

Characterization of the murine B lymphocyte response to  
*Serpulina hyodysenteriae* following oral  
immunization and challenge

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Microbiology, Immunology, and Preventive Medicine

Major: Microbiology

Signatures have been redacted for privacy

Iowa State University  
Ames, Iowa

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## GENERAL INTRODUCTION

Swine dysentery is a mucohemorrhagic diarrheal disease affecting mainly feeder pigs (29). Its etiologic agent has been demonstrated to be *Serpulina hyodysenteriae*, a gram negative spirochete (27, 29, 106). The costs associated with morbidity and mortality (79) due to swine dysentery have recently totaled 180 million dollars annually for the U.S. pork industry (47).

Though antibiotics are effective in treating the disease, no effective vaccine against swine dysentery has been developed for pigs nor do swine develop complete immunity once recovered from the disease (42, 47, 106). While specific antibody against *S. hyodysenteriae* is seen in serum and intestinal secretions upon infection, it is not wholly protective (90, 122).

Cholera toxin used as an oral adjuvant has been shown to affect a greater specific immune response at mucosal surfaces for some antigens (14, 31). Protection against Sendai virus infection has also been shown with this model (61).

The research described in this thesis was performed to ascertain if oral immunization with cholera toxin and *S. hyodysenteriae* cellular antigens in mice would promote a greater, more efficient antibody immune response against *S. hyodysenteriae* than immunization and/or infection without cholera toxin and if a stronger antibody response than that generated by conventional immunization and/or infection allowed for protection against the cecitis induced by the bacteria. This was accomplished through enzyme-linked immunosorbant assay of serum antibody and ELISPOT examination of antibody secreting cells (ASC) in the spleen and mesenteric lymph nodes.

**Explanation of thesis format:** This thesis includes one manuscript written for Infection and Immunity. A general introduction and a general review of the literature precede the manuscript. A general summary immediately follows the manuscript. Literature cited in the literature review, general introduction, and general summary then follows.



## LITERATURE REVIEW

Swine Dysentery

Swine dysentery is an intestinal disease affecting pigs primarily during the growing-finishing period. Clinical signs generally include a mucohemorrhagic diarrhea resulting from lesions of the large intestine. Alternately, swine dysentery has been referred to as vibronic dysentery, bloody scours, bloody dysentery, black scours, necrotic enteritis and mucohemorrhagic diarrhea (29). Its importance lies in the economic toll of an estimated 180 million dollars exacted annually from the U.S. pork industry (47). This value includes monetary losses due to mortality, decreased rate of gain and preventative use of antibiotics in feed (79). Swine dysentery has been reported in most pig-rearing countries of the world (91).

Whiting, Doyle, and Spray first described swine dysentery at Purdue University in 1921 (116). Though no etiologic agent was described for the disease at that time, an infectious etiology was suspected based on the following observations. Outbreaks of swine dysentery were documented after the introduction of new animals onto farms from stock yards. Also, the disease was transferred to naive swine via feces, stomach tissue or large intestine from sick pigs (117, 118). In the following years *Bacillus suispestifer*, *Vibrio* spp., *Salmonella* spp., paratyphoid organisms, "protozoal" spirochetes, and balantidia were all implicated as the causative agent of swine dysentery, but none were ever conclusively demonstrated as such (2, 16, 39, 117).

It was not until 1971 that two groups simultaneously identified a large spirochete (*Treponema hyodysenteriae*) as the etiologic agent of swine dysentery. Harris et. al. at Iowa State University (27) and Taylor & Alexander (106) at

Cambridge University were able to isolate *T. hyodysenteriae* and reproduce disease by administration of the bacteria to pigs. Twenty years later the genus name was changed to *Serpula* based on DNA-DNA reassociation experiments, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell proteins, and 16S rRNA sequence analysis. The results of these tests showed *S. hyodysenteriae* and a closely related nonpathogenic spirochete, *S. innocens*, to be unique from various other identified spirochete genera including *Treponema*, *Leptospira*, *Spirochaeta*, and *Borrelia* (87, 102). The genus was subsequently changed to *Serpulina* due to an oversight that *Serpula* was already a genus of wood rot fungus (103).

*Serpulina hyodysenteriae* is a motile Gram negative-staining, loosely coiled spirochete measuring 6 to 8.5  $\mu\text{m}$  in length and 320 to 380 nm in diameter with a guanine-plus-cytosine content of 25.8% and 7 to 13 endoflagella. The flagella are inserted at each end of the cell and overlap in the middle. An outer membrane covers the entire organism (27).

Substrate amounts of oxygen are consumed by *S. hyodysenteriae* with growth of the bacteria being enhanced by the presence of 1% oxygen in the media (100). *Serpulina hyodysenteriae* utilizes a limited number of carbohydrates for energy, namely glucose and maltose, the end products of fermentation being acetate, butyrate,  $\text{H}_2$ , and  $\text{CO}_2$ . However, the bacteria has also been shown to utilize fructose, sucrose, galactose, trehalose, N-acetylglucosamine, glucosamine, mannose, and pyruvate as growth substrates (100). Biochemically, the organism is negative for catalase, cytochrome oxidase, the production of hydrogen sulfide, gelatin hydrolysis, meat hydrolysis, glycine tolerance, starch hydrolysis, and urease while being positive for bile tolerance,

esculin hydrolysis, iodacetic acid tolerance, and indole production (29).

*Serpulina hyodysenteriae* has also been shown to use cholesterol in membrane synthesis (99). The bacteria can be propagated both on solid and in liquid media (51).

Most pathogenic strains of *S. hyodysenteriae* produce a beta hemolysin as opposed to a incomplete hemolysis produced by *S. innocens*. This feature along with enteropathogenicity in mice or pigs is considered to be the definitive test routinely used to differentiate between the two species (29).

Infection of pigs with *S. hyodysenteriae* is most often accomplished by ingestion of feces from other pigs that are currently shedding the bacteria. Asymptomatic pigs have been demonstrated to shed *S. hyodysenteriae* for as long as 89 days post infection (41). However, this is not the sole route of transmission. Any number of mechanical means can spread the disease including contaminated boots, tires, and animals. Once established in a herd, *S. hyodysenteriae* can persist for over two weeks in soil and over two months in water with colder temperatures (4°C) supporting the longest periods of viability. In addition, mice are considered to be reservoirs of the disease since they have been shown to shed the bacteria for long periods of time (over four months) and their feces have also been shown to cause infection upon ingestion by pigs (29, 41). Long term infection of mice with *S. hyodysenteriae* and the accompanying cecitis make mice a common choice for the laboratory model of swine dysentery (41, 75). Also used as models are chicks (106), rabbits (53), and guinea pigs (40).

In the porcine large intestine, *S. hyodysenteriae* is attracted to the mucus covering the gut via chemotaxis (48). Because of this phenomenon and the



ability of the organism to move efficiently through mucus, the bacteria are able to directly interact with the epithelium. It is in this location that organisms multiply, particularly in the crypts of Lieberkuehn, and trigger the detrimental effects seen in the host (119, 120).

Potential virulence factors of *S. hyodysenteriae* have been described. These include the aforementioned hemolysin (49, 50, 54, 58, 93-96), lipopolysaccharide (LPS) (29), enzymes (100), and motility (29).

The beta hemolysin (or possibly the beta hemolysins (35)) of *S. hyodysenteriae* has been suggested to be a protein associated with lipids or a lipoprotein (94). Adhesive and lytic capabilities have been ascribed to this molecule (74) and its probable substrate is a lipophilic molecule (47). The hemolysin is oxygen stable and heat labile (94). Estimates of molecular mass have varied including 74 kilodaltons (kDa), 26.9 kDa, and 19 kDa (50, 74, 94). A hemolysin gene termed *tly* has been identified for *S. hyodysenteriae*, but may not be the sole hemolysin present (35). The molecule is cytotoxic for several types of cell cultures (29) including fibroblasts and porcine lymphocytes (49). Colonic loops from germfree pigs have been shown to sustain epithelial cell damage in the intercrypt zone when injected with high levels *S. hyodysenteriae* hemolysin (100,000 hemolytic units) (64). The lytic process is temperature dependent for red blood cells and appears to be associated with swelling for both red blood cells and porcine epithelial cells (29, 93). Lipolytic and proteolytic actions or the requirement for divalent cations are likely not involved in the hemolysin's lytic mechanism (94). The action causing red blood cell lysis has been suggested to be a change in cell permeability due to digestion or disruption of a membrane component. Production of beta hemolysin by *S. hyodysenteriae*

has been reported to peak during the late-logarithmic phase and extracellular amounts can be increased with supplementation of yeast core RNA or sodium RNA (54, 58, 88). The beta hemolysin of *S. hyodysenteriae* and the alpha hemolysin of *S. innocens* have been found to differ in phospholipid sensitivity, hemolytic spectra, specific activity on rabbit erythrocytes and lysing efficiency of bovine, ovine, equine, or porcine erythrocytes where the beta hemolysin was a more effective lytic agent (95). No homologies have been found between the hemolysin of *S. hyodysenteriae* and the nucleic acid and protein characteristics of other reported hemolysins (74).

In 1979, Baum and Joens first described an LPS for *S. hyodysenteriae* (29). This molecule is dissimilar to that extracted from other Gram negative organisms, such as *Escherichia coli*, due to the absence of lipid A (25, 47). The LPS has been shown to be endotoxic with possible direct action on porcine epithelial cells of the large intestine (83). It has been reported to be toxic for murine macrophages plus having the ability to promote greater complement (C3) and immunoglobulin G-Fc receptor-mediated phagocytosis (23, 81). The LPS is an agent of chemotaxis both for neutrophils and monocytes while also causing lymphocyte proliferation and increased Ia expression on murine macrophages (47). In the presence of *S. hyodysenteriae* endotoxin (LPS + protein) murine peritoneal exudate cells (PEC) show increased production of interleukin-1 (IL-1) and tumor necrosis factor (TNF) (23, 29). These two substances can up-regulate many more products involved in inflammation and general immune system activity such as prostaglandin E<sub>2</sub>, increased numbers of MHC class I or II molecules, intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1), platelet activating factor (PAF), IL-2,

granulocyte/macrophage colony stimulating factor (GM-CSF), and IL-6 (79). It has also been suggested that the LPS/endotoxin of *S. hyodysenteriae* may augment natural killer (NK) cell activity and immunosuppression in some tissues (23). Work with the mouse model has demonstrated differential susceptibility to disease based on LPS sensitivity. Lipopolysaccharide hyporesponsive mice are more refractive to the development of cecal lesions than LPS normal responder mice upon infection with *S. hyodysenteriae*. However, at challenge doses of  $1 \times 10^6$  *S. hyodysenteriae* or greater, even LPS hyporesponsive mice (C3H/HeJ) develop lesions (78, 83). Due to these traits, LPS has been implicated in participating in inflammation during the course of disease by inducing the release of lymphokines, prostaglandins and histamine from infiltrating cells (47). However, when compared to the LPS of nonpathogenic *S. innocens*, it has not been conclusively proven that the difference in virulence between the two bacteria lies in the biologic activity associated with the the LPS or endotoxin moiety (22).

Enzymes cannot be discounted as possible virulence mechanisms of *S. hyodysenteriae*. This is not an area that has received much study. However, it has been shown that *S. hyodysenteriae* in the utilization of minute amounts of oxygen produces the enzymes NADH oxidase and superoxide dismutase (100). The role of these two enzymes has not been investigated in relation to the pathogenesis of swine dysentery.

Motility almost certainly plays a significant role in the development of disease. As stated above, *S. hyodysenteriae* moves swiftly and efficiently through mucus. This characteristic may make the bacteria more difficult to phagocytize and to eliminate from the gut by excess mucoid secretions. This is



also an area that has received minimal attention.

The progression of swine dysentery, though, is much more complex than one or two significantly toxic products from a bacterial species destroying tissue. Also implicated in the induction of swine dysentery are other general groups of bacteria, an inappropriate host immune response, environmental stresses, diet, and genetic predisposition towards susceptibility.

One of the early pathologic responses seen in conventional swine following colonization of *S. hyodysenteriae* is an increase in mucus secretion from goblet cells in the basilar crypts of the large intestine (119). This is accompanied by coagulative necrosis of the mucosal epithelium and the superficial lamina propria. Such areas are associated with large numbers of spirochetes (34). Also initially observed in infection by scanning electron microscopy is a rough, corrugated appearance of the cecal and colonic mucosa (107). Plus, superficial vascular congestion in cecal and colonic lesions and edema of the lamina propria have been demonstrated (1). As the disease progresses, crypt dilatation (47), separation of epithelial cells in the crypt shoulders (1), mucosal hemorrhage (29), fibrin thrombi within superficial lamina propria vessels (34), hepatic congestion (29), and eroded gut surfaces (34) are observed. Covering the eroded gut are pseudomembranes consisting of mucus, erythrocytes, bacteria and fibrin (34). Polymorphonuclear cells infiltrating under the eroded surface and mononuclear cells infiltrating into the lamina propria and submucosa also occur at this time (34). Primarily at this point the clinical signs of swine dysentery are exhibited. *Serpulina hyodysenteriae* has been observed to be present within and between epithelial cells of the large intestine, but not beyond the lamina propria. However, tissue invasion is not believed to be a

necessary requirement for lesion development (1, 48, 108). It has not been conclusively proven whether or not receptor/ligand attachment of *S. hyodysenteriae* to enterocytes is prerequisite to lesion progression (4, 37, 48). Similarly, mice infected with *S. hyodysenteriae* show mucoid feces, hyperemia of the cecal and colonic mucosa, varying incidence of cecal petechial hemorrhage, plus cecal mucosal edema, dilatation of crypt areas, and multifocal epithelial erosions (41). In addition, studies with mice have indicated that mast cells do not play a role in the pathogenesis of the disease affected by *S. hyodysenteriae* (77). Enlarged local lymph nodes often accompany infection both in pigs and in mice (29, personal observation).

Ultimately diarrhea resulting from *S. hyodysenteriae* is due to colonic malabsorption. This occurs because of the failure of the epithelial transport mechanisms to move sodium and chlorine from lumen to blood. Also, responses of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are altered. This evidence indicates swine dysentery's pathogenesis is not similar to that of enterotoxigenic *Escherichia coli* or *Salmonella* spp. (29). Most animals die of dehydration, acidosis, and hyperkalemia (29).

Gnotobiotic animals are often used to study diseases where pre-existing microflora may have varied and controversial effects on the pathogenesis. Swine dysentery is such a disease. *Serpulina hyodysenteriae* has been monoassociated (being the only microorganism present) or biassociated (being one of only two microorganisms present) with gnotobiotic pigs or mice (28, 30, 44, 70, 71, 76, 79, 114). Swine monoassociated with *S. hyodysenteriae* show the organism invading goblet cells as early as two days post infection and adjacent enterocytes

being invaded by the fourth day. This was accompanied with mucus hypersecretion and hyperplasia of immature basilar crypt cells. In addition, minor mucosal erosion, but little inflammation, was observed in this model (79). When selected gram negative or gram positive microorganisms are biassociated with *S. hyodysenteriae* in the gnotobiotic pig or mouse model, disease follows. Initially four anaerobes were demonstrated to affect clinical disease when biassociated with *S. hyodysenteriae* in gnotobiotic pigs. Though not characterized, these bacteria were likely bacteroides and/or fusobacteria (70, 71, 79). In fact, high numbers of *Bacteriodes vulgatus* and *Fusobacterium necrophorum* have been found in the colonic mucosa of conventional swine during the initial stages of swine dysentery (2). Subsequently, *Fusobacterium necrophorum*, *Bacteroides vulgatus*, a *Clostridium* species, or *Listeria denitrificans* biassociated with *S. hyodysenteriae* have been shown to facilitate the induction of dysenteric lesions in gnotobiotic swine (28, 114). None of the above mentioned organisms affected disease when monoassociated. Clinical signs were similar regardless of the secondary bacteria with which *S. hyodysenteriae* was co-infected. It has been suggested that the secondary bacteria may assist *S. hyodysenteriae* in growing to greater numbers in the intestine (114). Despite the reports of indicating the need for a second bacterial species in the development of swine dysentery (28, 44, 69, 114), there has been one report of monoassociated gnotobiotic pigs showing signs of disease (115). Gnotobiotic mice, also, have not exhibited lesions when monoassociated with *S. hyodysenteriae*, but have been shown to develop lesions when biassociated with *S. hyodysenteriae* and either *Bacteroides vulgatus* or *Bacteriodes uniformis* (30, 44, 76). These experiments indicate a synergistic role of existing microflora in



the development of swine dysentery (69). *Serpulina hyodysenteriae*, however, still appears to be the sole etiologic agent associated with every naturally occurring outbreak in pig herds.

Stress is another important factor in the development of swine dysentery. Different stressors include change in feed, overcrowding, castration, handling, transportation, other infectious diseases, and exposure to extreme temperatures (29). When stressed, carrier animals become more likely to develop clinical signs of disease.

Additionally, diet seems to affect the development of disease. In pigs a higher fibre diet decreases the likelihood of disease (29). On the other hand, an egg-white-based diet in mice increases the susceptibility of *S. hyodysenteriae* affected cecitis. This increased susceptibility was accompanied by increased numbers of gram negative and gram positive organisms in the gut (78).

Genetic background has also been shown to affect susceptibility of mice to *S. hyodysenteriae* induced cecitis. Lipopolysaccharide responsiveness, as described above, and specific mouse strain are known factors in disease development (78, 104). Presumably, genetic background would affect swine susceptibility, also, to swine dysentery.

If a pig is multiply exposed to *S. hyodysenteriae*, increased, often serotype specific, resistance is seen to redevelopment of swine dysentery, but not complete protection (42, 47, 86). This lack of protective immunity is not well understood. Various methods have been used to demonstrate specific immune responses to *S. hyodysenteriae* in the pig and mouse which include microtitration agglutination and *in vitro* growth inhibition of *S. hyodysenteriae*, both performed using convalescent serum (18, 29). Specific immunity is also

seen lactogenically with piglets (29). However, the majority of experiments showing specific immune responses to *S. hyodysenteriae* have utilized either the enzyme linked immunosorbant assay (ELISA) technique or the western immunoblot technique.

A rise in specific immunoglobulin M (IgM), IgG, and IgA has been observed in mice and pigs exposed to *S. hyodysenteriae* in both the serum and intestinal secretions (90, 122). A rise in the titre of specific IgA, the major immunoglobulin at mucosal surfaces, has not been shown to correlate with protection against swine dysentery. Rather, increased levels of *S. hyodysenteriae* specific IgA reflects recent exposure to the bacteria (90). Paradoxically, specific IgA levels in gut secretions were demonstrated to be higher in swine during primary infection than during tertiary infection with *S. hyodysenteriae* (90). Further, it has been demonstrated that the colonic lamina propria of swine is a site for specific IgM and IgA production while the colonic lymph nodes are sites for specific IgG and IgA production. Systemically, IgG is the major antibody present. Conflicting reports exist pertaining to the correlation of specific IgG titres to protection against swine dysentery. Rees et. al. (90) have reported specific IgG titre to be related to prolonged exposure to *S. hyodysenteriae* and not protection. However, Wright et. al. (122) reported higher specific IgG titres in *S. hyodysenteriae* infected swine showing no clinical signs of swine dysentery as opposed to swine with clinical signs. Convalescent swine sera has also been shown to have anti-*S. hyodysenteriae* LPS antibody that affects opsonization and partial disease protection in a strain specific manner (82). This strain specific anti-LPS antibody is routinely used to separate *S. hyodysenteriae* isolates

into serotypes and serogroups (29, 65). To date, at least nine serogroups have been demonstrated many of which contain different serotypes (26, 59, 60).

The western immunoblot technique has been used extensively in the characterization of the humoral immune response of mice and swine to *S. hyodysenteriae*. Much of the antibody response seen using this method is cross-reactive with *S. innocens* (79, 47). However, three cell antigens at 31, 36, and 68 kDa were found to be specific for *S. hyodysenteriae* by western immunoblot using hyperimmune rabbit serum raised against *S. hyodysenteriae* (10). Additionally, as shown by western immunoblot, cellular antigens with molecular weights from 29 to 45 kDa were conserved between different serotypes of *S. hyodysenteriae* using sera from convalescent or vaccination protected pigs (10, 56). This is in contrast to cellular antigens with molecular weights from 14 to 24 kDa which differed between strains when hyperimmune rabbit serum raised against *S. hyodysenteriae* was used in the immunoblot (10). The conserved bands were probably components of the flagella (56, 52) and the nonconserved associated with the LPS (10). Both flagellar and LPS antigen are associated with the cell envelope of *S. hyodysenteriae* which has been shown through immune electron microscopy to contain the major antigens recognized on western immunoblots (113). Therefore, the outer membrane has been separately characterized through western immunoblot. Protein antigens were found ranging in sized from 14 kDa to over 100 kDa (47, 113). A band at 16 kDa has been demonstrated as species specific for *S. hyodysenteriae* and endotoxin- or LPS-like in character as shown by loss of immunoblot activity when convalescent pig serum was absorbed with *S. hyodysenteriae* endotoxin (113). The 16 kDa antigen has also been shown to have antibactericidal activity to *S. hyodysenteriae*



*in vitro* and has been found in sera and colonic secretions of convalescent swine. This indicates a possible importance in stimulating a protective immune response (97). When cell components were treated with metaperiodate or proteinase K, loss of the 14-19 kDa band was demonstrated on immunoblots only with the metaperiodate treated preparation. This would indicate that the species specific antigen has carbohydrate and/or lipid moieties (47, 113). The gene encoding the 16 kDa molecule has been identified and termed *smpA* (109). In addition, a unique 45 kDa antigen has also been demonstrated in the outer membrane of two *S. hyodysenteriae* strains, P18A and MLC52/80 (97). Despite the above demonstrated specific immune responses, it has been hypothesized that resistance also requires a low level chronic colonization with *S. hyodysenteriae* (42). A better understanding of the immune response to *S. hyodysenteriae* may explain its largely ineffective protective qualities for feeder pigs.

#### Mucosal Immune System

The immune system response occurs at different sites where, first, antigen is encountered and initial responses occur (inductive sites), second, the physical path activated cells follow while proliferating and differentiating, and third, sites where mature, stimulated, fully differentiated immune cells are found (effector sites) (67). The signals received in these three steps are a complex network that in great part defines the immune response.

Because *S. hyodysenteriae* doesn't invade beyond the lamina propria, most of the specific immune response, theoretically, would be stimulated via the mucosal route. Antigen mainly, though not exclusively, exits the gut through dome cells also called microfold (M) cells or follicle-associated epithelial (FAE) cells. These cover the gut associated lymphoid tissue (GALT) termed Peyer's

patches. The dome cells have short microvilli, small cytoplasmic vesicles, and few lysosomes. They can easily transport luminal antigens such as proteins and particulates like bacteria, viruses and small parasites via nondenaturing pinocytosis (67, 92, 110).

Once in the Peyer's patches, the antigen acts in its unmodified form as the first of two signals to stimulate mature, specific B lymphocytes by binding to membrane bound immunoglobulin (Ig) receptors. The majority of these B cells are located in germinal centers of distinct follicles. Switching to the IgA isotype and affinity maturation are considered to occur at this site, but significant numbers of plasma cells do not develop in the Peyer's patches (67). Adjacent to the follicles are areas containing mature T lymphocytes. These cells contain the pan T cell marker, CD3, and have T cell receptors (TCR) mainly of the  $\alpha\beta$  phenotype (95%). This T cell population is also characterized by  $CD4^+CD8^-$ ,  $CD4^-CD8^+$ , and  $CD4^-CD8^-$  subsets (67). In addition, accessory cells are present in the Peyer's patches, namely major histocompatibility antigen (MHC) class II<sup>+</sup> B cells, dendritic cells, and macrophages (67). These cells present partially degraded antigen associated with MHC class II antigen to act as the first of two activation signals for specific T lymphocytes. These two molecules interact with CD3, TCR, and CD4 markers on the target T lymphocyte (67). This T cell antigen recognition is probably more limited than B cell antigen recognition and represents sites unique from those bound by B cells. T cell recognized antigens are often "unfolded" portions of protein with which B cells don't naturally have contact (17).

The antigen presenting cells can also provide lymphokines, namely interleukin-1(IL-1) from macrophages, that function as the second activation

signal for T cells. This is particularly effective in the presence of suboptimal mitogen levels (17). As stated above, IL-1 is a proinflammatory cytokine. It has been shown to affect fever, release of neutrophils from the bone marrow, elevation of acute phase reactants, and lowering of plasma iron and zinc concentrations (17). The activated T cells, in turn, secrete lymphokines that autostimulate such as IL-2 (17) and act as the second activation signal for B cells such as IL-2, IL-4, and transforming growth factor beta (TGF- $\beta$ ) (17, 67). Interleukin-2 is essential for T cell proliferation, differentiation, and clonal expansion (17).

Upon original exposure to an antigen, IgM is produced. Later, with continued antigen exposure or antigen re-exposure, an isotype switch occurs resulting in IgG, IgA, or IgE phenotype. The antibodies also become more specific for the stimulating antigen(s) through a process referred to as affinity maturation. Affinity maturation is due to somatic point mutations in the immunoglobulin variable region genes (89). Such activities occur before the plasma cell (terminal antibody secreting cell) stage and are often attributed to memory B cells. Whether or not T cells go through an analogous process has not been demonstrated. However, preferential expansion of different T cell subsets has been observed. Switch T cells, cells that cause a change in antibody isotype, that are specific for IgA have been demonstrated in the Peyer's patches (17). These differ from switch T cells found in the spleen which upon stimulation with concanavalin-A suppressed IgA synthesis (6, 67). Switch T cells appear to be auto reactive and triggered directly by MHC class II determinants (6). The switch mechanism probably involves deletion and rearrangement of the heavy chain gene of B lymphocytes (110). Transforming growth factor- $\beta$  has



been implicated in the switch of IgM<sup>+</sup> cells to IgA<sup>+</sup> cells (63). Also, many other effects are attributed to TGF- $\beta$ . These include inhibition of cytotoxic T cell generation, NK cell function, and T and B cell proliferation, monocyte chemotaxis, and suppression of IgG and IgM secretion (98, 112). However, switch T cells don't support terminal differentiation into IgA plasma cells (6, 67).

Post switch T helper (Th) cells favoring IgA production have been cloned from murine Peyer's Patches (6). The presence of these post switch T cells is often accompanied by suppression of a systemic IgG reaction to the stimulating antigen(s), also called oral tolerance (92). The phenomenon has been attributed to T suppressor (Ts) cells which leave the Peyer's patches and seed peripheral tissues (110). Oral tolerance abrogation has, in fact, been seen when T contrasuppressor (Tcs) cells are passively transferred to tolerized mice (110). Immunoglobulin A binding factor has also been implicated in oral tolerance induction (110). Oral tolerance may be a protective response of the host against harmful systemic reactions elicited by IgG, IgE, and T cell mediated delayed type hypersensitivity (6). There is evidence that oral tolerance may also be abrogated by abnormal expression of MHC class II antigens on epithelial cells. This may cause preferential activation of CD4<sup>+</sup> T cells instead of CD8<sup>+</sup> T cells. Such a situation was observed when T cells were exposed to colonic epithelial cells from patients with inflammatory bowel disease (IBD) (6).

Due to the actions of Th, Ts, and Tcs cells present in the Peyer's patches, initially stimulated B lymphocytes will begin proliferating and differentiating into the dimeric IgA-secreting cells found at mucosal sites (110). Once specific lymphocytes are activated, they move out of the Peyer's patches

via the efferent lymphatics and move into the blood stream through the thoracic duct (67). The cells then progress to the spleen where they probably receive additional developmental signals; they then migrate back to mucosal sites and mucosal associated lymph nodes including the lamina propria of the respiratory, gastrointestinal (GI) and reproductive tract, and mammary, salivary and lacrimal glands where more developmental signals are likely received (IL-5, IL-6, interferon gamma (INF- $\gamma$ ), TNF, additional antigen stimulation) (67). Specific IgA antibody-forming cells have been observed transiently in peripheral blood mononuclear cells at 7-12 days post oral immunization and their presence preceded the appearance of specific secretory-IgA (S-IgA) antibodies in saliva and tears (67). It has been estimated that approximately eighty percent of immunoglobulin producing cells are located in the intestinal mucosa alone (6).

Switch T cells have receptors for the Fc (FcR) portion of IgA. Other nonswitch T cell bear FcR for IgA, too. It is likely such cells play a role in isotype regulation (17). There is evidence that IgA binding factor secreted from T cell hybridomas stimulates IgA responses at low doses and inhibits at high doses (17). Another factor in the development of IgA antibody secreting cells (ASC) are the ratios and levels of CD4 versus CD8 positive cells and Th1 versus Th2 cells. The former are differentiated by their receptor expression and latter are differentiated on their cytokine secretion profiles.

T cells expressing CD4 and CD8 markers interact with MHC class II and class I antigens, respectively (17). Cells that are CD4<sup>+</sup> demonstrate mainly helper cell functions such as supporting antibody secretion or expansion of other T cell populations. Cells that are CD8<sup>+</sup> usually exhibit cytotoxic or suppressor functions (67).

T helper 1 cells typically secrete IL-2, IL-3, IFN- $\gamma$ , and TNF- $\beta$ .

Interleukin-3 is a differentiation factor for immature T-cells and it serves as a growth factor for murine basophils and mast cells (15). Interferon- $\gamma$  has a wide range of effects which includes local and systemic antiviral effects, cellular differentiation, growth, surface antigen expression, morphology, immunoregulation, cell mediated immunity, augmentation of natural killer cell activity, activation of macrophages for tumoricidal and bacteriocidal activity, and induction of IL-2 receptors and T cells effects (17). T helper 1 cells have been implicated in cell-mediated immunity by secreting cytokines that activate macrophages to more effective killing of intracellular bacteria (67).

T helper 2 cells typically secrete IL-3, IL-4, IL-5, IL-6, and IL-10 (63, 115). There has been shown to be higher levels of Th2 cells in mucosa associated tissues which may in turn support IgA production (57, 67).

Interleukin-4 alone affects LPS activated mouse spleen cells to switch isotype to IgG1 or IgE (63). This lymphokine is a B cell growth factor which stimulates activated B lymphocytes to proliferate (9). Interleukin-4 can also up-regulate MHC class II expression on B cells (56).

Interleukin-5 will synergize with several different lymphokines for IgA production (57). Transforming growth factor- $\beta$  has been shown to act in conjunction with IL-5 to cause IgA production additively in LPS stimulated B cell cultures (98). In this same system, IL-2, in place of IL-5, was found to have a synergistic effect on IgA production (98). There is also evidence that the presence of IL-5 suppresses the production of antibody isotypes other than IgA (98). Interleukin-5 has been shown to increase numbers of IgA secreting cells and to induce actual secretion, but not cell division in culture (6, 67).



Interleukin-4 in conjunction with IL-5 has been shown to enhance IgA secretion in splenic B cells. In this situation IL-4 probably acted as a switch factor (98, 110). With Peyer's patch cells, IL-5 and IL-6, but not IL-4 seem to affect IgA synthesis. Interleukin-5 also up-regulates IL-2 receptors on B cells further indicating a role for IL-2 in IgA synthesis (110).

Interleukin-6 is a potent cytokine in conjunction with IL-5, as indicated above, in conjunction with other factors, and separately. It has been shown that IL-6 acts with IL-1 to promote IgM secretion and with IL-5 to promote IgA secretion in Peyer's patch B cells in a synergistic manner (57). Interleukin-6 is considered to be a relatively late-acting lymphokine while IL-1 and IL-5 are considered to be early-acting lymphokines. Interleukin-6 can also act to induce synthesis of acute phase proteins in liver cells, induce the central nervous system (CNS) to cause neuronal differentiation and to induce fever (57). Interleukin-6 may have an autocrine effect since it is also produced by plasma cells. Additionally, macrophages, fibroblasts, endothelial cells, and epithelial cells produce IL-6 (57, 65, 110). Interleukin-6 has also been found to act on T cells in concert with other stimuli to induce IL-2 production (57). Interleukin-6 is a potent cofactor in mitogen-induced T cell proliferation and can enhance the cytolytic T cell differentiation in the presence of IL-2 (57). The above observations, then, may represent a step wise stimulation of B cells by different lymphokines for antibody secretion (57). Alternately, IL-6 may simply cause an increase in the number of cells secreting IgA via proliferation (57).

Interleukin-10 production has been reported in mice during parasitic infection. The presence of IL-10 is often accompanied by INF- $\gamma$  suppression (73). Interleukin-10 has also been shown to inhibit production of IL-1, IL-6,

and TNF- $\alpha$  while up-regulating expression of MHC class II molecules on B cells. In addition to T cells, B cells, macrophages, and keratinocytes have also been demonstrated to secrete IL-10 (56).

Different phenotypic characteristics between systemic immune sites, bronchus associated lymphoid tissue (BALT), and GALT have been implicated in, and may be partially explained by, the different cell populations and microenvironments found (6). It has been suggested that Ia<sup>+</sup> antigen on epithelial cells increases the number of CD8<sup>+</sup> cells as opposed to antigen presented by monocytes to CD4<sup>+</sup> cells and may make the difference between tolerance and responsiveness, respectively (110).

In the lamina propria of the gut, IL-5<sup>+</sup> cells have been shown to outnumber INF $\gamma$ <sup>+</sup> cells by two or three times as opposed to Peyer's patch T cells where less total cytokine-producing cell numbers were present and the IL-5<sup>+</sup> and INF $\gamma$ <sup>+</sup> cells were equal in number (67). The same ratios were seen with intraepithelial lymphocytes (IEL) as in the Peyer's patches except that at least some of the IL-5 and INF- $\gamma$  secretion was due to CD8 positive cells (67). Additionally, it may be that mucosa associated lymphoid tissue (MALT) preferentially attracts and activates T cells secreting IL-5 (6). Secondary signals for terminal differentiation into Ig-producing plasma cells have been implicated to be provided at final secretory tissue sites (6). It has been further suggested that HLA-DR determinants not only of lamina proprial dendritic cells, but also on epithelial may contribute to B cell differentiation (6).

Once activated, the specific homing of committed immune cells to mucosal sites has been attributed to cell surface markers both on lymphocytes (homing receptors) and high endothelial venules (HEV) (addressins) adjacent to

mucosal sites. Different markers have been implicated to serve the same function for peripheral lymphoid tissue (38, 110). In humans, lymphocyte function-associated antigen 1 (LFA-1) and very late activation antigen-4 (VLA-4) are two mucosal homing receptors and intercellular adhesion molecules (ICAM-1 and ICAM-2) are two possible complementary addressins for LFA-1 at the mucosal sites. Similarly, vascular cell adhesion molecule-1 (VCAM-1) may serve as the addressin for VLA-4. During inflammation, ICAM-1, VCAM-1 and endothelial leukocyte adhesion molecule-1 (ELAM-1), another addressin, have their surface expression up-regulated (38).

Bacterial LPS/endotoxin is a substance that has an immunomodulatory effect on lymphocytes and, therefore, regulatory cells (110). Its presence can cause an increase in local cytokine levels and expression of surface activation markers (3, 6). At the gut surface where so many bacteria reside LPS's effects on immune responses play an important role.

In the gut, the B cells and CD4<sup>+</sup> T cells are mainly in the lamina propria (6, 17). In swine the largest population of plasma cells was found to reside in the lamina propria of the intestinal tract for IgM, IgG, and IgA isotypes. The porcine spleen and mesenteric lymph nodes were thought to play a minor role in immunoglobulin synthesis (7). Secreted pentameric IgM or dimeric IgA is assembled in plasma cells with an associated J-chain then it is transported from the basolateral surface of epithelial cells to their mucosal surface via the secretory component receptor in vesicles. In the pig IgA and IgM are, in fact, found in intestinal crypt epithelial cells indicating a similar intracellular secretion pathway (7). When the multimer is released into the gut lumen, part of the secretory component remains to anchor the complex into the



mucus layer and to protect against degradation (92, 110). Interferon gamma and TNF- $\alpha$  have been implicated in up-regulation of the membrane expression of functional secretory component (6, 110). Secretory Ig-A represents over eighty percent of the antibody produced in mucosa associated tissues (67).

The presence of IgA has the potential to cause neutrophil accumulation. The subsequent neutrophils are destroyed and release lactoferrin, lysozyme, and cationic proteins which may contribute to immunity against bacteria (92). Immunoglobulin-A also has the affect of neutralizing virus before it crosses the mucosal barrier. Immunoglobulin-A can inhibit adherence of some bacteria to the mucosal epithelium by specific and nonspecific binding. Bacteria covered with IgA are at a survival disadvantage in the highly competitive microenvironment of the gut. Continuous desquamation of epithelial cells also plays a protective role against pathogenic bacterial colonization (67). The lactoferrin and lactoperoxidase systems that act against several mucosal pathogens are enhanced by the presence of IgA. Immunoglobulin-A can also act with CD3<sup>+</sup>CD4<sup>+</sup>Leu8<sup>+</sup> T cells to affect antibody dependent cell cytotoxicity (ADCC) against *Salmonella* and *Shigella* species. In fact, such T cells can be seen to increase in individuals orally immunized with a *Salmonella* vaccine. Immunoglobulin A can act to immune exclude antigens that the host has already been exposed to and, owing to its reduced ability to activate the complement cascade as compared to IgG or IgE, reduce the likelihood of a severe anaphylactic or inflammatory reaction (67). However, some bacteria can degrade IgA then use the resulting Fab portion for coating which affects protection from the destructive action of other antibodies (110).

The classical view of IEL populations states that T lymphocytes (mainly CD8<sup>+</sup>) home to mucosal epithelium. They mainly exhibit cytotoxic activity, contain a high percentage of  $\gamma\delta$  TCR's, and may not require thymic processing (6, 12, 92). With age, MHC class II (Ia) expression is increased. Along with increased Ia antigen expression by enterocytes, comes increased numbers of IEL's (17). However, there is evidence that T cell populations between the small and large intestine vary significantly. Small intestine IEL's fit more the classical description, while large intestine IEL's do not. The IEL's present in the large intestine have been shown in mice to be less cytolytic and to contain homing receptors like L-selectin which is a traditional lymph node homing receptor (8).

What exact developmental pathway a cell takes depends on its microenvironment and includes site of activation. It has also been shown that significant numbers of IgA plasma cells in the murine GI tract are derived from self-renewing Ly-1<sup>+</sup> (CD5<sup>+</sup>) B cells which reside in the peritoneal cavity. These B cells have been shown to be the origin of serum IgM against naturally occurring antigens and some autoantigens. It is not known if B cells from the Peyer's patches contribute to this population or not. It has also been postulated that CD5<sup>+</sup> B cells represent memory cells (67).

Additionally, memory T cells have been demonstrated in the lamina propria of humans. These cells showed a high expression of CD45RO marker, a typical characteristic of systemic memory T cells. However, these cells differed from systemic memory T cells in that they did not show a high expression of CD29 and they directly secreted B cell helper factors (124).

As stated above, IgA is not the only immunoglobulin isotype synthesized during swine dysentery. However, IgG production may, at least in part, be generated by direct contact of antigen with blood cells. Any IgG present in the gut lumen, also, comes from the blood. Its presence may augment inflammation via the complement cascade. This cascade could also be activated via the alternative pathway with blood contacting certain key organisms in the microflora. Release of C5a, C3a, and C4a with complement activation causes smooth muscle contraction, vascular permeability, and chemotactic influx of phagocytic cells and other leukocytes. Both IgG and IgA can also act as opsonization factors, particularly in the presence of C3 and C4 on the same cells. Fibronectin, a serum protein, can also affect opsonization in the presence of another opsonin. There is also evidence that activated complement receptors on B lymphocytes may cause them to proliferate and mature into plasma cells. In vitro C3a and C5a have antagonistic effects on antibody secretion and lymphocyte proliferation with C5a enhancing the two events. Also, complement activation has been suggested to be important in lymphocyte memory (72).

Mast cells may also play a significant role in inflammation. Cells susceptible to the often proinflammatory or anaphylactic signals of mast cells include lymphocytes, macrophages, neutrophils, eosinophils, platelets, bone marrow stem cells, smooth muscle cells, goblet cells, and neurons (17). However, as stated above, mast cells do not likely play a significant role in the pathogenesis of swine dysentery.

Cytotoxicity is another important protective immune response demonstrated by certain lymphocytes and is important in combatting viral infections and eliminating transformed cells (17, 67). It can either be due to



antibody dependent cell cytotoxicity (ADCC), defined receptor/ligand interactions such as MHC class I antigen with viral antigen contacting the CD3, TCR, and CD8 markers of cytotoxic T cells or undefined receptor/ligand interactions such as that seen with natural killer cells. Natural killer cells are large lymphocytes containing azurophilic cytoplasmic granules and are not T or B cells (17). In addition to direct killing of cells, NK cells may play a role in stem cell development and immunoglobulin regulation. There is also evidence that NK cells can be inhibited by the inflammatory process (17).

Another important consideration in the mucosal immune response is what occurs upon a break in the epithelium. The following process has been outlined. When there is a break, undue stimulation of antigen presenting cells (APC) and/or enhanced epithelial expression of MHC class II determinants occurs. These events are followed by increased presentation of luminal antigens and general overstimulation of CD4<sup>+</sup> cells. This can cause increased production of dimeric IgA, excessive IgG and IgE responses, and delayed type hypersensitivity (6).

In swine dysentery, lymphocytic cells accumulate and are specific, but not very protective. Different medications are effective in eliminating *S. hyodysenteriae* from pigs. These include tiamulin, lincomycin and carbadox given in water (29). However, immunization attempts either parenterally or orally with naive swine using inactivated *S. hyodysenteriae* or attenuated strains have had little or no success in generating a protective immune response (21, 32, 33). With the exception of a report showing protection with a cloned flagellar fragment, minimal vaccination effectiveness has also been seen in the mouse model (5). However, another report found no correlation between vaccination

with a flagellar protein and protection in the mouse from *S. hyodysenteriae* induced cecitis (19).

Found to be a factor in many inflammatory diseases, though not always representative of recovery from disease, is a rise in antibody production systemically and/or locally. Adult periodontitis is such a disease where a large number of local specific plasma cells are seen against *Porphyromonas gingivalis*, but recovery from disease is not related to these numbers. Rather, chronic inflammation with associated host tissue destruction is seen (84, 85). Rotavirus infection is another inflammatory disease where local specific ASC have been demonstrated (68). An increase in nonspecific plasma cells in the intestine, particularly IgG positive cells, is seen in inflammatory bowel disease (IBD), a general category of chronic intestinal inflammation including Crohn's disease and ulcerative colitis (123).

#### Cholera Toxin

It may be possible that not a great enough or an appropriate immune response can be generated either parenterally or without oral adjuvants against *S. hyodysenteriae* for protection against swine dysentery. Several different adjuvants have been used orally with other diseases including biodegradable microspheres, cholera toxin and heat labile toxin of enterotoxigenic *E. coli* (58, 63). Also, selective delivery of protective antigens has been attempted using recombinant bacteria (67).

Cholera toxin is one of the most frequently used oral adjuvants. It consists of five B subunits and one A subunit. The B subunits bind to monoasialoganglioside, GM1, present on all nucleated cells. This binding causes a conformational change that allows the A subunit to penetrate into the cell and

disrupt the cAMP cycle (67). Cholera toxin in microgram amounts will induce an antibody response to itself consisting of specific S-IgA and serum IgG both which originate in the GALT. Cholera toxin and its B subunit do not induce oral tolerance. Oral immunization with cholera toxin also results in prolonged memory in the intestinal lamina propria (67). Cholera toxin is not a typical adjuvant because it stimulates an immune response against itself; however, this trait may be what allows cholera toxin to be used as an adjuvant (67). Cholera toxin may provide the appropriate signals that alter the regulatory environment of the GALT from suppression to responsiveness (67). Cholera toxin may also stimulate Th2 cells in the gut and suppress Th1 cells (121) which would ultimately lead to the expansion of T cell subsets supporting B cell proliferation and differentiation. Additionally, cholera toxin's adjuvanticity may be a fairly complex event involving increased uptake of antigen into mucosal follicles, enhancement of IL-1 production by APC's, altered regulation by T cells, inhibition of CD8<sup>+</sup> suppressor cells, stimulation of B cell switching to IgA and IgG positive cells, and possible enhancement of B cell clonal expansion (67).

Cholera toxin plus keyhole limpet haemocyanin (KLH) has been shown to enhance KLH sensitivity upon re-exposure in the spleen, mesenteric lymph nodes (MLN), lamina propria, and Peyer's patch lymphocyte populations as evidenced by proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> cells and their production of IL-2 and IL-4 (11). Cholera toxin plus a streptococcal antigen has evoked substantial specific antibody responses in the MLN, spleen, salivary glands, and serum (14). Ovalbumin conjugated to cholera toxin and presented to the intestinal mucosa also shows an enhanced antibody responses in the lamina propria of mice (31). However, cholera toxin does not just stimulate the



immune system. It can have more practical application. Cholera toxin conjugated to noninfectious Sendai virus given orally to mice then given as an intranasal booster conferred protection. This was directly related to virus specific titres of IgA in nasal secretions (61). Oral immunization with cholera toxin and tetanus toxoid was also shown to protect experimental mice against a later normally lethal dose of tetanus toxoid. This effect was attributed to neutralizing systemic antibodies (36). The above antigens can not achieve the same level of immune stimulation seen when administered without cholera toxin. It has also been suggested that the ability of certain antigens to bind to intestinal epithelium may dictate the strength of the antibody response elicited by cholera toxin adjuvant. In other words, strong immunogens in the presence of cholera toxin are weak tolerogens in its absence and vice versa (111). Thus, exploration into the effects of oral adjuvants against the development of swine dysentery along with a better understanding of immune events in the gut could prove useful in future generation of an effective vaccine.

PAPER: CHARACTERIZATION OF THE MURINE B LYMPHOCYTE  
RESPONSE TO SERPULINA HYODYSENTERIAE FOLLOWING ORAL  
IMMUNIZATION AND CHALLENGE

## ABSTRACT

Using the ELISPOT technique, antibody secreting cell (ASC) responses to antigens of *Serpulina hyodysenteriae* were explored. Mice were either parenterally or orally immunized with a cellular lysate of *S. hyodysenteriae*. Orally immunized mice received 500 µg of the spirochetal lysate with cholera toxin as an adjuvant or 1 mg of the spirochetal lysate alone. Subsequent infection of immunized mice with *S. hyodysenteriae* showed that oral immunization had primed for a greater specific systemic and mucosal ASC response as compared to parenteral immunization. This was seen both at ten and 17 days post infection. In addition, the use of cholera toxin as an adjuvant increased systemic IgA ASC's at 17 days post infection. Using the ELISA technique, it was demonstrated that oral immunization effectively primed for both the serum IgG and IgA responses while parenteral immunization only primed for the serum IgG response. Even though oral immunization primed for specific immune response to *S. hyodysenteriae*, there was no correlation between the level, specificity, or isotype of the immune response and protection from disease.



## INTRODUCTION

The etiologic agent of swine dysentery is *Serpulina hyodysenteriae*, formerly *Treponema hyodysenteriae* (10, 54). *Serpulina hyodysenteriae* is a motile, gram negative spirochete that grows well in the presence of 1% oxygen (52).

Swine dysentery is a mucohemorrhagic diarrheal disease affecting primarily, though not exclusively, feeder pigs (12). Annually, this disease has been estimated to cost the U.S. pork industry 180 million dollars (22) due to the monetary losses associated with morbidity and mortality (41).

Pigs become infected with *S. hyodysenteriae* most commonly by ingesting feces from pigs that are currently shedding the bacteria. Asymptomatic pigs can shed *S. hyodysenteriae* as has been demonstrated by Joens et. al. (19). In addition, *S. hyodysenteriae* can be spread mechanically or exist in reservoir mouse populations (18). In the porcine large intestine, the site where *S. hyodysenteriae* causes lesions, the organism is chemotactically attracted to mucus and subsequently multiplies in the crypts of Lieberkuehn (23, 60, 61). Because mice can maintain a long term infection with *S. hyodysenteriae* and will show an accompanying cecitis, they are often used as a laboratory model for swine dysentery (18, 38).

A hemolysin and the lipopolysaccharide (LPS) of *S. hyodysenteriae* have been implicated in the pathogenesis of swine dysentery (12, 22, 24, 25, 26, 41, 42, 43, 50, 51). However, it is likely that development of disease involves more than these two factors. Other bacteria, an inappropriate host immune response, environmental stresses, diet, and genetic background have also been implicated

in the development of or susceptibility to swine dysentery (9, 12, 17, 20, 21, 34, 35, 40, 53).

Pigs multiply exposed to *S. hyodysenteriae* show increased, often serotype specific, resistance to reinfection. Despite this, complete immunity to *S. hyodysenteriae* has not been demonstrated (19, 20, 46).

A rise in antibody production systemically and/or locally has been reported for some mucosal inflammatory conditions. Adult periodontitis is such a disease. Specific local and systemic antibody against *Porphyromonas gingivalis* has been demonstrated which does not correlate with recovery from disease (44, 45). Inflammatory bowel disease (IBD) is another chronic inflammatory condition associated with a rise in local antibody production. An increase in the number of nonspecific plasma cells located in the human large intestine has been associated with the pathogenesis of IBD (64). A rise in local specific antibody secreting cells (ASC) has also been reported for rotavirus infections (32).

During swine dysentery, *S. hyodysenteriae* specific IgM, IgG, and IgA have been demonstrated both in serum and intestinal secretions (48, 63). An increase in IgA, the major immunoglobulin at mucosal surfaces, has been reported to reflect recent exposure to *S. hyodysenteriae*, but has not been correlated with protection against disease (48). An increase in specific serum IgG has been reported to be related to prolonged exposure to *S. hyodysenteriae* (48). Relatively higher specific IgG titres have been demonstrated in asymptomatic, *S. hyodysenteriae* infected pigs as opposed to infected swine showing clinical disease (63). However, immunological prevention of the clinical symptoms of swine dysentery, in a predictable manner, has not been achieved. In general, vaccination either parenterally or orally of naive swine

with preparations of *S. hyodysenteriae* or with attenuated strains have had minimal success at inducing protection (8, 15, 16). A similar situation has been observed in mice with the exception of using cloned flagellar fragments. Immunization with these fragments may protect mice against *S. hyodysenteriae* induced cecitis (2, 7).

Mucosal adjuvants may enhance the mucosal immune response and protection from disease caused by mucosal pathogens. Biodegradable microspheres, cholera toxin, and heat labile toxin of enterotoxigenic *Escherichia coli* have all been used as oral adjuvants (29). In other systems, cholera toxin has proven to enhance antibody production at mucosal surfaces against poorly immunogenic proteins (6, 14). Cholera toxin in an inactivated Sendai virus immunization trial of mucosal surfaces has been shown to enhance specific IgA that prevented infection by live Sendai virus (28).

In the following experiments, the ability of cholera toxin to enhance the immune response of mice to *S. hyodysenteriae* antigens was explored. This was accomplished by measuring specific ASC via the ELISPOT technique in mesenteric lymph nodes (MLN) and spleen of variously immunized and/or infected animals. Specific serum antibody levels were also determined using the ELISA technique and antibody specificity was determined using the western immunoblot technique.



## MATERIALS AND METHODS

**Animals:** The mice used were strain C3H/HeO<sub>u</sub>J. These mice were obtained from a breeding colony maintained at the College of Veterinary Medicine, Iowa State University. Original breeders were purchased from Jackson Laboratories, Bar Harbor, Maine.

**Bacterial Strains:** *Serpulina hyodysenteriae* B204 with less than 20 *in vitro* passages was grown anaerobically at 37°C in trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% horse serum (HyClone Laboratories, Logan, Utah), 0.5% yeast extract (BBL), and 1% VPI salt solutions as previously described (58). Highly motile (motility, >90%) log-phase cultures were obtained by inoculating warm (37°C) broth with a 33% inoculum from an overnight culture and incubating the inoculated broth for approximately 5 h. Bacteria were enumerated by using a Petroff-Hauser counting chamber.

**Antigen Preparation:** *Serpulina hyodysenteriae* B204 cells in log phase were collected by centrifugation. The cells were washed with 0.1 M phosphate buffered saline (PBS), pH 7.2, and suspended in a final volume of 10 ml. The cells were disrupted via sonication and the mixture was harvested by centrifugation (10,000 x g). The subsequent pellet was then assayed for total protein by the method of Lowry et. al. (30) and found to be 20%. For storage the lysate was lyophilized and stored at -20°C.

**Vaccination Protocol:** In Experiment One mice were either intraperitoneally (I.P.) or orally (P.O.) immunized. Mice I.P. immunized were injected with 300 µg *S. hyodysenteriae* B204 whole cell lysate in normal pyrogen

free saline on days one and 13. Mice P.O. immunized were orally intubated with a total of 500 µg of *S. hyodysenteriae B204* whole cell lysate, 30 µg of cholera toxin subunit B, and 1.5 µg of whole cholera toxin (62) in 0.1 M PBS, pH 7.2, on days one, two, and three; this immunization was repeated on days 13, 14, and 15.

In Experiment Two, mice were either orally intubated with 1mg of *S. hyodysenteriae B204* whole cell lysate or orally intubated with 500 µg of *S. hyodysenteriae B204* whole cell lysate, 30 µg of cholera toxin subunit B, and 1.5 µg of whole cholera toxin (62) in 0.1 M PBS, pH 7.2. The above two vaccination protocols were performed over a period of three days (days one, two, and three); these immunizations were repeated on days 13, 14, and 15.

In Experiment Three, the mice were orally intubated with a total of 500 µg of *S. hyodysenteriae B204* whole cell lysate, 30 µg cholera toxin subunit B, and 1.5 µg of whole cholera toxin (62) in 0.1 M PBS, pH 7.2, on days one, two, and three; this immunization was repeated on days 13, 14, and 15.

**Infection Protocol:** In all experiments vaccinated mice along with an unvaccinated group were infected with  $1 \times 10^8$  *S. hyodysenteriae B204* orally on day 25. Three days prior to challenge all mice involved in the experiment were fed a Teklad 85420 Diet (Harlan Sprague Dawley) on which they remained for the duration of the experiment. Mice were sacrificed on day three, ten, 17, or 30 post infection. Spleen, MLN, serum, and cecum were collected from each mouse. At the time of sacrifice, mice were between ten and 18 weeks of age.

**Tissues:** Blood samples were collected following CO<sub>2</sub> asphyxiation and cardiac puncture and allowed to clot for 24 hours at 4°C. Serum was then separated from the clot by a three minute centrifugation in a microfuge,

collected, and stored at -20°C until the samples were used in either western immunoblot analysis or an enzyme linked immunosorbant assay (ELISA).

Ceca were given a score indicating severity of the *S. hyodysenteriae* induced cecitis then were preserved in formalin. Within the scoring system, zero indicated no excess mucus or cecal atrophy, one indicated excess mucus with no cecal atrophy, two indicated excess mucus with partial cecal atrophy, three indicated excess mucus with total cecal atrophy, and four indicated excess mucus with total cecal atrophy and blood.

The mesenteric lymph nodes and spleen were collected and minced through 60 mesh wire screens into RPMI 1640 Media (Sigma) containing 10 mM HEPES buffer, 2 mM L-glutamine, penicillin/streptomycin (100 U/ml), gentamycin (0.05 mg/ml). The subsequent single cell suspensions were then immediately used in an ELISPOT assay.

**Western Immunoblot:** A whole cell lysate of *S. hyodysenteriae* B204 (100 µg) was denatured by boiling for two minutes with sodium dodecyl sulfate then electrophoresed on a 12.5% sodium dodecyl sulfate polyacrylamide gel using a Bio-rad Mini-Protean II Cell (165-2940) for 45 minutes. The resulting gel was then assemble into a Bio-rad Mini Trans-blot Electrophoretic Transfer Cell (170-3930) and blotted onto a nylon membrane (Immobilon-P Transfer Membrane, Millipore) for one hour. The membrane was blocked with 5% skim milk in tris buffered saline (TBS) (29 g NaCl/L, 2.42 g Tris/L), pH 7.5, for one hour at 37°C. The membrane was then cut into strips and incubated for two hours with a mouse serum sample diluted 1:50 in TBS plus 0.2% skim milk. Alkaline phosphatase conjugated goat antibodies specific for mouse IgM, IgG, and IgA isotypes were then incubated with the membrane strips for one hour at



37°C at a 1:500 dilution in TBS plus 0.2% skim milk. Finally, the membrane strips were developed using Fast Red (4-chloro-2-methylbenzenediazonium) (1.5 mg/ml) and Naphthol-AS-MX phosphate ( $C_{19}H_{16}NO_5PNa_2$ ) (0.75 mg/ml) suspended in tris buffer (2.5 g Tris-HCl/L), pH 8.0 (36, 37).

**ELISA Assay:** Immulon-4 flat bottom plates (Dynatech Laboratories) were coated with a whole cell lysate of *S. hyodysenteriae B204* (100 µg/ml) in 0.1 M PBS, pH 7.2, overnight at 4°C. Serum samples from individual mice described above were diluted 1:100 in normal saline solution plus 0.025% Tween 20 (TS) then incubated on the antigen coated plates for two hours at 37°C. After this incubation period, alkaline phosphatase conjugated goat antibody specific for mouse antibodies of either the IgM, IgG, or IgA isotype at a 1:2000 dilution in TS were incubated on the plate for one hour at 37°C so that each serum sample was tested for relative levels of the above mentioned isotypes. Lastly, p-nitrophenyl phosphate disodium (Sigma, 104-0) (1mg/ml) in a 0.05 M sodium carbonate and 1 mM magnesium chloride solution, pH 9.3, was added to the plates as substrate. The plates were incubated for 75 to 90 minutes and read on a Microplate Autoreader EL 310 (Biotek Instruments) (57).

**ELISPOT Assay:** Mesenteric lymph node and spleen single cell suspensions from the mice described above were put into 96-well plates with nitrocellulose backing (Millipore Multiscreen HV Filtration Plates). The wells had been previously coated with *S. hyodysenteriae B204* (100 µg/ml) whole cell lysate, mouse anti-immunoglobulin (2 µg/ml) or whole cholera toxin (20 µg/ml) suspended in 0.1 M PBS, pH 7.2, 100 µl per well, and incubated at 4°C for eight hours. The cells were then incubated at 37°C and 7.5% CO<sub>2</sub> in the above described RPMI media also containing 5% fetal calf serum, 1:50 dilution of

P388D<sub>1</sub> cell supernatant (source of murine IL-6), and *Escherichia coli* K235 lipopolysaccharide (1 µg/ml) for 21 hours. The plates were washed and a mouse specific alkaline phosphatase conjugated secondary goat antibody diluted 1:500 in TBS containing 0.05% Tween 20, pH 7.5, was incubated on the plates for 18 hours at 4°C. Lastly, the plates were developed, as described above for western immunoblot, by the addition of Fast Red (1.5 mg/ml) and Naphthol (0.75mg/ml) suspended in tris buffer as a substrate. The subsequent spots (Figure 1.) were counted using a dissecting microscope (4, 5). Each single cell suspension was tested for numbers of IgM, IgG, and IgA producing cells.



FIGURE 1. The above photograph shows ELISPOT's in one well of a ninety-six well nitrocellulose-backed culture plate. Each spot in the well represents one antibody secreting cell (ASC) specific for the antigen with which the well was coated.



## RESULTS

Spleen cells, MLN cells, and serum were assayed for antibody production. This was done to ascertain if previous per os (P.O.) immunization with *S. hyodysenteriae* B204 cellular antigens, employing cholera toxin as an adjuvant, resulted in a subsequent increase in antibody production over control mice or mice intraperitoneally (I.P.) immunized. Mesenteric lymph node and spleen cells were assayed from i) naive control, ii) infected only, iii) intraperitoneally (I.P.) immunized and infected, and iv) P.O. immunized and infected mice in duplicate trials (Experiment One) using the ELISPOT technique. Samples were analysed at day ten or 17 post infection. Table 1 shows the number of specific ASC for *S. hyodysenteriae* B204 antigens per million cells from either MLN or spleen at ten days post infection. An increase in specific ASC over naive control mice can be seen in all other groups for both IgG and IgA. The greatest increase in specific ASC over control was observed in the orally immunized and infected group. Similar results were also seen at day 17 (see Table 2). Specific serum antibody levels against *S. hyodysenteriae* B204 antigens were also assayed by ELISA. At ten days post infection, only the previously immunized mice showed increased levels of specific serum IgG over control. Also at this time point, only the P.O. immunized plus infected mice showed an increased level of specific serum IgA. At 17 days post infection, elevated levels of specific serum IgA had expanded to include all infected animals with the P.O. immunized group having the highest level (see Table 3).

Cholera toxin was used as an oral adjuvant in conjunction with

TABLE 1. Antibody Secreting Cells Specific for *Serpulina hyodysenteriae*, Ten Days Post Infection<sup>a</sup>

		Naive <sup>b</sup>	Infected <sup>c</sup>	I.P. Immunized and Infected <sup>d</sup>	P.O. Immunized and Infected <sup>e</sup>
IgG	MLN <sup>f</sup>	0	23 ± 5.6	18 ± 9.3	335 ± 50.4
	Spleen	2 ± 1	33 ± 19	348 ± 81.1	1227 ± 335.6
IgA	MLN	2 ± 0.9	125 ± 63.4	27 ± 16	826 ± 15.2
	Spleen	0	137 ± 22.3	92 ± 63	1767 ± 94.93

<sup>a</sup> The values in this table are representative of two duplicate experiments and are expressed as *S. hyodysenteriae* B204 specific antibody secreting cells per million cells harvested from C3H/HeOuj mice plus or minus the standard error of the mean (S.E.M.). These values are obtained from samples collected ten days post infection with  $1 \times 10^8$  *S. hyodysenteriae* B204 and were determined using the ELISPOT technique. n=4.

<sup>b</sup> The animals in the "Naive" group were neither immunized nor infected.

<sup>c</sup> The animals in the "Infected" group were not immunized, but were infected.

<sup>d</sup> The animals in the "I.P. Immunized and Infected" group were injected twice intraperitoneally with 300 µg of a *S. hyodysenteriae* B204 cell lysate (days one and 13). The mice were infected 12 days after the last injection (day 25).

<sup>e</sup> The animals in the "P.O. Immunized and Infected" group were immunized in the following manner. On days one, two, and three mice were intubated with a total of 500 µg of a *S. hyodysenteriae* B204 cell lysate, 30 µg of the cholera toxin B subunit, and 1.5 µg of whole cholera toxin; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).

<sup>f</sup> MLN = mesenteric lymph node.

TABLE 2. Antibody Secreting Cells Specific for *Serpulina hyodysenteriae*, Seventeen Days Post Infection<sup>a</sup>

		Naive <sup>b</sup>	Infected <sup>c</sup>	I.P. Immunized and Infected <sup>d</sup>	P.O. Immunized and Infected <sup>e</sup>
IgG	MLN <sup>f</sup>	1 ± 0.5	13 ± 5.4	6 ± 2	166 ± 68
	Spleen	6 ± 5	25 ± 8.1	237 ± 55.7	166 ± 31.9
IgA	MLN	1 ± 0.4	34 ± 4.5	40 ± 11	198 ± 36.8
	Spleen	1 ± 0.6	152 ± 6.46	269 ± 76.4	749 ± 59.9

<sup>a</sup> The values in this table are representative of two duplicate experiments and are expressed as *S. hyodysenteriae* B204 specific antibody secreting cells per million cells harvested from C3H/HeOuJ mice plus or minus the standard error of the mean (S.E.M.). These values are obtained from samples collected 17 days post infection with  $1 \times 10^8$  *S. hyodysenteriae* B204 and were determined using the ELISPOT technique. n=4.

<sup>b</sup> The animals in the "Naive" group were neither immunized nor infected.

<sup>c</sup> The animals in the "Infected" group were not immunized, but were infected.

<sup>d</sup> The animals in the "I.P. Immunized and Infected" group were twice intraperitoneally injected with 300 µg of a *S. hyodysenteriae* B204 cell lysate (days one and 13). The mice were infected 12 days after the last injection (day 25).

<sup>e</sup> The animals in the "P.O. Immunized and Infected" group were immunized in the following manner. On days one, two, and three, mice were intubated with a total of 500 µg of a *S. hyodysenteriae* B204 cell lysate, 30 µg of the cholera toxin B subunit, and 1.5 µg of whole cholera toxin; this immunization was repeated on days 13, 14, and 15. The mice were then infected ten days after the last intubation (day 25).

<sup>f</sup> MLN = mesenteric lymph node.



TABLE 3. Specific Serum Antibody Level for *Serpulina hyodysenteriae*<sup>a</sup>

		Naive <sup>b</sup>	Infected <sup>c</sup>	I.P. Immunized and Infected <sup>d</sup>	P.O. Immunized and Infected <sup>e</sup>
IgG	10 Day <sup>f</sup>	0.119 ± 0.008	0.134 ± 0.002	0.543 ± 0.031	0.454 ± 0.016
	17 Day <sup>g</sup>	0.141 ± 0.002	0.241 ± 0.014	0.468 ± 0.015	0.412 ± 0.017
IgA	10 Day	0.022 ± 0.003	0.062 ± 0.012	0.027 ± 0.001	0.322 ± 0.046
	17 Day	0.090 ± 0.007	0.232 ± 0.031	0.157 ± 0.028	0.632 ± 0.013

<sup>a</sup> The values in this table are representative of two duplicate experiments and reflect the level of serum antibody specific for *S. hyodysenteriae* B204 collected from C3H/HeOuJ mice at ten or 17 days post infection with  $1 \times 10^8$  *S. hyodysenteriae* B204 organisms. The values were measured via the ELISA technique and subsequent absorbance was recorded at 405 nm. Each absorbance value is given plus or minus its standard error of the mean (S.E.M.). Serum was diluted 1:100. Duplicate wells were done for each sample. n=4.

<sup>b</sup> The animals in the "Naive" group were neither immunized nor infected.

<sup>c</sup> The animals in the "Infected" group were not immunized, but were infected.

<sup>d</sup> The animals in the "I.P. Immunized and Infected" group were twice intraperitoneally injected with 300 µg of a *S. hyodysenteriae* B204 cell lysate (days one and 13). The mice were infected 12 days after the last injection (day 25).

<sup>e</sup> The animals in the "P.O. Immunized and Infected" group were twice immunized in the following manner. On days one, two, and three, mice were intubated with a total of 500 µg of a *S. hyodysenteriae* B204 cell lysate, 30 µg of the cholera toxin B subunit, and 1.5 µg of whole cholera toxin; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).

<sup>f</sup> Day 10 = ten days post infection.

<sup>g</sup> Day 17 = 17 days post infection.

*S. hyodysenteriae B204* cellular lysate to determine if its use enhanced antibody production in mice. Mesenteric lymph node and spleen cells were assayed in duplicate trials (Experiment Three) using the ELISPOT technique from the following four groups: i) naive control mice, ii) infected control mice, iii) P.O. immunized (1mg cellular antigens) without cholera toxin (C.T.) and infected, and iv) P.O immunized (500 µg cellular antigens) with C.T. and infected. Tables 4 and 5 show the specific ASC at 17 days post infection for *S. hyodysenteriae B204* per million cells of the MLN or spleen, respectively. In trial one, immunization with cholera toxin enhanced specific antibody production in comparison to all other groups (Table 4). In trial two, cholera toxin only enhanced the specific systemic IgA response (Table 5). Table 6 shows the specific serum antibody levels against *S. hyodysenteriae B204* in both experiments by using the ELISA technique. An increase in specific serum antibody IgG and IgA isotypes was observed in all groups of mice relative to naive controls and the greatest increase was demonstrated in mice orally immunized with cholera toxin and spirochetal antigen.

Spleen, MLN, and serum were assayed for antibody responses specific for *S. hyodysenteriae B204* over a period of 30 days. This was done to determine if the enhanced antibody production seen at ten and 17 days represented an enhanced antibody response over infected only mice or just a faster one (Experiment Three). Mesenteric lymph node and spleen cells from naive control mice, infected control mice, and P.O. immunized and infected mice were assayed for specific ASC against *S. hyodysenteriae B204*. Figures 2 and 3 show the resulting specific IgA ASC for the MLN and spleen, respectively, in the infected control and orally immunized groups. The results

TABLE 4. Antibody Secreting Cells from Mesenteric Lymph Node Specific for *Serpulina hyodysenteriae*<sup>a</sup>

		Naive <sup>b</sup>	Infected <sup>c</sup>	P.O. Immunized and Infected No C.T. <sup>d</sup>	P.O. Immunized and Infected With C.T. <sup>e</sup>
IgG	Trial One	4 ± 3	22 ± 30	52 ± 18	328 ± 7.32
	Trial Two	1 ± 1	2 ± 1	23 ± 9.2	7 ± 4
IgA	Trial One	7 ± 3	120 ± 7.79	125 ± 8.5	524 ± 92.2
	Trial Two	2 ± 1	26 ± 3.7	205 ± 67.1	92 ± 19.2

<sup>a</sup> The values in this table are from two duplicate trials and are expressed as *S. hyodysenteriae* B204 specific mesenteric lymph node antibody secreting cells per million cells harvested from C3H/HeOuJ mice plus or minus the standard error of the mean (S.E.M.). These values are obtained from samples collected 17 days post infection with  $1 \times 10^8$  *S. hyodysenteriae* B204 as determined using the ELISPOT technique. n=3.

<sup>b</sup> The animals in the "Naive" group were neither immunized nor infected.

<sup>c</sup> The animals in the "Infected" group were not immunized, but were infected.

<sup>d</sup> The animals in the "P.O. Immunized and Infected No C.T." group were immunized in the following manner. On days one, two, and three, mice were intubated with a total of 1 mg of a *S. hyodysenteriae* B204 cell lysate; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).

<sup>e</sup> The animals in the "P.O. Immunized and Infected With C.T." group were immunized in the following manner. On days one, two, and three mice were intubated with a total of 500 µg of a *S. hyodysenteriae* B204 cell lysate, 30 µg of the cholera toxin B subunit, and 1.5 µg of whole cholera toxin; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).



TABLE 5. Antibody Secreting Cells from Spleen Specific for *Serpulina hyodysenteriae*<sup>a</sup>

		Naive <sup>b</sup>	Infected <sup>c</sup>	P.O. Immunized and Infected No C.T. <sup>d</sup>	P.O. Immunized and Infected With C.T. <sup>e</sup>
IgG	Trial One	1 ± 1	11 ± 3.6	27 ± 1.6	151 ± 32
	Trial Two	0	1 ± 1	11 ± 3.7	37 ± 22
IgA	Trial One	6 ± 5	301 ± 86.3	291 ± 37.7	1031 ± 99.78
	Trial Two	0	84 ± 20	213 ± 32.1	593 ± 106

<sup>a</sup> The values in this table are from two duplicate trials and are expressed as *S. hyodysenteriae* B204 specific spleen antibody secreting cells per million cells harvested from C3H/HeOuJ mice plus or minus the standard error of the mean (S.E.M.). These values are obtained from samples collected 17 days post infection with  $1 \times 10^8$  *S. hyodysenteriae* B204 as determined using the ELISPOT technique. n=3.

<sup>b</sup> The animals in the "Naive" group were neither immunized nor infected.

<sup>c</sup> The animals in the "Infected" group were not immunized, but were infected.

<sup>d</sup> The animals in the "P.O. Immunized and Infected No C.T." group were immunized in the following manner. On days one, two, and three mice were intubated with a total of 1 mg of a *S. hyodysenteriae* B204 cell lysate; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).

<sup>e</sup> The animals in the "P.O. Immunized and Infected With C.T." group were immunized in the following manner. On days one, two, and three, mice were intubated with a total of 500 µg of a *S. hyodysenteriae* B204 cell lysate, 30 µg of the cholera toxin B subunit, and 1.5 µg of whole cholera toxin; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).

TABLE 6. Serum Antibody Level Specific for *Serpulina hyodysenteriae*<sup>a</sup>

		Naive <sup>b</sup>	Infected <sup>c</sup>	P.O. Immunized and Infected No C.T. <sup>d</sup>	P.O. Immunized and Infected With C.T. <sup>e</sup>
IgG	Trial One	0.120 ± 0.027	0.218 ± 0.037	0.326 ± 0.007	0.946 ± 0.064
	Trial Two	0.171 ± 0.110	0.283 ± 0.018	0.409 ± 0.020	0.343 ± 0.020
IgA	Trial One	0.105 ± 0.031	0.169 ± 0.012	0.206 ± 0.021	1.356 ± 0.140
	Trial Two	0.060 ± 0.004	0.137 ± 0.004	0.200 ± 0.025	0.306 ± 0.017

<sup>a</sup> The values in this table are from two duplicate trials and reflect the level of serum antibody specific for *S. hyodysenteriae* B204 collected from C3H/HeOuJ mice at 17 days post infection with  $1 \times 10^8$  *S. hyodysenteriae* B204. The values were determined via the ELISA technique and the subsequent light absorbance was recorded at 405 nm. Each absorbance value is given plus or minus its standard error of the mean (S.E.M.). Serum was diluted 1:100. Duplicate wells were done for each sample. n=3.

<sup>b</sup> The animals in the "Naive" group were neither immunized nor infected.

<sup>c</sup> The animals in the "Infected" group were not immunized, but were infected.

<sup>d</sup> The animals in the "P.O. Immunized and Infected No C.T." group were immunized in the following manner. On days one, two, and three, mice were intubated with a total of 1 mg of a *S. hyodysenteriae* B204 cell lysate; this immunization was repeated on days 13, 14, and 15. The mice were then infected ten days after the last intubation (day 25).

<sup>e</sup> The animals in the "P.O. Immunized and Infected With C.T." group were immunized in the following manner. On days one, two, and three mice were intubated with a total of 500 µg of a *S. hyodysenteriae* B204 cell lysate, 30 µg of the cholera toxin B subunit, and 1.5 µg of whole cholera toxin; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).

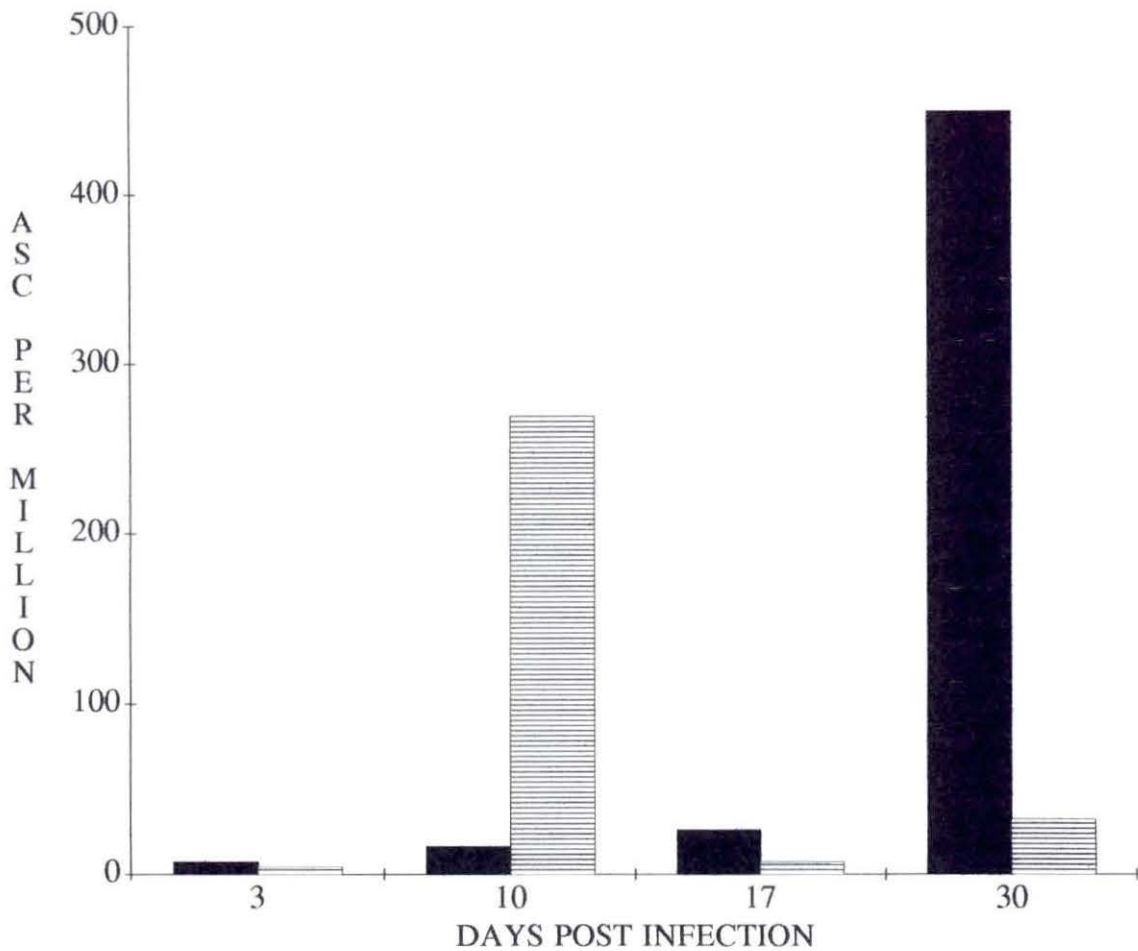


FIGURE 2. Detection of IgA antibody secreting cells (ASC) which are specific for a whole cell lysate of *Serpulina hyodyaenteriae B204*. Results are depicted as the average of ASC per million mesenteric lymph node cells. These results were determined by ELISPOT at four time points post infection with  $1 \times 10^8$  organisms of *S. hyodysenteriae B204*. The solid bars represent control infected mice. The hatched bars represent mice immunized in the following manner. On days one, two, and three, mice were orally intubated with a total of  $500 \mu\text{g}$  of *S. hyodysenteriae B204* cell lysate,  $30 \mu\text{g}$  of the cholera toxin B subunit, and  $1.5 \mu\text{g}$  of whole cholera toxin in a total volume of  $0.3 \text{ ml}$ ; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).



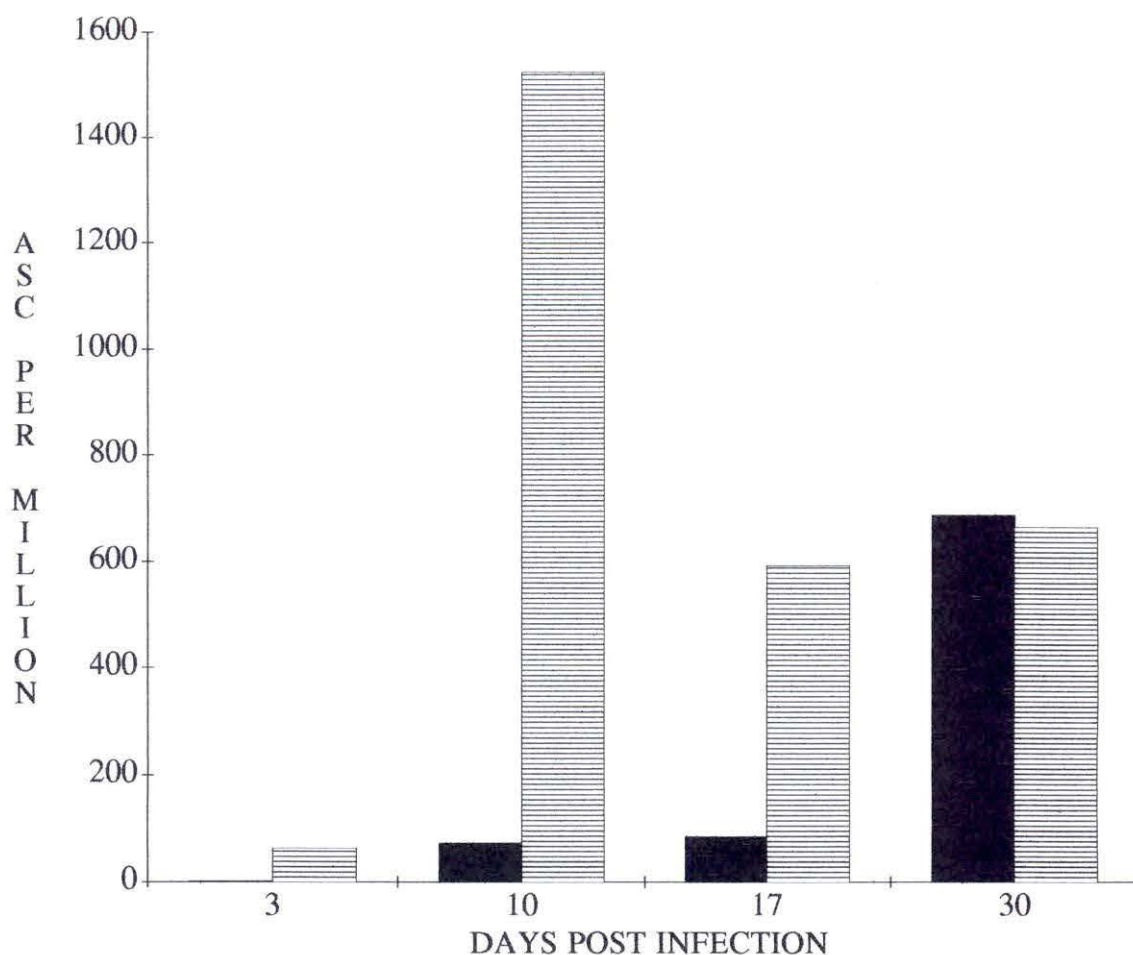


FIGURE 3. Detection of IgA antibody secreting cells (ASC) which are specific for a whole cell lysate of *Serpulina hyodysenteriae B204*. Results are depicted as the average of ASC per million spleen cells. These results were determined by ELISPOT at four time points post infection with  $1 \times 10^8$  organisms of *S. hyodysenteriae B204*. The solid bars represent control infected mice. The hatched bars represent mice immunized in the following manner. On days one, two, and three mice were orally intubated with a total of  $500 \mu\text{g}$  of *S. hyodysenteriae B204* cell lysate,  $30 \mu\text{g}$  of the cholera toxin B subunit, and  $1.5 \mu\text{g}$  of whole cholera toxin in a total volume of  $0.3 \text{ ml}$ ; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).

of specific IgG ASC mirror the IgA results (data not shown). Orally immunized mice show the highest ASC numbers at ten days post infection while infected control mice show highest ASC numbers at 30 days post infection. Specific serum antibody levels against *S. hyodysenteriae* B204 were also assayed. Figures 4 and 5 show these results for IgA and IgG, respectively, in the infected control and orally immunized groups. In general, antibody levels continued to rise in both groups over the experimental period. Naive mice continued to have negative ELISA responses to *S. hyodysenteriae* B204 antigens over the entire assaying period.

The western immunoblot technique was also used to analyze collected serum for specificity against whole cell antigens of *S. hyodysenteriae* B204. Western blot reactivity was subsequently observed for previously immunized mice or mice that had been infected with *S. hyodysenteriae* for at least ten days. However, no unique antigen bands were identified that related to protection from disease (Figure 6).

Ceca from mice were scored for severity of *S. hyodysenteriae* induced cecitis. No consistent difference in disease was found between the immunized and control mouse groups following challenge (Table 7).

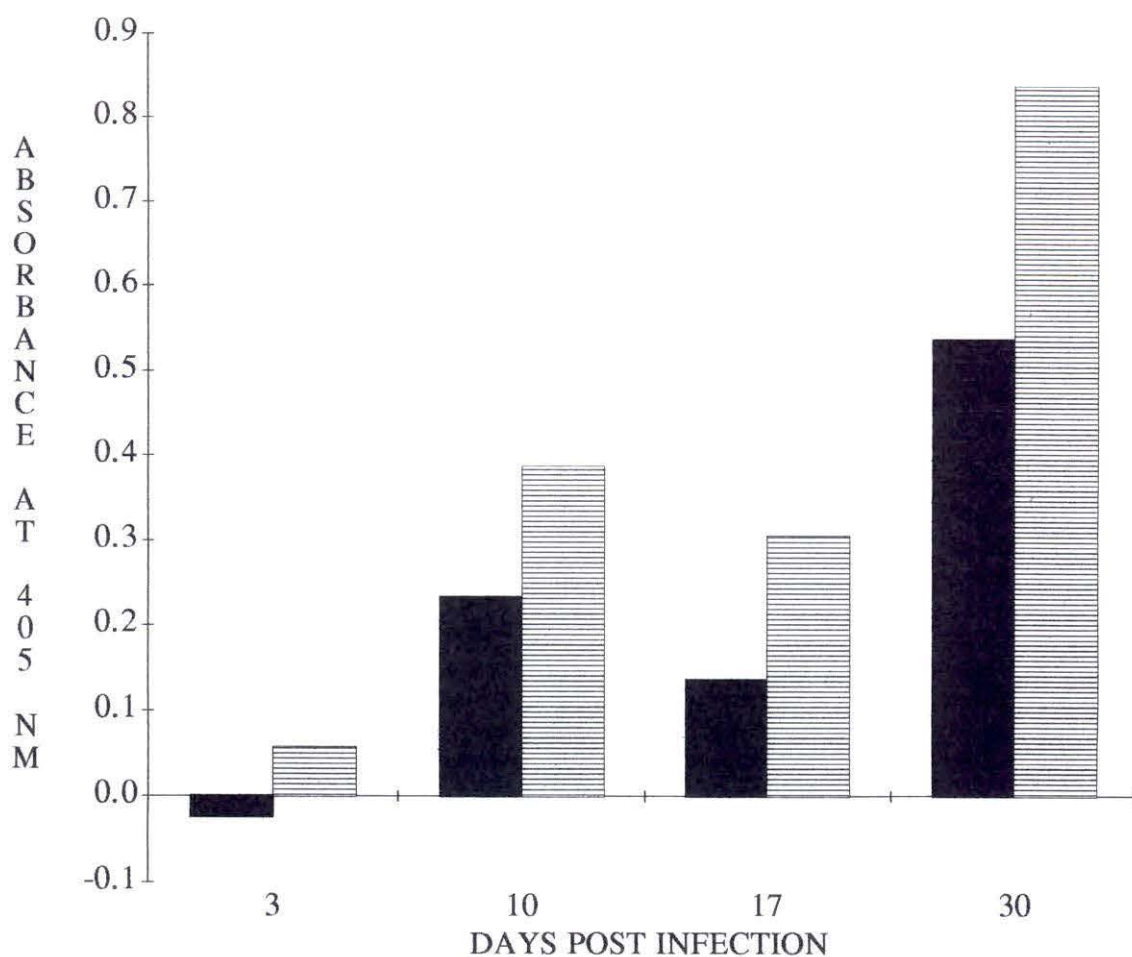


FIGURE 4. Measurement of *Serpulina hyodysenteriae B204* specific IgA serum antibody responses at various times post challenge. Antibody levels were measured by ELISA (405 nm) at four time points post infection with  $1 \times 10^8$  *S. hyodysenteriae B204*. The solid bars represent the response of mice which had been infected, but not vaccinated. The hatched bars represent mice immunized in the following manner. On days one, two, and three mice were orally intubated with a total of 500  $\mu\text{g}$  of *S. hyodysenteriae B204* cell lysate, 30  $\mu\text{g}$  of the cholera toxin B subunit, and 1.5  $\mu\text{g}$  of whole cholera toxin in a total volume of 0.3 ml; this immunization was repeated on days 13, 14, and 15. The mice immunized were infected ten days after the last intubation (day 25).



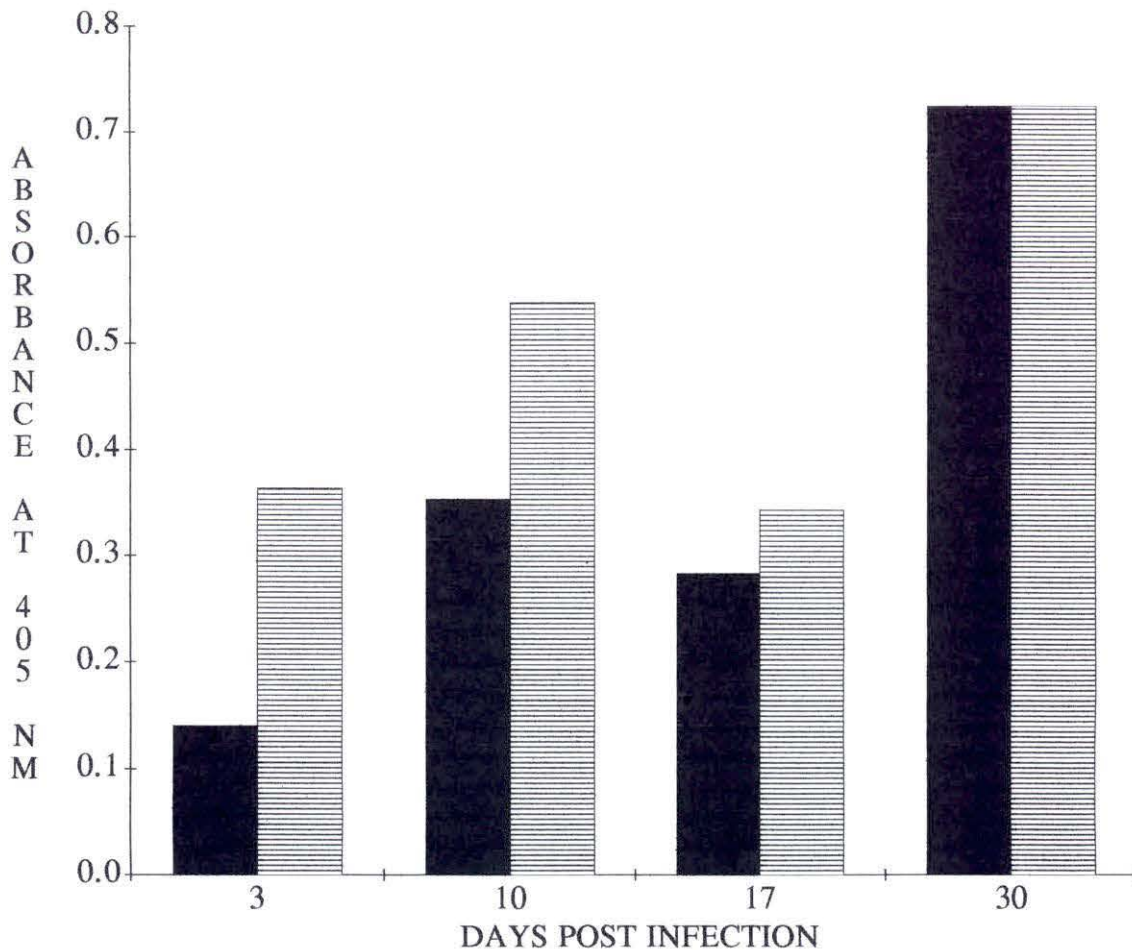


FIGURE 5. Measurement of *S. hyodysenteriae B204* specific IgG serum antibody responses at various times post challenge. Antibody levels were measured by ELISA (405 nm) at four time points post infection with  $1 \times 10^8$  *S. hyodysenteriae B204*. The solid bars represent the response of mice which had been infected, but not vaccinated. The hatched bars represent mice immunized in the following manner. On days one, two, and three, mice were orally intubated with a total of 500  $\mu\text{g}$  of a *S. hyodysenteriae B204* cell lysate, 30  $\mu\text{g}$  of the cholera toxin B subunit, and 1.5  $\mu\text{g}$  of whole cholera toxin in a total volume of 0.3 ml; this immunization was repeated on days 13, 14, and 15. The immunized mice were infected ten days after the last intubation (day 25).

FIGURE 6. Photograph of a western immunoblot. The antigen was a *Serpulina hyodysenteriae B204* cellular lysate separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Lanes are numbered from left to right. Lane one shows bands recognized by a positive control serum (a mouse hyperimmunized intraperitoneally with *S. hyodysenteriae B204* cellular lysate). Lanes two through five were developed with serum from naive control mice. Lanes six through nine were developed from mice treated in the following manner. On days one, two, and three mice were orally intubated with a total of 500 µg of a *S. hyodysenteriae B204* cellular lysate, 30 µg of cholera toxin subunit B, and 1.5 µg whole cholera toxin; this immunization was repeated on days 13, 14, and 15. Mice were infected with  $1 \times 10^8$  *S. hyodysenteriae B204* on day 25. Lastly, these mice were sacrificed 17 days post infection. Lane 10 shows molecular weight standards. Bands represent cellular lysate molecules of *S. hyodysenteriae B204* for which mice have formed specific antibodies.

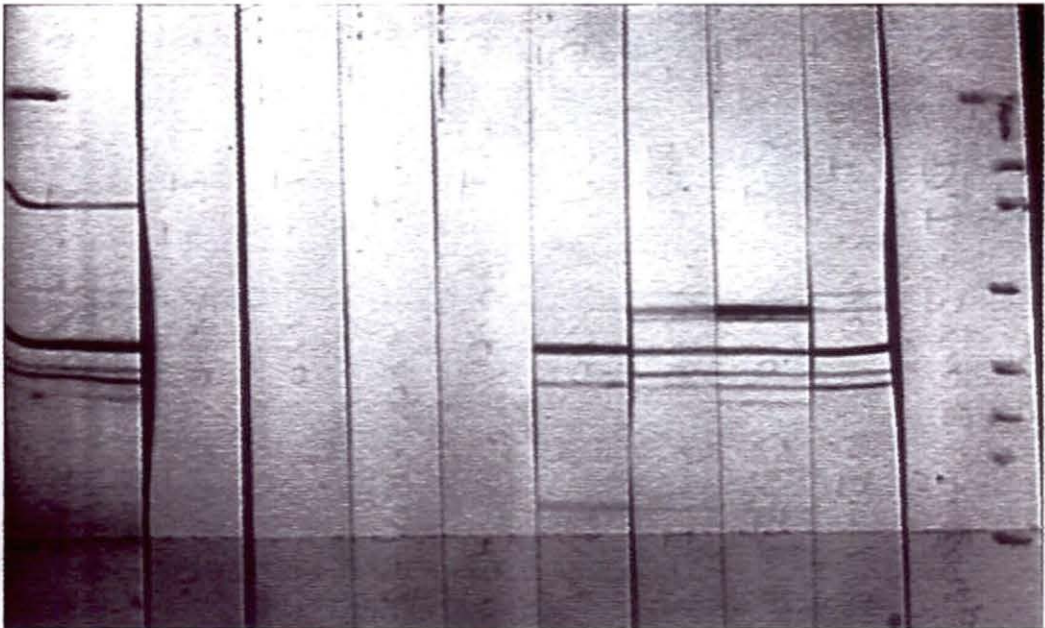




TABLE 7. Average Cecal Scores<sup>a</sup> of *Serpulina hyodysenteriae* Infected Mice

	Naive <sup>b</sup>	Infected <sup>c</sup>	I.P. Immunized <sup>d</sup> and Infected	P.O. Immunized <sup>e</sup> and Infected No C.T.	P.O. Immunized <sup>f</sup> and Infected With C.T.
Day 10 <sup>g</sup>	0.21 ± 0.14	2.07 ± 0.36	2.21 ± 0.22	ND <sup>h</sup>	2.50 ± 0.23
Day 17 <sup>i</sup>	0.14 ± 0.09	2.36 ± 0.22	2.00 ± 0.34	2.58 ± 0.45	2.32 ± 0.19

<sup>a</sup> Severity of *S. hyodysenteriae* B204 induced cecitis in mice were rated in the following manner. The score zero represents no excess mucus or cecal atrophy, one represents excess mucus, but no cecal atrophy, two represents excess mucus with complete cecal atrophy and blood. Mice were infected with 1 x 10<sup>8</sup> *S. hyodysenteriae* B204.

<sup>b</sup> The animals in the "Naive" group were neither immunized nor infected. n=7 (ten days post infection). n=14 (17 day post infection).

<sup>c</sup> The animals in the "Infected" group were not immunized, but were infected. n=7 (ten days post infection). n=14 (17 days post infection).

<sup>d</sup> The animals in the "I.P. Immunized and Infected" group were injected twice intraperitoneally with 300 µg of a *S. hyodysenteriae* B204 cell lysate (days one and 13). The mice were infected 12 days after the last injection (day 25). n=7 (ten days post infection) n=8 (17 days post infection).

<sup>e</sup> The animals in the "P.O. Immunized and Infected No C.T." group were immunized in the following manner. On days one, two, and three, mice were intubated with a total of 1 mg of a *S. hyodysenteriae* B204 cell lysate; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25). n=6.

- f The animals in the "P.O. Immunized and Infected With C.T." group were immunized in the following manner. On days one, two, and three, mice were intubated with a total of 500  $\mu\text{g}$  of a *S. dysenteriae* B204 cell lysate, 30  $\mu\text{g}$  of the cholera toxin B subunit, and 1.5  $\mu\text{g}$  of whole cholera toxin; this immunization was repeated on days 13, 14, and 15. The mice were infected ten days after the last intubation (day 25).  
n=7 (ten days post infection) n=14 (17 days post infection).
- g These mice were sacrificed ten days post infection.
- h ND = not determined.
- i These mice were sacrificed 17 days post infection.

## DISCUSSION

Swine dysentery has been difficult to control by immunization. Neither the parenteral route nor the oral route have been successful (8, 15, 16). *Serpulina hyodysenteriae* does not invade past the lamina propria (1, 23, 52). Therefore, it is likely that parenteral immunization may not adequately prime the mucosal immune system for a protective response against *S. hyodysenteriae*. In addition, the oral route may be an inefficient method of presenting *S. hyodysenteriae* antigens. Previously, it has been shown that cholera toxin (C.T.) can enhance the immune response at mucosal surfaces to substances that are poor immunogens such as ovalbumin and some streptococcal antigens (6, 14). In addition, protection to Sendai virus infection has been demonstrated when C.T. was used as an adjuvant. Protection was directly related to specific IgA titres at the mucosal surface (28). In the present study, the effect of C.T. on the murine B lymphocyte response to *S. hyodysenteriae* antigens presented orally was explored.

Cholera toxin given orally with a whole cell lysate of *S. hyodysenteriae* six times before infection was shown to prime for enhanced antibody production both in the spleen and MLN. This was more efficacious than I.P. immunization or infection (Tables 1 & 2). The antibody production was determined by assaying specific antibody secreting cell (ASC) numbers against *S. hyodysenteriae* at ten and 17 days post infection. However, infected control mice and I.P. immunized mice were not unresponsive to immunization and/or infection. These two groups did develop increased specific IgG and IgA ASC as



compared to naive mice. Because of the proximity of the MLN to the gut it is probable that the immune response in this tissue reflects an equally vigorous or even greater response in the intestinal lamina propria (3, 32). However, this response did not result in immune protection or resolution of lesions (Table 7). Assaying for specific serum antibody levels at ten days post infection indicated that the I.P. immunization mainly primed the systemic immune system while oral immunization primed both the systemic and mucosal immune response (Table 3). This would be consistent with the results obtained by other investigators (33).

The ability of oral immunization to augment mucosal immunity to *S. hyodysenteriae* B204 was examined. Mice were immunized P.O. with a whole cell lysate of *S. hyodysenteriae* B204 in combination with or without C.T. The ASC response in MLN and spleen, as well as serum antibody, was evaluated. In the spleen, oral immunization with C.T. enhanced antibody production over oral immunization without C.T. in duplicate trials. This was demonstrated by elevated numbers of *S. hyodysenteriae* specific ASC 17 days post infection. This was most pronounced for specific IgA ASC (Table 5). However, measurement of specific ASC in the MLN did not give similar results in the duplicate trials. One trial showed an increased number of specific ASC in the mice orally immunized with C.T. while the other did not (Table 4). The differences seen may reflect the variability of the specific ASC numbers with time (Figures 2 & 3). Additionally, the increased number of specific IgG and IgA ASC in the MLN of mice orally immunized with C.T. in one trial was accompanied by a high level of specific IgG and IgA antibody in the serum. In the other trial, lower numbers of *S. hyodysenteriae* specific ASC in the MLN were also reflected by a lower specific serum antibody level (Table 6). This indicates that the serum antibody

detected in orally intubated animals may in large part come from stimulated mucosal sites and not systemic sites. *Serpulina hyodysenteiae* specific serum antibody was present in all the infected groups. These results indicate that while cholera toxin can augment the induction of an immune response to *S. hyodysenteriae* antigens at the gut surface, the antigens themselves are also significantly immunogenic (Tables 4 & 5).

Mice infected with *S. hyodysenteriae* were assayed for specific serum antibody levels and specific ASC responses against *S. hyodysenteriae* antigens at various times post infection for 30 days. Specific ASC in orally immunized and infected mice peaked at ten days post infection while the ASC responses of infected control mice were maximal on day 30 (Figures 2 & 3). Both specific IgG and IgA serum antibody levels continued to rise over the experimental period (Figures 4 & 5). These results indicate oral intubation primed the immune system for a strong secondary immune response to *S. hyodysenteriae* antigens. It has not been conclusively proven, however, to enhance the level of the antibody secreted from the intestinal site during the course of the disease. The different peak times for specific ASC numbers seen in the MLN and spleen between orally immunized and infected control mice may simply be a measure of the B lymphocyte trafficking to the lamina propria. The actual numbers of specific ASC become similar over time as evidenced by the specific IgG serum response and to a lesser extent the specific IgA serum response (Figures 4 & 5).

*Serpulina hyodysenteriae* B204 specific IgM ASC in the spleen and MLN and specific IgM serum antibody was also assayed in the above experiments (data not shown). Specific IgM ASC were seen early in the spleen after challenge and was followed by specific IgM in the serum. Whole cholera

toxin specific IgM ASC were also observed in the spleen after challenge, but were at lower numbers compared to *S. hyodysenteriae* specific ASC (data not shown).

A general increase in nonspecific ASC was also observed (data not shown), particularly with IgA. These nonspecific ASC may recognize antigens of normal microflora. If this is the case, the role of secondary bacteria in the development of swine dysentery, as demonstrated with gnotobiotic animals, would be further supported (11, 12, 21, 37, 55).

Regardless of the immunization protocol, no predictor of protection against *S. hyodysenteriae* induced cecitis in the mouse model for swine dysentery could be found. This may indicate that antibody mediated immunity will be unprotective against *S. hyodysenteriae* even though IgA levels become elevated. This may be because the antigenic sites on the bacteria do not affect neutralization and/or opsonization. Alternately, the damaged epithelium may not effectively express and attach secretory component to dimeric IgA. This could make the antibody multimer more susceptible to degradation. If the initial lesion begins with affected epithelial cells, a large amount of intact, dimeric IgA bound to secretory component might effectively prevent swine dysentery. The presence of lactogenic immunity and partial immunity from recent exposure to *S. hyodysenteriae* supports this hypothesis (12).

In the mouse model, flagellar antigen from *S. hyodysenteriae* may confer immunity (2, 7) and *S. hyodysenteriae* LPS has also been implicated in immunity to swine dysentery (12, 22, 42, 43). Therefore, it is possible that C.T. in combination with the appropriate immunogens may affect a protective immune response. However, it must be noted that western blot analysis of specific serum



antibody against *S. hyodysenteriae* B204 LPS and flagella were detected in the present studies; therefore, the presence of these specific antibodies do not support the protective role of serum antibody specific for flagella or LPS (Figure 6).

In conclusion, a whole cell lysate of *S. hyodysenteriae* B204 orally intubated with C.T. primed the systemic and mucosal immune systems for a stronger secondary antibody response over I.P. immunization. Oral immunization without C.T. did not induce a systemic IgA response as well as oral immunization with C.T., but it may have primed the mucosal immune system equally well. No correlation between increased ASC in the MLN or spleen of mice could be found with protection against *S. hyodysenteriae* induced colitis nor could serum antibody specificity or level be correlated to protection.

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## GENERAL SUMMARY

The mucohemorrhagic, diarrheal disease of pigs, swine dysentery, has had its etiologic agent, *Serpulina hyodysenteriae*, identified for over twenty years (27, 29, 106). Serotype specific antibody recognition of the LPS or endotoxin moiety has been demonstrated to be related to disease resistance (82). Also, in the mouse, immunization with cloned flagellar fragments may confer protection against *S. hyodysenteriae* induced cecitis (5, 19). Unfortunately, actual immunization attempts against swine dysentery have not been successful (21, 32, 33). Because *S. hyodysenteriae* does not invade beyond the lamina propria (1, 48, 107), parenteral immunization may not induce mucosal immune protection against the bacteria and subsequent disease. Additionally, oral immunization with *S. hyodysenteriae* antigens may not be an efficient method of presentation to the gut lymphoid tissue.

Recently, mucosal adjuvants have been used to enhance immune responses to different antigens (62, 67). Cholera toxin is one of these substances. Ovalbumin and some streptococcal antigens are poor immunogens, but when administered with cholera toxin they elicit an enhanced mucosal antibody response (14, 31). Protection has also been demonstrated against Sendai virus when cholera toxin was used as an adjuvant in vaccination. This protection was directly related to specific IgA titres in nasal secretions (61). Based on this information, the ability of cholera toxin to enhance the immune response of mice to *S. hyodysenteriae* was explored using ELISA and ELISPOT.

In the present studies, oral immunization with a *S. hyodysenteriae* cellular lysate primed the B lymphocyte response better than parenteral immunization when analysed at ten and 17 days post infection with *S. hyodysenteriae*. The B cell response was measured by assaying specific serum antibody titres and by assaying numbers of specific antibody secreting cells (ASC) in the spleen and MLN. While cholera toxin could enhance the immune response seen with oral immunization, serpulina antigens proved to be significantly immunogenic alone as demonstrated by increased numbers of specific ASC in the spleen and MLN over control infected mice. Further, at 30 days post infection, infected only mice demonstrated as great a B lymphocyte response against *S. hyodysenteriae* as that observed in orally immunized mice. Also, western blot analysis of serum collected at ten days post infection or later showed antibody reactivity to flagellar and endotoxin molecules of *S. hyodysenteriae*. However, the increase in specific immunoglobulin did not correlate with protection against disease.

Specific antibody against *S. hyodysenteriae* may not be protective because it is not generated against appropriate antigens that would cause neutralization and/or opsonization of the bacteria. It is also possible that the ineffectiveness of *S. hyodysenteriae* specific antibody may be related to the damage epithelial cells receive from disease development. During swine dysentery, the damaged epithelium may not efficiently attach secretory component to dimeric IgA. The dimer is then more susceptible to enzymatic degradation. Swine do show serotype specific resistance to reinfection, as mentioned above, and lactogenic immunity (29) can be demonstrated against *S.*



*hyodysenteriae*. This would support the theory that a large amount of intact secretory IgA (S-IgA), is protective against disease.

Cholera toxin may also not be an effective adjuvant for *S. hyodysenteriae*. Most other antigens cholera toxin has been used with were poor immunogens. *Serpulina hyodysenteriae* antigens appear to be good immunogens. What may be needed is not greater antibody production over natural infection, rather a low-level constant stimulation with antigen to ensure that there is enough intact S-IgA in the intestine at all times to prevent disease.

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## ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Wannemuehler for his kind help and support that made this research possible. I also wish to acknowledge my other committee members, Dr. Joan Cunnick & Dr. John Mayfield for their useful suggestions; the lab. technicians, Linda (Vandemark) Ritland, Lori Gau, & Toni Bryant for their excellent, much appreciated technical support; and Haa Young Lee, Jeff Galvin, David Hutto, & Stu Nibbelink for listening to my theories on swine dysentery (even the crazy ones). Lastly, I would like to thank my family for their faithful support - most especially my parents, Kenneth and Mary Haggerty who unconditionally believed in me.