204 serotype 2
Experiment 2				
Control n=4	25%	3.3 $\times 10^3$	100%	B78 serotype 1
5 ug Ab 18.2 n=4	25%	7.0 $\times 10^3$	75%	"
50ug Ab 18.2 n=4	25%	7.8 $\times 10^3$	75%	"
100ug Ab 18.2 n=4	0**	0.6 $\times 10^2$	25%	"
100ug Ab 41B.7 n=4	25%	4.3 $\times 10^3$	75%	"
(not serotype specific)				

^aA= % mice with cecitis, B= # *T. hyodysenteriae* per gm cecum, C= % mice culture positive, D= strain and serotype of *T. hyodysenteriae* used for infection.

*Significant at $P < .025$, Chi-square test.

**Significant at $P < .05$, Chi-square test.

Table 3. In vitro growth of *T. hyodysenteriae* with or without complement and MAB^a

Treatment ^b	1h-A	B	2h-A	B	3h-A	B	C
Cont.w/oC' n=4	.115	96.5%	.132	92%	.128	87%,	2.4
Cont.w/C' n=4	.112	92%	.127	92.5%	.130	87%	1.3
5ugAb18.2 w/oC' n=2	.134	90%	.148	86%	.148	84%	3.9
5ugAb18.2 w/C' n=2	.101	84%	.116	77%	.122	79%	.37
50ugAb18.2 w/oC' n=4	.092	78%	.114	75%	.116	67%	3.4
50ugAb18.2 w/C' n=4	.117	70%	.118	62%	.123	60%	.51
50ugAb41B.7 w/oC' n=2	.098	85%	.130	88%	.138	80%	4.9
50ugAb41B.7 w/C' n=2	.113	44%	.135	58%	.150	32%	.05

^aSerotype 1 specific MAB - Ab 18.2.

Non-serotype specific MAB - Ab 41B.7.

^bA Columns - Optical Density at 600nm.

B Columns - Average percent motile organisms.

C Column - Colony forming units x 10⁶ after 3 hrs incubation.

GENERAL SUMMARY

Outer membrane proteins (OMP) of Treponema hyodysenteriae were extracted by a number of methods. Extraction with N-lauryl sarcosine, followed by SDS-PAGE, allowed for the differentiation of T. hyodysenteriae from T. innocens. These T. hyodysenteriae profiles were stable between serotypes or after many in vitro passages. After growth in the presence of an iron chelator, desferal mesylate, a new OMP was visible after SDS-PAGE which had a m.w. of 70 kDa. OMP profiles of T. innocens revealed this group to be heterogeneous.

Western blotting of OMP extracts was used to examine the immune response of swine to infection or immunization with T. hyodysenteriae. Pigs were immunized with sarcosine extracted OMPs and protected from challenge. Immune and convalescent swine serum contained antibody reactive to antigens not recognized by antibodies from non-protected, diseased pigs. Antibody to these antigens, which migrate as 14 and 19kDa polypeptides, may be protective and these antigens may be virulence factors of the organism. Enzyme treatment showed these antigens were sensitive to proteinase K and lipase. Serum from protected pigs had antibody to LPS and hemolysin isolated from T. hyodysenteriae. These antigens had similar migration patterns in SDS-PAGE as the protective

antigens. Colonic antibody and antibody from protective sow milk, react with several OMP antigens, including the suspected protective antigens.

Finally, monoclonal antibodies (MAB) were developed that react with T. hyodysenteriae. Serotype specific MAB was shown to passively protect mice from subsequent challenge with homologous but not heterologous T. hyodysenteriae. A non-serotype specific MAB did not protect mice. The LPS molecule is the basis for serospecificity. These results indicate that LPS may be an important virulence attribute of T. hyodysenteriae and that serum, colonic, or milk antibody to this antigen may be important in protecting pigs from swine dysentery.

LITERATURE CITED

1. Albassam, M. A., H. J. Olander, H. L. Thacker, and J. J. Turek. 1985. Ultrastructural Characterization of colonic lesions in pigs inoculated with Treponema hyodysenteriae. Can. J. Comp. Med. 49:384-390.
2. Alexander, T. J. L., and D. J. Taylor. 1969. The clinical signs, diagnosis and control of swine dysentery. Vet. Rec. 85:59-63.
3. Argenzio, R. A., S. C. Whipp, and R. D. Glock. 1980. Pathophysiology of swine dysentery: Colonic transport and permeability studies. J. Infect. Dis. 142:676-684.
4. Baum, D. H., and L. A. Joens. 1979. Serotypes of beta-hemolytic Treponema hyodysenteriae. Infect. Immun. 25:792-796.
5. Benson, S. A., and A. Decloux. 1985. Isolation and characterization of outer membrane permeability mutants in Escherichia coli K-12. J. Bacteriol. 161:361-367.
6. Binek, M., and Z. M. Szykiewicz. 1984. Physiological properties and classification of strains of Treponema sp. isolated from pigs in Poland. Comp. Immunol. Micro. and Inf. Dis. 7:141-148.
7. Blake, M. S., and E. C. Gotschlich. 1982. Purification and partial characterization of the major outer membrane protein of Neisseria gonorrhoeae. Infect. Immun. 36:277-283.
8. Donowitz, M., and H. J. Binder. 1976. Effect of enterotoxins of Vibrio cholera, Escherichia coli and Shigella dysenteriae type 1 on fluid and electrolyte transport in the the colon. J. Infect. Dis. 134:135-143.
9. Doyle, L. P. 1944. A vibrio associated with swine dysentery. Amer. J. Vet. Res. 5:3-5.

10. Dunne, H. W., and A. D. Leman. 1975. Swine Dysentery. P. 541. In, Diseases of Swine, 4th. ed. ISU Press, Ames, Ia.
11. Egan, I. T. 1981. Epidemiological aspects of swine dysentery in the midwestern United States. Ph.D. Dissertation. Iowa State Univ., Ames, Iowa.
12. Ezzell, J. W., W. J. Dobrogosz, W. E. Kloos, and C. R. Manclark. 1981. Phase-shift markers in Bordetella: alterations in envelope proteins. J. Infect. Dis. 143:562-569.
13. Filip, C., G. Fletcher, J. L. Wuluff, and C. F. Earhart. 1977. Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115:717-722.
14. Fitzgerald, T. J., C. A. Repesh, D. R. Blanos, and J. N. Miller. 1984. Attachment of Treponema pallidum to fibronectin, laminin, collagen 5 and collagen 1, and blockage of attachment by immune rabbit IgG. Br. J. Vener. Dis. 60:357-363.
15. Genho, P. C., L. E. Orme, D. N. Wright, and C. M. Mortenson. 1974. Prophylactic use of globulins in swine dysentery. J. Anim. Sci. 34 (Abstract 132).
16. Glauert, A. M., and M. J. Thornley. 1969. The topography of the bacterial cell wall. Annu. Rev. Microbiol. 23:159-198.
17. Glock, R. D., and D. L. Harris. 1972. Swine dysentery II. Characterization of lesions in pigs inoculated with Treponema hyodysenteriae in pure and mixed culture. Vet. Med. Small Anim. Clin. 67:65-68.
18. Glock, R. D., D. L. Harris, and J. P. Kluge. 1974. Localization of spirochetes with the structural characteristics of Treponema hyodysenteriae in the lesions of swine dysentery. Infect. Immun. 9:167-178.
19. Glock, R. D., and K. J. Schwartz. 1975. Swine waste lagoons as potential disease reservoirs. Proc. Int. Symposium Livestock Wastes 3:334-335.

20. Glock, R. D., K. J. Schwartz, and D. L. Harris. 1976. Experimental immunization of pigs against Treponema hyodysenteriae. Proc. 4th Int. Pig Vet. Soc. Congress, Ames, Iowa. Abstract L 11.
21. Granoff, D. M., S. J. Barenkamp, and R. S. Munson, Jr. 1982. Outer membrane protein subtypes for epidemiologic investigation of Haemophilus influenzae type b disease. Pp. 43-55. In, S. H. Sell and P. F. Wright, eds. Haemophilus influenzae: epidemiology, immunology and prevention of disease. Elsevier-Science Publishing Co., New York.
22. Hanff, P. A., J. N. Miller, and M. A. Lovett. 1983. Molecular characterization of common treponemal antigens. Infect. Immun. 40:825-828.
23. Harris, D. L. 1984. The epidemiology of swine dysentery as it relates to the eradication of the disease. The Comp. on Cont. Ed. 6:83-88.
24. Harris, D. L., and R. D. Glock. 1973. Swine dysentery. Vet. Scope 17:27.
25. Harris, D. L., and R. D. Glock. 1971. Swine dysentery: a review. Iowa State Univ. Vet. No.1-4-11.
26. Harris, D. L., and R. D. Glock. 1981. Swine dysentery. Pp. 432-444. In, A. D. Leman, R. D. Glock, W. L. Mengeling, R. H. C. Penny, E. School and B. Straw, eds. Diseases of Swine. 5th ed. Iowa State Univ. Press, Ames, Iowa.
27. Harris, D. L., R. D. Glock, C. R. Christensen, and J. M. Kinyon. 1972. Swine dysentery. Innoculation of pigs with Treponema hyodysenteriae (new species) and reproduction of the disease. Vet. Med. Small Anim. Clin. 67:61-64.
28. Harris, D. L., T. J. L. Alexander, S. C. Whipp, I. M. Robinson, and D. J. Matthews. 1978. Swine dysentery: studies of gnotobiotic pigs inoculated with Treponema hyodysenteriae, Bacteroides vulgatus, and Fusobacterium necrophorum. J. Am. Vet. Med. Assoc. 172: 468-471.
29. Hirota, Y., H. Suzuki, Y. Nishimura, and S. Yasuda. 1977. On the process of cellular division in Escherichia coli: a mutant of E. coli lacking a

murein-lipoprotein. Proc. Natl. Acad. Sci. USA 74:1417-1420.

30. Houlind-Hougen, K., and P. Hough. 1984. Morphological and serological heterogeneity among strongly hemolytic spirochetes isolated from pigs with swine dysentery. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 181.
31. Hryniewicz, W., and J. Pryjma. 1977. Effect of Streptolysin S on human and mouse T and B lymphocytes. Infect. Immun. 16:730-733.
32. Hudson, M. J., T. J. L. Alexander, R. J. Lyons, and P. D. Wellslead. 1974. Swine dysentery: Failure of an attenuated strain of spirochete given orally to protect pigs against subsequent challenge. Brit. Vet. J. 130:37-40.
33. Hughes, R., H. J. Olander, C. L. Kanitz, and A. Qureshi. 1977. A study of swine dysentery by immunofluorescence and histology. Vet. Pathol. 14:490-507.
34. Hunter, D., and C. N. Saunders. 1973. Serum agglutination test for swine dysentery. Vet. Res. 93:93-107.
35. Hunter, D., and C. N. Saunders. 1977. Diagnosis of swine dysentery using an absorbed fluorescent antiserum. Vet. Rec. 101:303-304.
36. Hunter, D., and T. Wood. 1979. An evaluation of the APIZYM system as a means of classifying spirochetes associated with swine dysentery. Vet. Rec. 104:383-384.
37. Ivanov, O. V. 1985. Influence of chemotherapy on the formation of antibodies to the swine dysentery spirochete. Mosk. Vet. Akad. 1985:29-32.
38. Jacob, E., and R. K. Nauman. 1982. Common antigens of Treponema denticola: chemical, physical and serological characterization. Infect. Immun. 37:474-480.
39. Joens, L. A., D. L. Harris, and D. H. Baum. 1979. Immunity to swine dysentery in recovered pigs. Am. J. Vet. Res. 40:1352-1354.

40. Joens, L. A., D. L. Harris, J. M. Kinyon, and M. L. Kaerberle. 1978. Microtitration agglutination for detection of Treponema hyodysenteriae antibody. J. Clin. Microbiol. 8:293-298.
41. Joens, L. A., D. W. DeYoung, J. C. Cramer, and R. D. Glock. 1984. The immune response of the porcine colon to swine dysentery. Proceedings 8th Int. Pig. Vet. Soc. Congress, Ghent, Belgium. Abstr. 177.
42. Joens, L. A., D. W. DeYoung, R. D. Glock, M. E. Mapother, J. D. Cramer, and E. Willcox. 1985. Passive protection of segmented swine colonic loops against swine dysentery. Am. J. Vet. Res. 46:2369-2371.
43. Joens, L. A., N. A. Nord, J. M. Kinyon, and I. T. Egan. 1982. Enzyme-linked immunosorbent assay for detection of antibody to Treponema hyodysenteriae antigens. J. Clin. Microbiol. 15:249-252.
44. Joens, L. A., and R. B. Marquez. 1986. Molecular characterization of proteins from porcine spirochetes. Infect. Immun. 54:893-896.
45. Joens, L. A., R. D. Glock, and J. M. Kinyon. 1980. Differentiation of Treponema hyodysenteriae from Treponema innocens by enteropathogenicity testing in CFI mice. Vet. Rec. 105:527-529.
46. Joens, L. A., S. C. Whipp, and R. D. Glock. 1983. Specifics of immunity to swine dysentery. pp. 19-23. In, Proc. 1983 Swine Dysentery Symposium.
47. Kent, K. A., and R. M. Lemcke. 1984. Purification and cytotoxic activity of a haemolysin produced by Treponema hyodysenteriae. Proc. 8th Int. Pig Vet Soc Congress, Ghent, Belgium. Abstr. 185.
48. Kinyon, J. M. 1974. Characterization of Treponema hyodysenteriae isolated from outbreaks of swine dysentery. M.S. Thesis, Iowa State Univ. Ames, Iowa.
49. Kinyon, J. M., and H. L. Harris. 1979. Treponema innocens, a new species of intestinal bacteria and amended description of the type strain of Treponema hyodysenteriae. Int. Journal of Systemic Bacteriology 29:102-109.

50. Kinyon J. M., D. L. Harris, and R. D. Glock. 1977. Enteropathogenicity of various isolates of Treponema hyodysenteriae. Infect. Immun. 15:638-646.
51. Kinyon, J. M., J. G. Songer, M. Jane, and D. L. Harris. 1976. Isolation and identification of Treponema hyodysenteriae: aid to the diagnosis and treatment of swine dysentery. p. 65 In, Proc. Am. Assoc. Vet. Lab. Diag.
52. Knoop, F. C. 1981. Investigation of a hemolysin produced by enteropathogenic Treponema hyodysenteriae. Infect. Immun. 31:193-198.
53. Kunkle, R. A., and J. M. Kinyon. 1986. BJ; selective medium for isolation of Treponema hyodysenteriae. 67th Conference of Research Workers in Animal Diseases, Chicago, Ill. Abstr. 166.
54. Lamden, P. R., J. E. Heckels, L. T. James, and P. J. Watt. 1979. Variations in surface protein composition associated with virulence properties in opacity types of Neisseria gonorrhoeae. J. Gen. Microbiol. 114:305-312.
55. Lee, C. H., L. D. Olson, and D. E. Rodabaugh. 1976. Influence of medication on development of serum antibody to swine dysentery as detected with indirect fluorescent antibody method. Am. J. Vet. Res. 37:1159-1163.
56. Lemcke, R. M., and J. Bew. 1984. Antigenic differences among isolates of Treponema hyodysenteriae. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 183.
57. Lemcke, R. M., and L. A. Williams. 1984. Modification of the selective medium for Treponema hyodysenteriae. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 184.
58. Lemcke, R. M., and M. R. Burrows. 1979. A disc growth-inhibition test for differentiating Treponema hyodysenteriae from other intestinal spirochetes. Vet. Rec. 104:548-551.
59. Lemcke, R. M., and M. R. Burrows. 1982. Studies on a haemolysin produced by Treponema hyodysenteriae. J. Med. Microbiol. 15:205-214.

60. Liven, E. 1979. Prevalence of Treponema hyodysenteriae in healthy pigs. ACTA Vet. Scand. 20:387-395.
61. Lysons, R. J., J. F. L. Pohlenz, D. L. Harris, S. C. Whipp, M. R. Burrows, and R. M. Lemcke. 1984. Treponema hyodysenteriae in large intestinal disease. Proc. 8th. Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 176.
62. Lysons, R. J., M. R. Gurrows, T. G. Debney, and J. Bew. 1980. Vaccination against swine dysentery - an effective novel method. Proc. 6th Int. Pig Vet. Soc. Congress. Abstr. 24.
63. Lysons, R. J., R. M. Lemcke, J. Bew, M. R. Burrows, and T. J. L. Alexander. 1982. Treponema hyodysenteriae like organism from herds free of swine dysentery. Proc. 7th Int. Pig Vet. Soc. Congress. Abstr. 22.
64. Mapother, M. E., and L. A. Joens. 1985. New serotypes of Treponema hyodysenteriae. J. Clin. Micro. 22:161-164.
65. Marchitto, K. S., S. A. Jones, R. F. Schell, P. L. Holmans, and M. V. Norgard. 1984. Monoclonal antibody analysis of specific antigenic similarities among pathogenic Treponema pallidum subspecies. Infect. Immun. 45:660-666.
66. Masuda, K., and T. Kawata. 1987. Cleavage and isolation of the outer sheath of Treponema phagedenis by treatment with N, N'-dicyclohexylcarbodiimide. FEMS Microbiol. Letters 41:73-78.
67. McCartney, A. C., and A. C. Wardlaw. 1985. Endotoxic activities of lipopolysaccharides. Pp. 203-238. In, D. E. S. Stewart-Tull and M. Davies, eds. Immunology of the Bacterial Cell Envelope. John Wiley and Sons Ltd., New York.
68. Moskophidis, M., and F. Muller. 1984. Molecular characterization of glycoprotein antigens on the surface of Treponema pallidum: Comparisons with non-pathogenic Treponema phagedenis Blotype Reiter. Infect. Immun. 46:867-869.

69. Nikaldo, H., and M. Vaara. 1986. Outer Membrane. Pp.3-22. In, F. C. Neidhardt, ed. Escherichia coli and Salmonella typhimurium cellular and molecular biology. Vol 1. ASM, Washington, DC.
70. Norris, S. J., and S. Sell. 1984. Antigenic complexity of Treponema pallidum: antigenicity and surface localization of major polypeptides. J. Immunol. 133:2686-2692.
71. Nuessen, M. E., and L. A. Joens. 1982. Serotype-specific opsonization of Treponema hyodysenteriae. Infect. Immun. 38:1029-1032.
72. Olson, L. D. 1974. Clinical and pathological observations on the experimental passage of swine dysentery. Can. J. Comp. Med. 38:7-13.
73. Olson, L. D. 1986. Tiamulin in water for treatment and development of immunity to swine dysentery. J. Am. Vet. Med. Assoc. 188:1165-1170.
74. Olson, L. D., and D. E. Rodabaugh. 1979. Clinical and pathological features of various drug-related problems in the control of swine dysentery. J. Am. Vet. Med. Assoc. 173:843-851.
75. Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of Salmonella typhimurium. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. 247:3962-3972.
76. Penn, C. W., A. Cockayne, and J. J. Balley. 1985. The outer membrane of Treponema pallidum: Biological significance and biochemical properties. J. Gen. Microbiol. 131:2349-2357.
77. Peterson, L. H. 1982. Immunologic response of swine to solubilized outer envelope components of Treponema hyodysenteriae. Ph.D. Dissertation. Iowa State Univ., Ames, Iowa.
78. Pohlenz, J. F., S. C. Whipp, I. M. Robinson, and J. A. Fagerland. 1984. A hypothesis on the pathogenesis of Treponema hyodysenteriae induced diarrhea in pigs. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 178.

79. Postle, K., and R. R. Good. 1983. DNA sequence of Escherichia coli ton B gene. Proc. Natl. Acad. Sci. USA. 80:5235-5239.
80. Radolf, J. D., T. E. Fehniger, F. J. Silverblatt, J. N. Miller, and M. A. Lovett. 1986. The surface of virulent Treponema pallidum: Resistance to antibody binding in the absence of complement and surface association of recombinant antigen 4D. Infect. Immun. 52:579-585.
81. Richardson, K., Jr., J. Michalski, and J. B. Kaper. 1986. Hemolysin production and cloning of two hemolysin determinants from classical Vibrio cholerae. Infect. Immun. 54:415-420.
82. Rout, W. R., S. B. Formal, R. A. Glanella, and G. J. Dammin. 1975. Pathophysiology of Shigella dysentery in the rhesus monkey: Intestinal transport, morphological, and bacteriological studies. Gastroenterology 68:276-278.
83. Saheb, S. A., and L. LaFleur. 1980. Characteristics of the interaction of a treponemal hemolysin and rabbit erythrocytes. Blochimie 62:787-793.
84. Saheb, S. A., L. Massicotte, and B. Picard. 1980. Purification and characterization of Treponema hyodysenteriae hemolysin. Blochimie 62:779-785.
85. Saheb, S. A., N. Daigneault-Sylvestre, and B. Picard. 1981. Comparative study of the hemolysins of Treponema hyodysenteriae and Treponema innocens. Curr. Microbiol. 5:87-90.
86. Saheb, S. A., R. Lallier, L. Massicotte, L. LaFleur, and S. Lemieux. 1981. Biological activity of Treponema hyodysenteriae hemolysin. Curr. Microbiol. 5:91-94.
87. Schmall, L. M., R. A. Argenzio, and S. C. Whipp. 1983. Pathophysiologic features of swine dysentery: Cyclic nucleotide-independent production of diarrhea. Am. J. Vet. Res. 44:1309-1316.
88. Schnaitman, C. A. 1973. Outer membrane proteins of Escherichia coli. I. Effect of preparation conditions on the migration of protein in

- polyacrylamide gels. Arch. Biochem. Biophys. 157:541-552.
89. Schwartz, K., and R. D. Glock. 1976. Resistance to Treponema hyodysenteriae in passively immunized pigs. Proc. 4th Int. Pig Vet. Soc. Congress, Ames, Iowa. Abstr. L12.
 90. Singh, S. K., S. Gurusiddalah, and J. W. Whalen. 1985. Treponemycin, a nitrite antibiotic active against Treponema hyodysenteriae. Antimicrob. Agents and Chemotherapy 27:239-245.
 91. Smyth, C. J. 1985. Immunology of Outer Membrane Proteins of Gram-Negative Bacteria. Pp. 177-201. In, D. E. S. Stewart-Tull, and M. Davies, eds. Immunology of the Bacterial Cell Envelope. John Wiley and Sons, Ltd. New York.
 92. Songer, J. G. 1976. Epidemiology of swine dysentery: Development and evaluation of cultural and serological methods for the detection of Treponema hyodysenteriae infection. Ph.D. Dissertation, Iowa State Univ., Ames, Iowa.
 93. Songer, J. G., J. M. Kinyon, and D. L. Harris. 1976. Selective medium for isolation of Treponema hyodysenteriae. J. Clin. Microbiol. 4:57-60.
 94. Taylor, D. J., and R. Stevenson. 1984. Studies of the relationship between the large intestinal mucosa and Treponema hyodysenteriae in swine dysentery. Proc. 8th Int. Pig Vet. Soc. Congress, Ghent, Belgium. Abstr. 180.
 95. Taylor, D. J., and R. Stevenson. 1986. ELISA test for Treponema hyodysenteriae in pig feces and slurry. Proc. 9th Int. Pig Vet. Soc. Congress, Barcelona, Spain. Abstr. 24.
 96. Taylor, D. J., and T. J. L. Alexander. 1971. The production of dysentery in swine by feeding cultures containing a spirochete. Br. Vet. J. 27:58-61.
 97. Taylor, D. J., and W. F. Blakemore. 1971. Spirochoetal invasion of the colonic epithelium in swine dysentery. Res. Vet. Sci. 12:177-179.
 98. Terpstra, J. J., J. P. W. M. Akkermans, and H. Ouwerkerk. 1968. Investigations into the etiology

- of vibronic dysentery (Doyle) in pigs. Neth. J. Vet. Sci. 1:5-13.
99. Thornburg, R. W., and J. B. Baseman. 1983. Comparison of major protein antigens and protein profiles of Treponema pallidum and Treponema pertense. Infect. Immun. 42:623-627.
 100. Tommassen, J., and B. Lugtenberg. 1980. Outer membrane protein e of Escherichia coli K-12 is co-regulated with alkaline phosphatase. J. Bacteriol. 143:151-157.
 101. Whipp, S. C., D. L. Harris, J. M. Kinyon, J. G. Songer, and R. D. Glock. 1978. Enteropathogenicity testing of Treponema hyodysenteriae in ligated colonic loops of swine. Am. J. Vet. Res. 39:1293-1296.
 102. Whipp, S. C., I. M. Robinson, D. L. Harris, R. D. Glock, P. J. Matthews, and T. J. L. Alexander. 1980. Pathogenic synergism between Treponema hyodysenteriae and selected anaerobes in gnotobiotic pigs. Infect. Immun. 26:1042-1047.
 103. Whiting, R. A., L. P. Doyle, and R. S. Spray. 1921. Swine Dysentery. Purdue Univ. Agric. Exp. Stn. Bull. 257:3-15.
 104. Wilcock, B. P., and H. J. Olander. 1979. Studies on the pathogenesis of swine dysentery 1. Characterization of the lesions in colons and colonic segments inoculated with pure cultures or colonic content containing Treponema hyodysenteriae. Vet. Pathol. 16:450-465.
 105. Williams, B. J., and J. E. Shively. 1978. In vitro anti-treponemal activities of carbadox, virginiamycin, olaquindox and tylosin as indices of their effectiveness for preventing swine dysentery. Vet. Med. Sm. Anim. Clin. 73:349-351.
 106. Yamazaki, T., N. Narukawa, I. Sueraga, and K. Takeda. 1986. Sedecamycin a 17-membered macrocyclic antibiotic active against Treponema hyodysenteriae. Proc. 9th Int. Pig Vet. Soc. Congress, Barcelona, Spain. Abstr. 25.

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APPENDIX I. MONOCLONAL ANTIBODIES REACTIVE TO Treponema
hyodysenteriae AND Treponema innocens

Monoclonal antibodies (MAB) to strains of T. hyodysenteriae were developed by the methods of Colwell et al. (1). The MABs were produced in the laboratory of Dr. M.J. Wannemuehler. MABs were tested for reactivity with five serotypes of T. hyodysenteriae and with four isolates of T. innocens. The MABs were also identified as to their isotype.

BALB/c mice were immunized with lipopolysaccharide or whole cells from T. hyodysenteriae strains intraperitoneally with 1 mg/ml antigen. Mice received a secondary immunization intravascularly (i.v.) 24 hr before the fusion. Mice were sacrificed by cervical dislocation, spleens were removed and passed through a 60 mesh stainless steel screen to form a single cell suspension. Myeloma cells, from the X63-Ag8.653 non-immunoglobulin secreting murine myeloma cell line, and spleen cells were washed separately in warm (37 C) RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.). Myeloma and spleen cells were mixed at a ratio of 1:5 (respectively) in a 50ml conical centrifuge tube and packed by centrifugation at 600 x g for 10 min. Supernatant fluid was discarded and the pellet was resuspended in the residual fluid to form a cell slurry

by gently tapping the bottom of the tube. Cells were fused by adding 1ml of warmed polyethylene glycol 1500 (PEG) (Boehringer Mannheim, West Germany) dropwise with gentle mixing. The mixture was incubated for 1 min at 37 C. The PEG and cell suspension was diluted by dropwise addition of 7 ml of RPMI 1640. Cells were centrifuged and the supernatant discarded. Cells were resuspended to a concentration of 4×10^5 spleen cells per ml in RPMI with 10% FCS (JR Scientific, Woodland, CA), 10% conditioned media, 20mM L-glutamine (Sigma), 10 units/ml penicillin (Sigma), 10 ug/ml streptomycin (Sigma), 100 ug/ml gentamicin (Sigma), 1 X MEM non-essential amino acids (Sigma) and 100 uM hypoxanthine, 0.4 uM aminopterin, 16 uM thymidine supplement (HAT supplement, Sigma). Cells were dispensed in 1 ml aliquots into 24 well flat-bottom plates (Costar, Cambridge, Mass.)

Conditioned medium was prepared by suspending normal mouse spleen cells at a concentration of 1×10^6 cells/ml in the above media, without HAT supplement, and then incubated for 24 hr at 37 C with a 5% carbon dioxide atmosphere. After incubation the suspension was centrifuged at 600 x g and the supernatant collected and frozen in 50 ml aliquots at -20 C until needed.

After one week of incubation, plates were observed daily for hybridoma growth. Culture supernatants, from

wells displaying hybridoma growth, were tested for the presence of antigen-specific antibody by the enzyme - labeled immunosorbent assay (ELISA) (2). Positive hybridomas were subcloned by limiting dilution in media without HAT supplement, and expanded after 1-2 wks incubation. Culture supernatants were collected and stored frozen at -20 C for later use or the immunoglobulin was precipitated with 40% cold saturated ammonium sulfate, followed by exhaustive dialysis against saline at 4 C.

The ELISA procedure was modification of the methods of Engvall and Perlmann (2). Plates were prepared by suspending whole, washed, lyophilized treponemal cells (100 ug/ml, dry weight) in fresh 0.1M sodium carbonate buffer (pH 9.6) and 100 ul of the suspension was added to each well (100 ul/ml) and incubated overnight at 4C. After incubation of plates, unabsorbed antigen was discarded and the plates were washed three times with a solution of 0.85% NaCl and 0.05% Tween 20 (Fisher Scientific, Fairlawn, NJ) (Tween-saline, TS). Plates were then incubated with 0.5% bovine serum albumin (BSA) in TS for 30 min at 37 C as a blocking agent. Plates were then washed three times with TS and blotted dry. Undiluted hybridoma culture supernatants were added to duplicate wells and incubated for 4hr at 37 C or overnight at 4 C.

Plates were then washed three times with TS, wells were filled with TS and allowed to incubate at room temp. for 5 min and then washed three more times and blotted dry. Affinity purified goat anti-mouse immunoglobulin, labeled with alkaline phosphatase or horse radish peroxidase (Southern Biotechnology Associates, Birmingham, Ala.) were added in 50 ul/well at a dilution of 1:2000 in TS. Plates were then incubated for 1hr at 37 C on a rocker platform (Bellco Glass Inc., Vineland, NJ) and then washed as before.

Substrate was prepared while the plates were incubating with the TS wash. Substrate for alkaline phosphatase was prepared by dissolving p-nitrophenyl phosphate (1 mg/ml; Sigma 104 phosphatase substrate) in 0.05M sodium carbonate buffer with 0.001M magnesium chloride (Ph 9.3). Substrate for horseradish peroxidase was prepared by adding 5 ul 30% hydrogen peroxide to 5 ml of 50mM citric acid pH 4 (solution A). Solution B was prepared by dissolving 128 mg of 2-2 azino-di-ethyl benzothiazolin sulfonic acid (ABTS, Sigma) in 5ml of distilled water. To make 15 ml of substrate solution 60 ul of solution A were added with 150 ul of solution B to 15 ml of 50mM citric acid pH 4. After the substrate was prepared it is added in 100 ul volume to each well of the plate, which was previously blotted dry. The alkaline

phosphatase substrate was incubated on the plate for 100 min before the absorbance of the wells was measured at 405nm. It was not generally necessary to stop the color reaction of this substrate. However, the reaction can be stopped by the addition of an equal volume of 0.1N NaOH. The peroxidase substrate was incubated on the plate for 20 min before the reaction was stopped by the addition of 100 ul of 0.1N hydrofluoric acid. The peroxidase reaction must be stopped for accurate readings. The resulting absorbance of the color reaction in the wells was measured at 405nm.

Monoclonal antibodies that showed reactivity to treponemal antigens were tested for reactivity to five serotypes of T. hyodysenteriae and four isolates of T. innocens. The isotype of the MABs was tested using the ELISA procedure discussed above. The MAB supernatants were reacted with plates coated with Sigma anti-mouse polyvalent serum (Sigma #M-8019) at a concentration of 0.1mg dry weight/ml in 0.1M sodium carbonate buffer, pH 9.6 with each well receiving a 0.1 ml volume. After incubation of the supernatant the plates were reacted with either affinity purified goat anti-mouse IgG, IgM or IgA labeled with horseradish peroxidase (Southern Biotech. Assoc. Inc., Birmingham, Ala.) at the appropriate dilutions. Serotype reactivity and isotype

of some of the MABs that were developed are listed in Table 1.

Table 1. MAB reactivity and isotype

MAB <i>T. hyodysenteriae</i>		<i>T. innocens</i> isolates									
Serotype		1	2	3	4	7	B256	4/71	B1555a	B6571	P43
Class ^a											
B78,7.7	? ^b	+	+	+	+	ND ^c	+	ND	ND	ND	-
B78,18.1	?	+	-	-	+	-	-	+	-	-	-
B78,18.2	M	+	-	-	-	-	-	-	-	-	-
B78,18.3	M	+	-	-	+	-	-	-	-	-	ND
B78,20.5	ND	+	+	+	+	ND	+	ND	ND	ND	+
B78,23.3	ND	+	-	-	-	ND	+	ND	ND	ND	-
B234,2.4	G	+	-	+	-	-	-	-	-	-	ND
B234,6.3	M	+	+	+	+	+	-	+	-	-	ND
B256,11.5	ND	+	+	+	+	+	+	ND	ND	ND	+
B169,27.3	M	+	+	+	+	+	+	ND	ND	ND	+
B169,27.5	ND	+	+	+	+	+	+	ND	ND	ND	+
41B.7.2	M	+	+	+	+	+	+	+	+	+	ND
41B.7.3	M	+	+	+	+	+	+	?	+	+	+
41B.7.8	M	+	+	+	+	+	+	+	+	+	ND
41B.7.14	M	+	+	+	?	+	+	+	+	+	ND
39B.3	G	+	+	+	+	+	+	+	-	-	ND
39B.4.1	M	+	+	+	+	?	?	?	-	-	ND
T18.4	M	+	+	+	?	?	?	ND	ND	ND	ND
T18.7	?	+	+	+	+	-	-	-	-	-	-
T18.10	?	+	+	-	-	-	-	-	-	-	ND
T18.11	M	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
T22b.2	M	+	+	+	+	+	?	?	?	?	ND
T22b.4	M	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
N11.1	M	+	+	+	+	+	?	?	?	?	ND
T34b.5	M	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
AA 6.2	M	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
AA 20.1	M	ND	+	ND	ND	ND	ND	ND	ND	ND	ND

^aClass = isotype (IgM, IgG or IgA).^b? = inconclusive results.^cND = not done.

1. Colwell, D. E., K. A. Gollahon, J. R. McGhee, and S. M. Michalek. 1982. IgA hybridomas: A method for generation in high numbers. *J. Immunol. Meth.* 54:259-266.
2. Engvall, E., and P. Perlmann. 1971. Enzyme-linked immunosorbant assay (ELISA): quantitative assay of immunoglobulin G. *Immunochemistry* 8:871-874.

APPENDIX II. APIZYM REACTIONS OF Treponema
hyodysenteriae AND Treponema innocens ISOLATES

The APIZYM system is a semi-quantitative technique designed for the detection of bacterial enzyme activities (1). The technique is applicable to many specimen sources, including microorganisms, for the rapid study of 19 enzymatic reactions. This technique assays for constitutive enzymes. Inducible enzymes can be detected by adding the corresponding inducer to the culture medium. In 1979, Hunter and Wood (2) published a report using the APIZYM system for the classification of spirochetes associated with swine dysentery. Their report, however, included only strains isolated in England.

This appendix is intended to provide further evidence for the usefulness of this technique to differentiate T. hyodysenteriae isolates from T. innocens. It was found that this system could differentiate between these species by a consistent difference in one enzyme. This enzyme, alpha - galactosidase (enzyme 13), was consistently present in T. innocens isolates, but consistently absent in T. hyodysenteriae isolates.

A bacterial suspension with a turbidity between a McFarland No. 5 and No. 6 standard was prepared. Pure growth from a centrifuged 10 ml, 18 hr log phase, broth culture was used to prepare the suspension in broth. APIZYM strips were placed in an incubation tray on top of 5 ml of water. With a Pasteur pipette, each microampule was inoculated with two drops of the specimen. After inoculation the lid was placed on the tray and incubated for 4 hrs at 37C, in the dark. After incubation one drop of ampule A (Tris-HCl and SDS in water), and ampule B (Fast Blue BB in 2-methoxyethanol) were added and the color was allowed to develop for 5 min.

After color development a value ranging from 0-5 (5 = maximum intensity) was assigned corresponding to the colors developed, compared to the color chart enclosed with the APIZYM strips. Microampule number and the enzyme assayed for are listed below:

<u>Ampule</u>	<u>Enzyme</u>	<u>Ampule</u>	<u>Enzyme</u>
1	control	16	Alpha-glucosidase
2	Alkaline phosphatase	17	Beta-glucosidase
3	Esterase (C4)	18	N-acetyl-beta-glucosaminidase
4	Esterase Lipase (C8)	19	Alpha-mannosidase
5	Lipase (C14)	20	Alpha-fucosidase
6	Leucine aminopeptidase		
7	Valine aminopeptidase		
8	Cystine aminopeptidase		
9	Trypsin		
10	Chymotrypsin		
11	Acid phosphatase		
12	Phosphoamidase		
13	Alpha-galactosidase		
14	Beta-galactosidase		
15	Beta-glucuronidase		

STRAIN ENZYMES

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
T. hyodysenteriae n=4

B204-L^a 3 1 3 0 0 0 0 0 0 0 3 2 0 5 0 3 4 0 0 0
 B204-H^b 3 1 3 0 0 0 0 0 0 0 3 2 0 5 0 3 2 0 0 0
 B204-M^c 4 1 2 0 0 0 0 0 0 0 3 3 0 5 0 3 5 0 0 0

B78-L 1 1 2 0 0 0 0 0 0 0 2 2 0 3 0 2 0 0 0 0
 B78-H 2 1 2 0 0 0 0 0 0 0 2 2 0 5 0 4 2 0 0 0

B169 4 1 1 0 0 0 0 0 0 0 3 2 0 5 0 5 3 0 0 0

A1 3 1 2 0 0 0 0 0 0 0 3 2 0 5 0 4 3 0 0 0

G 1 2 1 0 0 0 0 0 0 0 3 2 0 5 0 1 3 0 0 0

8430 3 1 2 0 0 0 0 0 0 0 4 2 0 3 0 2 1 0 0 0

T. innocens n=2

B256 1 1 1 0 0 0 0 0 0 0 2 2 4 5 0 3 4 0 0 0

4/71 1 1 1 0 0 0 0 0 0 0 2 2 4 5 0 3 3 0 0 0

B1555a 2 2 4 0 1 0 0 1 0 4 3 3 5 0 1 0 0 0 0 0

B6571 1 3 5 1 1 0 0 0 0 4 3 3 5 0 2 0 0 0 0 0

B359 2 3 4 0 2 0 0 0 0 4 4 3 5 0 0 0 0 0 0 0

B297 1 0 3 0 0 0 0 0 0 2 2 4 5 0 2 4 0 0 0 0

B6830 1 4 1 0 1 0 0 0 0 3 3 2 5 0 1 5 0 0 0 0

PA-5 1 0 3 0 0 0 0 0 0 2 2 2 4 0 3 2 0 0 0 0

P43 1 1 3 0 0 0 0 0 0 3 2 3 5 0 2 0 0 0 0 0

^aL = low passaged (< 16 in vitro passages)

^bH = high passaged (> 54 in vitro passages)

^cM = mouse reisolate (2 in vitro passages)

1. The APIZYM system. 1979. Analytab Products, Plainview, N.Y.
2. Hunter, D., and T. Wood. 1979. An evaluation of the APIZYM system as a means of classifying spirochetes associated with swine dysentery. Vet. Rec. 104:383-384.

APPENDIX III. INVESTIGATION OF AGE-RELATED RESPONSE TO ANTIGENS OF Treponema hyodysenteriae IN PIGS

This experiment was undertaken to evaluate the age-related response of pigs to apparently protective antigens of T. hyodysenteriae. Earlier work has shown that mature pigs respond to immunization with a T. hyodysenteriae subunit vaccine by producing antibodies that unvaccinated and also unprotected pigs do not develop. Subsequent experiments showed that young, just weaned, pigs did not respond to the vaccine by production of protective antibody. Therefore it became apparent that there may be an age-related ability to respond to vaccination by the production of apparently protective antibody. This experiment was to evaluate the efficacy of a subunit vaccine and the ability of three age groups of swine to respond to the immunization.

Materials and methods Three groups of specific pathogen free pigs were purchased from H&K Enterprises, Ames, Ia. Group one consisted of six 18 lb. pigs. Group two consisted of six 30 lb. pigs and group 3 of four 60 lb. pigs. Half of the pigs in each group were immunized with a T. hyodysenteriae subunit vaccine, consisting of sarcosine extracted outer membrane (OM) antigens, with Incomplete Freund's adjuvant (ICF). The other half of each group were immunized with vaccine alone. All pigs

received 1 mg of protein per vaccination. Groups of pigs were housed separately. All pigs were bled and sera collected at the following intervals and dates:

6/10/87 Pre-Bleed
6/26/87 11 days post first vaccination
7/8/87 8 days post second vaccination
7/22/87 13 days post infection

Pigs were weighed and vaccinated on 6/15/87 and 6/30/87. Pigs that had received ICF with the first immunization were boosted without adjuvant. All pigs were fasted and challenged on 7/9/87 and 7/10/87 with 10^9 T. hyodysenteriae, strain B204, serotype 2. All pigs were examined for signs of diarrhea daily after challenge. All pigs were exsanguinated at 13 days post infection, weighed, blood collected, examined by necropsy for gross lesions and swabs were taken for T. hyodysenteriae isolation. Fecal material was also taken for extraction of colonic antibody to T. hyodysenteriae. Antibody was measured by ELISA to T. hyodysenteriae whole cells. The antibody response to immunization was also evaluated by western blot analysis following antigen electrophoresis by SDS-PAGE and transfer to nitrocellulose. Antibody was visualized with rabbit anti-swine IgG, labeled with horse radish peroxidase, followed by o-dianisidine in 10mM Tris-HCl, pH 7.4 buffer.

Results The three age groups of pigs were each divided in half as (a) having received vaccine with ICF, (b) vaccine without ICF. Table 1 depicts the average optical density of the anti-T. hyodysenteriae ELISA reactions at a serum dilution of 1:200, and the average rate of gain of the groups while infected.

Data for individual pigs are given in Table 2. These data include total number of clinical signs observed during the experiment (CST), culture at necropsy for presence of T. hyodysenteriae (CAN), gross lesions observed at necropsy (GL), weight gained (lbs.) since infected (WG), antibody response to T. hyodysenteriae expressed as the optical density (O.D.) of the anti-T. hyodysenteriae ELISA from a 1:200 dilution of serum and colonic antibody O.D.

Western blot Serums from 7/8 and 7/22 pigs were reacted against transferred proteins from the vaccine preparation and against sonicated T. hyodysenteriae cells. Only pigs in groups 2(a) responded to the initial immunization by production of antibody to lower migrating antigens. After infection with T. hyodysenteriae one pig of each of the groups 1(a), 3(a) and 3(b) also developed antibody to these antigens as detected by western blotting with a serum dilution of 1:100.

Colonic antibody Colonic contents were examined for the presence of anti-T. hyodysenteriae antibody by ELISA. Optical densities of ELISA reactions are presented in Table 2. Only five of the animals had detectable T. hyodysenteriae-specific antibodies.

Discussion Pigs immunized with outer membrane antigens of T. hyodysenteriae, with or without ICF adjuvant, showed evidence of an age related ability to respond to immunization. In the youngest group 20% developed antibodies to lower migrating antigens believed to be important in protection. In the middle and older groups 50% developed antibody to these antigens.

The pigs given the vaccine in conjunction with ICF adjuvant developed higher antibody levels than did the pigs not immunized with ICF. The pigs given the vaccine with ICF also had a higher rate of gain. The younger group of pigs had more clinical signs, more gross lesions and more positive isolations of T. hyodysenteriae than the older groups. None of the pigs developed high amounts of detectable colonic antibody. Four of the six that had detectable colonic antibody were also culture positive.

In conclusion, it appears that the T. hyodysenteriae subunit vaccine is moderately efficacious, especially if given with ICF adjuvant. Pigs receiving the vaccine with

adjuvant had higher rates of gain, higher antibody responses, as detected by an anti-T. hyodysenteriae ELISA, more antibodies, as detected by the western blot procedure, and fewer clinical signs. There is also evidence for an age-related response to the probable protective antigens indicated by a decrease in the incidence of gross lesions, observed at necropsy, in the older pigs.

TABLE 1. Average optical density of the anti-T.
hyodysenteriae ELISA reaction from sera
diluted 1:200

DATE	1(a)	1(b)	2(a)	2(b)	3(a)	3(b)
6/10	.083	.003	.040	.099	.111	.082
6/26	.266	.144	.279	.177	.189	.339
7/8	.325	.233	.336	.331	.312	.324
7/22	.445	.338	.522	.447	.472	.378
Ave. Wt. Gain	8.66	5.66	12.66	11.66	14	13 (lbs.)

1(a). 18 lb. plgs immunized with Freund's
Incomplete adjuvant (FIA)
1(b). 18 lb. plgs immunized without FIA
2(a). 30 lb. plgs immunized with FIA
2(b). 30 lb. plgs immunized without FIA
3(a). 60 lb. plgs immunized with FIA
3(b). 60 lb. plgs immunized without FIA

TABLE 2. Pathogenic Effects of *T. hyodysenteriae*

Infection											
Group	Pig#	CST	CAN	WG	6/10	6/26	7/8	7/22	CAb 1:2	GL	
1(a)	1	1	-	9	.270	.406	.542	.613	-	mild hyp	
w/ICF	2	2	+	10	-	.224	.183	.568	-	"	"
	3	2	-	7	-	.167	.249	.312	-	"	"
1(b)	4	0	+	8	.056	.128	.277	.253	-	"	"
w/o ICF	5	3	+	1	-	.201	.281	.463	.113	"	"
	6	2	+	8	.011	.104	.141	.295	.145	"	"
2(a)	7	0	-	13	-	.316	.445	.617	-	"	"
w/ICF	8	0	-	18	.158	.258	.258	.680	.031	NGL	
	9	0	-	7	-	.264	.306	.350	-	NGL	
2(b)	10	0	+	10	.089	.212	.322	.596	-	mild hyp	
w/oICF	11	1	+	15	.113	.205	.375	.435	.100	"	"
	12	0	-	10	-	.114	.296	.400	.100	NGL	
3(a)	13	0	+	8	.136	.200	.436	.595	.010	NGL	
w/ICF	14	0	-	20	.086	.177	.187	.360	-	NGL	
3(b)	15	0	-	15	.127	.336	.250	.387	-	NGL	
w/oICF	16	0	-	11	.036	.342	.598	.675	-	NGL	

CST = clinical signs of diarrhea observed.

CAN = presence of *T. hyodysenteriae* detected
by culture at necropsy.

GL = gross lesions observed at necropsy.

WG = weight gained (lbs.) since date of challenge.