Effect of *Treponema hyodysenteriae* infection on the populations of mucosal mast cells and T cell subsets in the murine cecum

by

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TABLE OF CONTENTS

		Page
GENERAL INTRODUCTION		1
EXPLANATION OF THESIS FORMAT		5
LITERATURE REVIEW		7
LITERATURE CITED		36
MANUSCRIPT	EFFECT OF *Treponema hyodysenteriae* INFECTION ON THE POPULATIONS OF MUCOSAL MAST CELLS AND T CELL SUBSETS IN THE MURINE CECUM	61
	ABSTRACT	62
	INTRODUCTION	63
	MATERIALS AND METHODS	66
	RESULTS	70
	DISCUSSION	75
	REFERENCES	84
GENERAL SUMMARY		91

ACKNOWLEDGMENTS 94

APPENDIX 1 PROTECTION STUDIES IN THE PARENTERALLY IMMUNIZED MOUSE 95

APPENDIX 2 STUDIES OF POTENTIAL INVOLVEMENT OF MURINE H2 HAPLOTYPE ON CHALLENGE WITH Treponema hyodysenteriae 105

APPENDIX 3 MOUSE STRAIN CHALLENGE STUDIES 118

APPENDIX 4 INVOLVEMENT OF THE ity GENE IN THE PATHOGENESIS OF Treponema hyodysenteriae IN THE MURINE MODEL 127

GENERAL INTRODUCTION

Swine dysentery is a mucohemorrhagic diarrheal disease of growing swine caused by an anaerobic spirochete, Treponema hyodysenteriae. Swine dysentery is a costly disease for the pork producer, not only because of direct mortality loss, but also from the expense of suppressing and/or treating the disease with antibiotics incorporated in the feed. Diagnostic tests to identify carrier or asymptomatic pigs are not readily available, and standard diagnostic procedures for swine dysentery include clinical and pathological signs, bacteriological culture, and serology.

Though Treponema hyodysenteriae has been found to be the etiological agent of swine dysentery, pathogenetic mechanisms in the disease have not been elucidated. Many ultrastructural and histopathological studies have been performed on tissues from swine with dysentery under controlled conditions, and many observations of pathological changes have been described. However, no virulence mechanism(s) has been conclusively demonstrated in vivo. Initial lesions in tissues have been correlated with the presence of the spirochete, however, the progressive, fulminating, mucohemorrhagic diarrhea has

not been wholly attributed to the spirochete; this is evidenced by results obtained from experiments using gnotobiotic animals. If it is to be assumed that T. hyodysenteriae alone induces clinical disease, it would be expected that gnotobiotic animals would develop symptoms similar to those in conventional animals. However, since gnotobiotic animals develop a much less severe disease, the development of lesions and clinical signs may be attributed to secondary opportunistic bacterial invasion or toxin production as well as host inflammatory cell responses.

Convalescent swine appear to be refractory to reinfection with T. hyodysenteriae, but the basis for development of resistance has not been adequately defined. Efforts have been made to develop parenteral vaccines, but results have only been nominally successful. Little has been done to address the development of mucosal immunity to swine dysentery. This disease is primarily confined to the mucosal surface and the development of specific local immunity or enhanced non-specific immune mechanisms active at the mucosal surface needs to be examined.

The research described in this thesis deals with the effects of swine dysentery on the mucosal surface of infected animals. Specifically, the role of mucosal mast

cells in concert with T lymphocytes was examined in the development of the disease. It has been speculated that mast cells are involved in the disease process, either directly or indirectly. The following material details the population dynamics of mucosal mast cells and T lymphocyte subsets in the murine model of swine dysentery. The ability of MMC to directly effect the development of lesions was examined using a mast cell deficient mouse model.

The differences in rodent connective tissue mast cells (CTMC) and mucosal mast cell (MMC) populations have been well described. In the rodent, investigation of a cecitis/colitis would invariably involve research in the function of the MMC. However, porcine CTMC and MMC populations have not as yet been described in terms of mediators possessed or degranulation stimuli. Therefore, since it would be possible that different characteristics would be possessed by porcine MMC, the inclusion of CTMC information is also included in the literature review.

During the course of this research, the parameters of the disease in the mouse were defined, such as H2 involvement, parenteral immunization protection, and influence of lipopolysaccharide (LPS) genes. Since the convalescent pig appears to be refractory to challenge, and the immunologic basis of this finding is not as yet

known, the use of the mouse model facilitated the examination of various genetic parameters in relation to a T. hyodysenteriae infection.

The initial and primary goal of this research was to determine if the mucosal mast cell contributed to lesion formation in swine dysentery. In utilizing the murine model, other genotypic inbred traits were tested to determine the character of the disease. It is intended that results obtained from these experiments can be extrapolated to swine.

EXPLANATION OF THESIS FORMAT

This thesis is being submitted in the alternative format, which includes one manuscript to be submitted for publication and four appendices. The manuscript is entitled "Effect of *Treponema hyodysenteriae* Infection on the Populations of Mucosal Mast Cells and T Cell Subsets in the Murine Cecum", and will be prepared in the style of *Regional Immunology*. The appendices are entitled "Protection Studies in the Parenterally Immunized Mouse" (Appendix 1), "Studies of Potential Involvement of Murine H2 Haplotype on Challenge with *Treponema hyodysenteriae*" (Appendix 2), "Mouse Strain Challenge Studies" (Appendix 3), and "Involvement of the ity^r or ity^s Gene in the Pathogenesis of *Treponema hyodysenteriae* in the Murine Model" (Appendix 4).

A literature review precedes the manuscript, and cited sources are listed in the bibliography immediately following. Literature cited in the manuscript is listed in the bibliography section of the manuscript. Any literature citations in the appendices immediately follow the respective appendix. All citations are listed in the format of *The Journal of the American Medical Association*, the form required for manuscripts submitted

to the Journal Regional Immunology. A general summary follows the manuscript.

LITERATURE REVIEW

The presence of mast cells in connective tissue has been known for some time (1), however, mucosal mast cells were first described in the lamina propria of rats by Maximow (2). Enerback (4) and Kitamura et al. (3) have defined various aspects of the mucosal mast cell (MMC) population, including fixation properties, staining characteristics, and tissue location.

The connective tissue mast cell (CTMC) population in the rodent occurs in loose connective tissue and the peritoneal cavity. The intestinal MMC population, however, only occurs in the intestinal lamina propria, gut mucosal epithelium, and mesenteric lymph nodes (5). In contrast to CTMC, the MMC population in the rat has been shown to be refractory to staining after formalin fixation, to contain differing concentrations of vasoactive amines and proteases, and to be under the influence of Lyt 1+ 2- T cells. Both CTMCs and MMCs possess IgE receptors and are degranulated following specific antigen or anti-IgE treatment (6). The differing qualities of these two mast cell populations led investigators to investigate the ontogeny of these cells. A small amount of research has been reported on the origin and heterogeneity of mast cell populations in

the dog (7), but most of the ontological studies have been done in the rat and mouse (4,5,8,9).

The primary question that arises in a discussion of MMC origin is whether the terminally differentiated MMC is a thymus-derived lymphocyte, or directly derived from a bone marrow precursor cell. Currently, it is indeterminable whether CTMC and MMC arise from the same precursor cells and terminally differentiate due to *in situ* influences, or, alternatively, arise from different bone marrow precursor cells (6).

To determine MMC origin, Kitamura et al. (8,9) demonstrated that grafted bone marrow cells reconstitute mast cells in irradiated genetically normal mice, and also subsequently showed that homozygous recessive mast cell deficient W/W^v mice could also be mast cell reconstituted by bone marrow grafts from normal +/+ littermates (10). To carry this investigation further, Kitamura et al. (11) determined whether the bone marrow mast cell precursors also circulated via the bloodstream. In this study, a mouse to mouse skin parabiosis (two different histocompatible mice surgically attached) was surgically created between normal C57BL/6 mice and Chediak-Higashi beige C57BL/6 (bg/bg) mice. The Chediak-Higashi beige mice possess a granulated cell defect creating large "giant granules" within mast cells

which are easily recognized by light microscopy. After parabiosis, the normal mouse of the parabiont was lethally X-irradiated, while the bg/bg mouse was shielded. After irradiation, less "normal-type" mast cells appeared in the Chediak-Higashi mouse when compared to a non-irradiated parabiont. In non-irradiated parabionts, normal mast cells developed in the bg/bg mice, and Chediak-Higashi giant granulated mast cells developed in the normal mice. From these reports (8,9,10,11) it was concluded that mast cell precursors originate in the bone marrow and that they circulate in the bloodstream. In addition, Zucker-Franklin et al. (12) showed that collected mononuclear cell fractions from rat peripheral blood yielded colonies identified as mast cells in an _in vitro_ soft agar culture technique. The colonies exhibited IgE Fc receptors, contained histamine, and were ultrastructurally and histochemically similar to CTMC (12).

To specifically investigate the ontogeny of the MMC, the homozygous recessive W/Wv mouse has been used extensively. This mouse strain (to be discussed later) has less than 1% of the normal complement of both CTMC and MMC. It has been shown (3) that W/W^{v} may be MMC-reconstituted with bone marrow transplants. Also, Sonoda et al. (13) showed that the implantation of a

single peritoneal mast cell (CTMC) into the stomach mucosa of the W/W^v mouse resulted in the development of both MMC and CTMC populations. In addition, Kitamura et al. (3) developed MMC in vitro by culturing hematopoletic cells with a mast cell growth factor similar to interleukin 3 (IL 3). The cultured cells developed many MMC features (3), and, after injection into the peritoneal cavity of W/W^v mice, these mice developed CTMC cells in the peritoneal cavity. These data and that of others (13) suggest a common precursor cell, which, upon specific microenvironment maturation influences, will differentiate into either a CTMC or an MMC.

Connective tissue mast cells are found in varying numbers in loose connective tissue and are situated adjacent to most blood vessels. Subsequent to appropriate fixation, the granules of CTMC stain metachromatically with basic dye staining, i.e., with toluidine blue staining, at high pH the granules stain a reddish color rather than blue, and at a low pH the granules stain blue. Transmission electron microscopy (TEM) reveals that the CTMC population possesses a well developed Golgi apparatus, but a small number of mitochondria, free ribosomes, and granular endoplasmic reticulum (1).

The granules contained within the CTMC contain a variety of mediators and enzymes (14,15), including histamine, heparin, and varying amounts of serotonin (5 hydroxytryptamine, 5HT).

Connective tissue mast cells are activated to release their soluble mediators by either classical IgE-induced or non-specific mechanisms. Currently, degranulation is believed to be controlled by transient intracellular increases in cyclic adenosine monophosphate (cAMP) which is regulated by calcium (16). Although IgE-mediated (specific) degranulation appears to utilize cAMP-mediated pathways exclusively, non-specific (chemical or physical factors) degranulation appears to involve multiple biochemical pathways (17).

The classical model of CTMC immune-stimulated degranulation involves the binding of IgE (cytophilic antibody) via Fc receptors on the CTMC cell membrane. After cross linking of two or more antigen binding sites by specific antigen (allergen), the CTMC cell membrane is "perturbed", and granules are released. Stanworth (18), using synthetic peptides, has suggested that IgE is not a passive signalling immunoglobulin, but rather, after being cross-linked by antigen, possesses sites in the Fc region which then signal an adjacent regulatory protein on the cell membrane, thus effecting granule release.

Connective tissue mast cell granules contain histamine, and recent work suggests an interaction of this mediator with neutrophils. The CTMC functional interaction with neutrophils, in fact, might be bi-directionally controlled. Fantozzi et al. (19) have shown that passively sensitized rat serosal mast cells (i.e., CTMC incubated with IgE), when caused to degranulate by acetylcholine, decreased superoxide anion formation by N-formyl-methionyl-leucyl-phenylalanine (FMLP) stimulated human neutrophils. Conversely, incubating non-sensitized CTMC with FMLP-stimulated neutrophils caused histamine release. In view of this work, it is possible that CTMC (via histamine) have a regulatory role in the development of inflammation.

Histamine is a vasoactive amine autocoid secreted and produced during inflammation, and is primarily recognized for its role in atopic or allergic conditions (20). Histamine is a low molecular weight (formula weight 111) amine (20) formed by the decarboxylation of histidine by the enzyme histidine decarboxylase. In vivo, catabolism or tissue breakdown of histamine is either via methylation of the pyrole nitrogen by histamine methyl transferase (20,21), or else by oxidative deamination, yielding imidizoleacetic acid (IAA) by diamine oxidase (DAO) (20,21). Histamine

methyltransferase activity is found within monocytes and tissue fluids, while diamine oxidase activity is found within polymorphonuclear granulocytes (PMNs) (20) and eosinophils (22). In this capacity, eosinophils, neutrophils, and monocytes participate in the regulation of histamine-induced effects.

The major stores of histamine are found within tissue mast cells and circulating basophils. Although the major biogenic amine in these two cell types is histamine, these cells also contain varying amounts of heparin, chemotactic factors, leukotrienes, prostaglandins (23), and proteolytic enzymes (20). Degranulation of histamine-containing cells may be caused by a number of factors involving either immune or non-specific stimuli. Nonspecific stimuli for CTMC degranulation include various physical agents (e.g., trauma), and chemical compounds (e.g., compound 48/80 (24)). The immune stimulated degranulation of CTMCs and basophils is now being recognized as a complex process, involving any or all of the following: antigen-antibody reactions (20,25), anaphylatoxins (23,26,27), lymphokines (27,28,29), activated neutrophils (30), and low doses of esterase inhibitors, such as diisopropyl flourophosphate (31). Ultrastructurally, the histamine-containing cells appear to exocytose their granules after an increase of

intracellular cAMP (32,33) and calcium (34) followed by perturbations in the cell membrane caused by the fusion of secretory granules with the mast cell membrane (35). "Gating" of intracellular Ca^{+2} appears to be under the control of a guanine nucleotide binding protein (36), and actin filaments appear to be involved with the degranulation (37). Granule release is inhibited by various concentrations of zinc, which blocks calcium ion uptake (38). MMCs do possess surface and cytoplasmic IgE receptors and degranulate due to immune stimulation (6), but are less prone to nonspecifically degranulate than the CTMC.

Histamine has various *in vivo* effects on cells bearing H1 or H2 receptors. H1 tissue receptors mediate the classical and well known broncho-constriction, gut contraction, and fine blood vessel dilation (20,39). H2 class receptors regulate gastric acid secretion and are cardiac chronotropes. In addition, some physiological phenomena, such as vascular dilation, appear to be under the influence of both H1 and H2 receptor control (39,40).

The response of cells to histamine stimuli appear to be closely tied to changes in intracellular levels of cAMP and/or cGMP (20). *In vitro* keratinocyte cultures show that an induced increase in intracellular cAMP caused a reduced mitotic rate (20). However, other

research now shows that histamine, in fact, causes proliferation of human microvascular endothelial cells (41), and increases in vivo mesothelial cell, fibroblast, and jejunal mucosal epithelial cell mitosis via H2 receptors (42,43). Abnormal tissue fibrosis has also been associated with degranulating mast cells in conjunction with T lymphocytes (44).

In order to study histamine-immune system interactions, investigators looked at which lymphocyte populations possessed histamine receptors. It was found that diverse subpopulations of leukocytes possessed histamine receptors, including peripheral blood or lymph node lymphocytes (45,46) and thymic lymphocytes (45,47).

In functional assays, exogenously administered histamine increases intracellular cAMP in basophils and mast cells, causing a blockade of histamine secretion by these cells (negative feedback). Also, administered histamine has profound effects on PMN inflammatory cell functions as summarized in Table 1 (Adapted, 20).

TABLE 1. Effects of Histamine on Inflammatory Cells

Cell type	Function	Effect of histamine
neutrophils	chemokinesis	None
	chemokinesis, activated	increase
	chemotaxis, activated	decrease
	lysosomal enzyme release	decrease
	cyclic AMP	increase
	membrane potential, activated	decrease
	superoxide anion production	decrease
	peroxide formation	decrease

Histamine has also been proposed as an immunoregulatory molecule for various immune effector cells (48). A compilation of these known effects is summarized in Table 2 (Adapted, 20).

TABLE 2. Effects of Histamine on Inflammatory Cells

Cell type	Activity	Effect
T-cytotoxic lymphocyte	cytotoxicity	decrease
Natural Killer cell	cytolytic activity	decrease
T cell	blastogenesis	decrease
	lymphokine production	decrease
Suppressor T cell	blastogenesis	increase
Contrasuppressor cell	generation of hapten self-cytotoxic T cells	increase
B lymphocytes(mouse)	IgG response (SRC)	decrease
B lymphocytes(human)	Immunoglobulin response	decrease

Gut mucosal mast cells are located within the intestinal lamina propria. There is additional evidence to suggest that MMC are present in the mucosal epithelium and mesenteric lymph nodes (49). Recently, Kawanishi and Ihle (50) have also demonstrated probable MMC precursors in the Peyer's Patches (PP) of the mouse. After screening against T lymphocytes, B lymphocytes, and null cells, the resultant PP cell fraction was grown in the presence of interleukin 3 (IL 3). The predominant cell population was IL 3-dependent, which did not express Ia antigens, and did not have T, B, or macrophage cell surface markers. The cells released histamine in response to Ca^{2+} ionophores, but not to compound 48/80. All these characteristics are consistent with those of the MMC. In the PP, the MMC could be exposed and potentially interact with "new" antigens in conjunction with the lymphocytes composing the Gut-Associated Lymphoid Tissue (GALT). No research has been performed on gut MMC demonstrating a role in antigen processing, but regulation of the gut mucosal immune response might be possible through release of mediators, including histamine (20).

Utilizing transmission electron microscopy, no adequate or identifiable marker has been found for the MMC (51). Following infection with Nippostrongylus

brasiliensis, Greenwood et al. (51) examined rat intestinal tissues and described the rat MMC granules as being more electron dense and larger and fewer in number than those of CTMC. It was also observed that MMC have a higher nucleo-cytoplasmic ratio than the CTMC population. The cellular morphology of the rat MMC was similar to that of the human and primate (51).

The major differences between rodent MMC and CTMC are listed below:

1.) Degranulation stimuli: In contrast to CTMC, MMC do not respond to secretagouges such as compound 48/80 or bee venom peptide 401 (52,53). MMC are also hyporesponsive to anti-IgE, specific antigen (both specific stimulation), and some calcium ionophores (53).

2.) Degranulation blocking agents: Many pharmacologic agents exist which will block degranulation of CTMCs, including intracellular Ca^{+2} modifiers, such as theophylline and prostaglandins (54), flavonoids, which include disodium cromoglycate (53), colchicine-like drugs, and rat transferrin (55). Shanahan et al. (56) also reported that endogenous rat endorphins had a secretagouge effect on CTMC, but not MMC. Of all the blocking drugs available for the CTMC, only doxantrazole, a flavonoid, is effective in blocking both MMC and CTMC in a dose-dependent fashion (54).

3.) Granule enzymes and mediators: The CTMC population has been shown to possess many different enzymes and soluble mediators including elastases and cathepsin G (14), sulphated glycosaminoglycans (i.e., heparin) (57,58), histamine (59), 5HT (60), and, in the rat, rat mast cell protease I (RMCP I). MMC populations, however, possess a glycosaminoglycan with a lower degree of sulphation than the heparin of the CTMC (61). MMC possess an over-sulphated galactosaminoglycan described as chondroitin sulphate E (57). In the rat, MMC contain less histamine than CTMC (62,59), a relatively higher percent composition of 5HT (63), and a chymotrypsin-like serine protease known as rat mast cell protease II (RMCP II) which is known to selectively digest type IV collagen (6,64) and can be quantified in the blood (65).

4) T cell control: MMC appear to be under the differentiative control of T cells (6, 66). MMC are known to be under the proliferative control of a secreted IL-3 or IL-3-like molecule from an Lyt 1+ T cell (66). CTMC have not been shown to be regulated by T helper cells (67,68).

5.) Degranulation: CTMC may be degranulated either by non-immunologic or by immunologic mechanisms. Non-immunologic degranulation may be triggered by previously described substances or chemicals, and relies

on the mobilization of intracellular calcium (69). Immunologic degranulation is accomplished via antigen-IgE recognition and the cross linking of adjacent immunoglobulin molecules by antigens, is heavily dependent on extracellular calcium (69), and the response is slowly inactivated (69). MMCs in the rat may be stimulated to degranulate immunologically, but less non-specific stimulation is observed (6).

6.) Granular morphology: The CTMC possess more numerous and larger granules than the MMC (6). The CTMC was differentiated morphologically from other cell populations due to the presence of readily stainable granules which were observed in formalin fixed tissues. However, specific staining of fixed MMC granules has only recently been reported (70). Enerback (70) hypothesized that the poor staining of MMC resulted from the extraction of the soluble glycosaminoglycan during formalin fixation. More recently, Wingren and Enerback (59) demonstrated that long staining times (5-7 days) in alcian or toluidine blue after formalin fixation would result in stained MMC granules. The currrent hypothesis is that formalin and glutaraldehyde fixatives form protein crosslinks and protein diffusion barriers which mask or cover up the specific cationic binding sites of the MMC but not the CTMC granules (59,71). This

observation demonstrates differing spacial arrangements of glycosaminoglycans and protein in CTMC and MMC granules (71).

Most histologic work examining MMC is performed with tissue fixation in Carnoy's fluid (72,73), a coagulative fixative. Coagulative fixation does not form nitrogen cross links in proteins (as do glutaraldehyde and formalin), allowing the blue dye uptake by the MMC granules of toluidine blue, alcian blue, or astra blau (59) in an acid staining solution (pH 0.5). The MMC population has a postulated role in nematode expulsion from the rodent and ovine intestinal tissue (74,75). Most research deals with helminth expulsion in mice and rats, although Gustowska et al. (76) did report an increase in human jejunal MMC after infection by Trichinella spiralis.

In rodents, specifically the rat, mucosal mastocytosis as well as goblet cell proliferation (77,78) occurs at the time of T. spiralis worm expulsion. Following Nippostrongylus brasiliensis infection, the increase in MMC is reported to occur after worm expulsion (78). Huntley et al. (79) also reported that mucosal mastocytosis occurred in normal rats 14 days after infection with Eimeria nieschulzi oocysts. Nude rats, T cell deficient, did not develop this response. This work

has shown that gut mastocytosis may be induced with a unicellular parasite (79).

Immunoparasitologists also report that the chymotrypsin-like protease that MMC possess (in rats, RMCP II) which enzymatically degrades type IV collagen, increases concurrently with increases in the MMC population. King et al. (80) investigated RMCP II levels both in naive and in N. brasilensis-infected rats. After inducing anaphylaxis with worm antigen, systemic levels of RMCP II increased significantly, and MMC populations were depleted or degranulated in the jejunal, gastric, and colonic mucosa (80). These findings were interpreted to support the contention that MMC are the major source of RMCP II. Huntley et al. (79) also demonstrated that coccidial infections increased the systemic concentration of RMCP II enzyme and that the increase was dependent on the dose of oocysts given to the rats. Conversely, in the same study (79), nude rats did not exhibit increases in RMCP II in response to coccidial infection.

The strongest evidence for the participatory role of the MMC in parasite expulsion is illustrated by using the W/W^v, mast cell deficient, mouse strain. In 1978 it was reported that the W/W^v mouse strain was CTMC (10,81) and MMC deficient (82). This mutant mouse strain possesses a genetic defect in undifferentiated stem cells and has a

macrocytic anemia (83). W/W^v mice have low or undetectable levels of mast cell associated histamine or heparin (83), but interestingly have normal levels of (5HT) in the gut tissue (60).

Even though the W/W^v has a macrocytic anemia, Ha et al. (81) demonstrated that W/W^v mice were immunologically competent (i.e., immunoglobulin synthesis, contact hypersensitivity, and delayed type hypersensitivity). However, Ha et al. (84) demonstrated that W/W^v mice expelled adult T. spiralis slower than their normal littermates, and that after bone marrow grafts from normal littermates, expulsion of adult T. spiralis was accelerated (84). In 1984, Oku et al. (85) showed that MMC -reconstituted W/W^v mice expelled T. spiralis as fast as normal mice; in addition, lymphopoietic derived grafts failed to reconstitute MMC populations or allow normal expulsion of T. spiralis in the W/W^v mice (85). However, it was shown (86,87) that MMC reconstitution of W/W^v mice did not enhance the elimination of N. brasiliensis infection. Secondary or tertiary challenges of W/W^v mice with N. brasiliensis resulted in accelerated (same as control mice) expulsion; this was theorized to be due to higher specific IgE titers in the W/W^v mice (86).

The interaction of eosinophils and MMCs has been described by Kamiya et al. (88). In W/W^v mice,

eosinophil infiltration was identical in quantity as the normal littermates. In addition, comparable specific antibody responses were noted in both mouse strains (88). However, the increase in eosinophils was not associated with accelerated expulsion of T. spiralis from the W/W^v mice (78,84,85).

Numerous rodent models are available to investigate the role of mucosal mast cells in enteric diseases. Inbred strains of mice which are available for study include athymic nude mice, mast cell-deficient mice, beige mice, and germ free mice; in addition, rats have been used to investigate the role of MMCs in various mucosal diseases.

Wal et al. (89) investigated levels of histamine as well as MMC numbers in germ free and conventional rats. The total tissue histamine content as well as MMCs were increased in the conventional rat small intestine in comparison to germ free rats. Interestingly, the germ free rats had higher levels of tissue histamine in the colon and higher MMC numbers in the cecum and colon (89). These differences could be explained by the higher diamine oxidase activity (DAO, a histaminase) of the microflora-stimulated mucosal epithelium as well as that of the microflora of conventional rats (89).

The possible effects of MMC mediators (i.e., histamine, heparin, or serotonin) in the development of lesions are numerous. It has been suggested that mast cells promote endothelial cell migration and angiogenesis during venous ulcer granulation tissue formation (90). Also, histamine can modulate mesenteric basal cell proliferation *in vitro* (42) and has been shown to cause proliferation of human microvascular endothelial cells (41). Fine blood vessel dilation may be histamine-induced via an H1 receptor (20,39). *In vivo* fibroblast and jejunal mucosal epithelial cell mitosis has been shown to increase after stimulation of histamine H2 receptors (42,43); abnormal tissue fibrosis has also been associated with degranulating mast cells (44). Following immune complex induced inflammation, histamine has been shown to be the cause of post-capillary venule vascular leakage (91). Many neutrophil bacteriocidal functions are inhibited and suppressor T cell subpopulations appear to be increased as a result of increased histamine concentrations (20). Murine and human immunoglobulin responses are also inhibited in general by histamine (20).

Various bacterial components have been shown to induce histamine release from different histamine positive cells. Human basophils have been shown to

degranulate as a consequence of staphylococcal protein A (92,93) or peptidoglycan preparations (93) stimulation. In addition, Scheffer et al. (94) demonstrated that hemolytic strains of Escherichia coli induce higher histamine release from rat peritoneal mast cells (CTMCs) in comparison to non-hemolytic strains.

E. coli endotoxin, infused endoportally into rats (95), caused an immediate decrease in the number of serotonin-containing mast cells which was concurrent with changes in hepatic microcirculation. This observation suggests a direct relationship between bacterial lipopolysaccharide (LPS) and mast cell degranulation as well as subsequent pathologic changes of tissue organs.

Histamine also enhances natural killer (NK) cell activity in the presence of monocytes (96) while histamine decreases NK activity in the absence of monocytes (20). This increase in NK cell activity was not due to endogenously formed interferon (96) as interferon assays of the test system were found to be negative. Dimaprit, an H2 receptor agonist, mimicked the histamine potentiation, and H2 antagonists (cimetidine, ranitidine) abolished the effect. Thus, Hellstrand and Hermodsson (96) speculated that histamine directly or indirectly interferes with the regulation of the natural killer cell by the monocyte population.

A population of mucosal cells recently recognized as being similar to MMCs are intraepithelial lymphocytes (IELs). These cells reside between mucosal epithelial cells and possess a few granules which have staining characteristics similar to MMCs. The functions of these cells are not known, however, it is speculated that two subpopulations of IELs exist, one being T cell dependent, and the other being T cell independent (97). The IELs have been theorized to be degranulated MMC (98). It has been found that IELs possess granules containing histamine and sulphated glycosaminoglycan (99). However, the glycosaminoglycan of the IEL appears to be less sulphated than that of MMCs (100). As further evidence that IELs and MMCs have similar lineages, W/W^v mice do not exhibit IELs even upon nematode infection (101). If IELs are ontogenologically and functionally similar to MMCs, they would also be involved in mucosal responses and might directly affect the mucosal epithelial cells surrounding them.

No work has been performed on the role of MMCs or CTMCs in the pathogenesis of swine dysentery. However, researchers have observed colonic microvascular changes in pigs infected with T. hyodysenteriae suggestive of a histamine induced response (102).

Swine dysentery was first described by Whiting et al. in 1921 (103). The etiologic agent was initially suspected to be a Vibrio spp. (104), but was later isolated and described as a spirochete by Harris et al. (105,106,107) and Taylor and Alexander (108). The spirochete, T. hyodysenteriae (105,106,107), was found to be gram negative, motile by means of 7 to 9 periplasmic flagella, beta-hemolytic, anaerobic, oxidase and catalase negative, and weakly glucose fermentative (105,106,107). Microscopically, the organism is spiral-shaped and loosely coiled with one to three coils per bacterium.

Taylor and Alexander (108) also isolated a morphologically similar bacterium which did not produce clinical signs in pigs, and could be differentiated from pathogenic species by its weaker beta-hemolytic activity. In 1979, Kinyon and Harris (109) named this non-pathogenic species Treponema innocens.

Diagnostically, the pathogenic T. hyodysenteriae may be differentiated from T. innocens by its strong beta-hemolysis, its enteropathogenicity in mice (110), and by differences in APIZYME biochemical tests (111,112).

The diagnosis of swine dysentery is based on clinical signs, dark field microscopy, Victoria blue-stained fecal smears (113), and culture of the organism on selective

media (114). Differential diagnoses should include Trichuris suis infestations (whipworms), enteric salmonellosis, necroproliferative enteritis (i.e., proliferative ileitis), coccidiosis, and clostridial enteritis (114). Control of the disease is achieved by environmental sanitation, carefully re-introducing disease free stock, and antibiotic therapy (i.e., lincomycin, tiamulin, and carbadox) (115).

Experimental animal models available for swine dysentery include pigs (105-108,116), mice (110,117-119), and chick poults (120). Also, ligated swine colonic loops have been used in pathology trials (121). In 1980, Joens et al. (110) described an enteropathogenicity test differentiating T. hyodysenteriae from T. innocens by per os infection of CF1 mice. The gross lesions noted in the murine cecum were as follows (110): serosal opacity, catarrhal enteritis with large quantities of intraluminal mucus, mucosal hyperemia, and occasional hemorrhage. Histologically, crypts exhibited cellular irregularities, goblet cell proliferation, and leukocytic infiltration in the submucosa (110). Adachi et al. (120) recently described an infection model utilizing young broiler chicks. After infection, T. hyodysenteriae was cultured from the feces and clinical signs of diarrhea were noted. Pathological and histological examination demonstrated

roughened cecal mucosa and epithelial erosion. These models exhibit similar pathological signs to that observed in swine dysentery, although clinical signs (mucohemorrhagic diarrhea and mortality) are seldom observed.

The pathologic condition in swine following infection with T. hyodysenteriae is classified as a severe catarrhal colitis, with intraluminal hemorrhage leading to a non-resorbtive condition in the cecum and large colon (106,121). The pathogenesis of the disease, however, is less well understood. To date, two or possibly three virulence determinants of T. hyodysenteriae have been described. These include a hemolysin (122-124), LPS (117,118), and a cytotoxin (125).

Saheb et al. (122) first extracted and semi-purified the hemolysin from T. hyodysenteriae. The hemolytic activity in broth cultures was greatest during log phase and then declined during stationary phase (126). The treponemal hemolysin appeared similar to the hemolysin of group A Streptococcus spp., streptolysin S, in that both hemolysins are oxygen resistant and production appears to increase when the culture is supplemented with sodium ribonucleate (122,123,127). The hemolytic activity of T. hyodysenteriae can be inhibited by sucrose and trypan

blue (123), which is also similar to inhibition of Streptolysin S (127). Streptolysin S has been shown to decrease T rosette formation, and, in a dose dependent fashion, show cytotoxicity for murine and human T lymphocytes (127).

Saheb et al. (128) further classified the hemolysin of T. hyodysenteriae as being oxygen and pH stable (122), heat labile (122), decreasing murine splenocyte mitogenesis (128), and causing fluid accumulation in ligated rat ileal loops (128). T. hyodysenteriae hemolytic activity was sensitive to lipase and pronase digestion (128). Additional studies (126) demonstrated that hemolysins from T. hyodysenteriae and T. innocens differed in their hemolytic activity and their sensitivities to various phospholipids.

In 1982, Lemcke and Burrows (124) reported that RNA is needed as a carrier molecule for the hemolysin and that sonicated cultures produced no hemolysin (thus suggesting that functional hemolysin is transported extracellularly). The hemolysin has been characterized as a lipoprotein which was very weakly immunogenic (128). By sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the hemolysin has an approximate molecular mass of 19 kilodaltons and stained poorly with Coomasie Blue (129). The hemolysin has been

reported to exhibit in vitro cytotoxicity towards a range of cell types, especially porcine lymphocytes (129).

The LPS moiety, present in gram negative bacterial membranes, is frequently involved in serotyping and pathogenesis of the organism (117,118,130). Nuessen et al. (117) found the LPS of T. hyodysenteriae killed murine macrophages, induced murine splenocyte mitogenesis, and generated a leukocyte migration factor by coincubating LPS with normal swine serum. In a subsequent study (118), it was theorized that T. hyodysenteriae LPS played a major role in the swine dysentery pathogenesis following the observation of infection in C3HeB/FeJ mice, but not in the LPS hypo-responsive C3H/HeJ mice.

The characteristics of a cytotoxin produced by T. hyodysenteriae are poorly defined. Bineck et al. (125) described the cytotoxic effect of some T. hyodysenteriae strains on porcine kidney (PK-15) cells, however, the potential in vivo pathogenic effects of a cytotoxin are not known.

One of the earliest histopathologic findings in the pathogenesis of swine dysentery was the expulsion of mucigen from goblet cells in the basilar crypts of the large intestine (121), concurrent with acute coagulative necrosis of the mucosal epithelium and superficial lamina

propria (131). Spirochetes appeared to concentrate at sites of coagulative necrosis, thus implicating the spirochete in the pathologic process (131). Vibrio spp.-like organisms are reported present in the lesions (131), which correlates with the findings of several researchers that pathogenic synergism exists between T. hyodysenteriae and other colonic anaerobes (132,133). By transmission electron microscopy, T. hyodysenteriae has been observed within necrotic mucosal epithelial cells, but tissue invasion was not necessary for lesion development (102). Pohlenz et al. (134), working with gnotobiotic pigs, observed T. hyodysenteriae in goblet cells in high numbers as early as 2 days post infection. At 4 days post infection, T. hyodysenteriae had invaded enterocytes adjacent to goblet cells. Following infection, the characteristics of the mucus appeared changed, in that it was more homogeneous, less electron dense, and less sulphated (134). The mucosal epithelium was mildly eroded and crypt hyperplasia was noted, but little inflammatory response was noted in the gnotobiotic pigs (134). It was concluded (134) that T. hyodysenteriae has a predilection for mucus and goblet cells, which it colonizes, and this colonization leads to mucus hypersecretion, changes in mucus composition, and hyperplasia of immature basilar crypt cells. In

addition, conventional pigs developed ultrastructural pathological changes later (6-9 days), and exhibited a marked inflammatory reaction with endothelial damage.

The microenvironment of the swine colon is significant in the pathogenesis of swine dysentery; changes in colonic microflora allow T. hyodysenteriae to colonize the mucosal epithelium and elaborate its hemolysin and possibly its cytotoxin (121). Albassam et al. hypothesized that after T. hyodysenteriae colonization, histamine-type mediators were released from local mast cells, causing mucosal dysfunction (102). Indeed, in the mouse, Galli et al. (135) suggest that mast cell derived mediators contribute to intracellular edema and leukocyte infiltration, in the absence of IgE-mediated stimulation. If a poorly diffusable and superficial T. hyodysenteriae-derived mediator (i.e., hemolysin or cytotoxin) was produced in close proximity to the colonic epithelium, it might have a direct effect on local MMCs, resulting in degranulation, vascular congestion, anoxia of the epithelium, and eventual sloughing of the epithelium. The ensuant pathological and clinical signs might then be caused by both the spirochete and other toxigenic colonic anaerobes (121,102).

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MANUSCRIPT

EFFECT OF *Treponema hyodysenteriae* INFECTION ON THE POPULATIONS OF MUCOSAL MAST CELLS AND T CELL SUBSETS IN THE MURINE CECUM

ABSTRACT

C3H/HeN mice were infected with the swine pathogenic spirochete, Treponema hyodysenteriae, and mucosal mast cell (MMC) numbers were quantified in infected and control cecal sections. An initial increase in MMC numbers to 22/50 crypt-villus units (CVU) was observed, but at twenty days post infection only 5 MMC/50 CVU were detected. This decrease was significantly different ($p<0.05$) than the numbers observed in control mice (13 MMC/50 CVU). Immunohistochemical analysis performed on cecal sections failed to show a significant shift in lamina proprial T lymphocyte subsets. Numbers of cecal T. hyodysenteriae colony forming units (CFU) were stable throughout the experiment. Mast cell-deficient W/W^v mice and their mast cell-sufficient littermates were infected to determine if MMC degranulation was necessary for the occurrence of T. hyodysenteriae-induced lesions. W/W^v mice were as susceptible to infection and developed similar macroscopic and microscopic lesions as their normal littermates. These results indicate that MMC populations are responsive to a persistent T. hyodysenteriae infection, but MMC do not appear to be required for lesion development in the murine model.

INTRODUCTION

Infections with Treponema hyodysenteriae have been shown to cause swine dysentery (1,2,3), a severe mucohemorrhagic diarrheal disease of pigs. Virulence has been attributed to the presence of a beta-hemolysin (4,5,6), a biologically active endotoxin (7,8), and a cytotoxin (9). The organism has also been reported to be weakly invasive (10) and to be chemotactically attracted to mucin (11). Infected swine appear to develop active immunity as evidenced by the development of serum antibodies and the inability to re-infect convalescent pigs (12).

Lesions are found in the cecum and large colon of swine infected with T. hyodysenteriae (13). Histologically, basilar colonic crypt goblet cells expulse mucus early in the disease (14) followed by acute coagulative necrosis of the mucosal epithelium and superficial lamina propria in areas where spirochete colonization is greatest (13). The contraction of mucosal subepithelial venules has been observed as an early histopathological finding (10), and is similar to histamine-induced endothelial cell contraction (15). This evidence suggests that there is a participatory role for MMC mediators in the development of dysenteric lesions.

Rodents have been shown to have increased numbers of intestinal mucosal mast cells following infection with various intestinal parasites (16) or graft-versus-host disease (17). The protective role of the intestinal MMC in nematode infections has not been established, however, some evidence suggests that the MMC may be involved in nematode expulsion. Utilizing the W/W^v (mast-cell deficient) (18) strain of mouse, Ha et al. (19) showed that W/W^v mice, which are immunologically competent (20), expelled adult T. spiralis more slowly than their normal +/+ littermates and suggested an effector function for the MMC in worm expulsion. However, infection of W/W^v mice with Nippostrongylus brasiliensis indicated that MMC responses were not the sole effector mechanism for nematode rejection (21,22). To date, there are no published reports describing the influence of a bacterial enteritis/colitis on MMC numbers or function.

Previous investigators demonstrated the enteropathogenicity of T. hyodysenteriae using a murine model (7,8,23). The present study examined the MMC response in C3H/HeN mice challenged with T. hyodysenteriae. To determine if products of MMC degranulation contribute to lesions associated with T. hyodysenteriae infection, mast cell deficient W/W^v mice, as well as their normal +/+ littermates, were also

challenged. Since it appears that MMC differentiate in the presence of interleukin 3 (IL 3) (24), T cell subpopulations in the cecal lamina propria and mucosal epithelium were also quantified. Potential cellular interactions between various lymphoreticular cell types will be discussed.

MATERIALS AND METHODS

Bacteria. T. hyodysenteriae strain B204 (serotype 2) were grown anaerobically in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% horse serum (HyClone labs, Logan, Utah) and 5% yeast extract (BBL) as previously described (25).

Animals. C3H/HeN mice were obtained from Harlan-Sprague Dawley (Madison, Wis.), and W/W^v , +/+ (WB/ReJW/+ x C57BL/6J-W^v/+ F1), BALB/cByJ, and DBA/1J mice were obtained from Jackson Laboratories (Bar Harbor, Maine). NFR/N mice were kindly provided by Dr. E. Jeska, Veterinary Medical Research Institute, Iowa State University. All animals were maintained on Mouse Lab Chow #5010 (Purina Mills, Inc., St. Louis, Mo.) under controlled conditions in the Laboratory Animal Resource Facility of the College of Veterinary Medicine, Iowa State University. Mice used in the challenge studies were 6 to 10 weeks of age.

Infection Protocol. Mice were infected on 2 consecutive days by intragastric inoculation of 1 ml of culture broth containing 1 X 10^8 organisms. Non-infected control mice were intragastrically administered 0.5 ml of sterile culture broth. Mice were fasted 6 hours prior to receiving the first challenge inoculum and were

maintained on restricted feed (approximately 1 gm feed/mouse/day) for the next 48 hours.

Bacteriology. Mice were sacrificed at days 10,15, and 20 post-infection (PI). Pathological changes were noted as either excess cecal intraluminal mucus and/or cecal atrophy. The cecal apex was removed and fixed in Carnoy's fluid fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) and later prepared for MMC enumeration (see below). The remaining portion of the cecum was placed in sterile WhirlPak bags (Baxter Co., Minneapolis, Minn.) and weighed. Ceca were suspended 1:100 (w/v) in sterile phosphate-buffered saline (PBS)(0.14 M NaCl, 0.008 M Na_2HPO_4, 0.0015 M KH_2PO_4, and 0.0027 M KCl, pH 7.2) and homogenized for 30 seconds in a Stomacher laboratory blender (A. J. Seward Co., London). The homogenate was directly inoculated onto BJ blood agar media (26) for isolation of T. hyodysenteriae and subjected to darkfield microscopy. Serial 10-fold dilutions of the homogenate were added to 5 ml of molten (45 degrees C) Trypticase soy agar containing 5% ovine blood and antibiotics (26). This mixture was poured into 60 mm Petri dishes (#25010, Corning Glass Works, Corning, N.Y.) and incubated anaerobically at 37 degrees C for 96 hours. The zones of beta-hemolysis were enumerated and

the number of T. hyodysenteriae colony forming units (CFU) per gram of cecal tissue was calculated.

Histological Examination. Following fixation in Carnoy's fluid for two hours, cecal tissue was transferred to 70% ethanol. The tissues were routinely processed, embedded in paraffin, and cut at 5 microns thickness. The tissues were cleared, hydrated, and immersed for 12 hours in a solution of 0.1g Astrablau FM (Schmid G.M.B.H. and Co., Stuttgart, West Germany), 5 ml 12 N HCl, and 100 ml deionized water (pH 0.5) (27). The tissues were then rinsed in tap water and counterstained for 10 minutes with a solution of 0.1 gm Nuclear Fast Red (Schmid G.M.B.H. and Co., Stuttgart, West Germany), 5 gm $Al_2(SO_4)_3$-$18H_2O$, and 95 ml of deionized water. Examination of the section reveals blue mast cell granules while surrounding tissue stains red. Lamina proprial MMCs in 50 crypt-villus units (defined as 1 villus and 2 "shoulders") were counted for each mouse.

T Cell Quantification. Lamina propria and epithelial T lymphocyte subsets were visualized using an anti-rat Streptavidin-biotin immunoperoxidase reagent (Zymed Labs, San Francisco, Calif.). Primary monoclonal antibodies (rat anti-mouse) directed against Lyt 1+ and Lyt 2+ T lymphocyte markers were obtained from Becton Dickinson, Inc., Mountain View, Calif. Quantification

was accomplished using a Zeiss Image Analyzer, Department of Veterinary Anatomy, Iowa State University. The area occupied by stained cell(s) was expressed as a percentage of the total area of lamina propria and epithelium. Four fields per cecum were analyzed.

Statistical Methods. The Student's t distribution (28) was utilized to determine significance.

RESULTS

Dynamics of Mucosal Mast Cell Responses Following Infection with Treponema hyodysenteriae. While developing the murine model to examine T. hyodysenteriae infections, several inbred strains of mice were utilized (i.e., C3H/HeN, BALB/cByJ, NFR/N, DBA/1J, C57BL/6J, and C3H/HeJ). All of the mouse strains examined were susceptible to infection and developed macroscopic lesions detectable as early as 5 days PI and lasting as long as 70 days PI (data not shown).

Treponema hyodysenteriae was isolated from the ceca of infected mice at each time point examined (Fig. 1). Pathological signs (excess mucus and cecal atrophy) were noted in the ceca at 10, 15, and 20 days PI. Histopathologic examination of affected ceca showed villus hypertrophy and edema, mild mucosal epithelial sloughing, and focal areas of inflammatory cell infiltration within the lamina propria.

The numbers of T. hyodysenteriae in the ceca were correlated with changes in the numbers of MMC in infected mice (Fig. 1). The CFU per gram cecum at 10, 15, and 20 days PI were 3.3×10^8, 0.61×10^8, and 1.3×10^8 T. hyodysenteriae, respectively. Though the spirochetal numbers varied slightly, there was a significant decrease

($p<0.05$) in the numbers of MMC from day 10 PI to day 20 PI (Fig. 1). At 20 days PI, challenged mice also showed a significant decrease in MMC numbers ($p<0.05$) in comparison to control mice and to mice infected with the non-pathogenic porcine spirochete, T. innocens (Fig. 2).

At 10 days PI, the number of cecal MMCs increased from a control average of 12 MMC/50 CVUs to 22 MMC/50 CVUs (Fig. 2). At 15 days PI, a decrease in MMC numbers was observed, and at 20 days PI, the tissues contained fewer MMC ($p<.05$) than non-infected controls. In addition, numbers of MMCs in the ceca of mice infected with T. innocens did not change in comparison to controls (Fig. 2).

Immunohistochemical staining and image analysis quantification was performed on the C3H/HeN cecal tissues in the control and challenged mice as described in materials and methods. No significant T cell subpopulation shift was detected following T. hyodysenteriae infection.

Role of Mucosal Mast Cells in T. hyodysenteriae Induced Cecal Lesions. The functional role of the MMC during an infection with T. hyodysenteriae was examined in W/W^v mice and their +/+ normal littermates. The results in Table 1 indicate that the W/W^v mice were

colonized with T. hyodysenteriae and developed lesions as well or more frequently than their +/+ littermates. At 20 days PI, all the W/W^v and +/+ mice demonstrated macroscopic lesions associated with a T. hyodysenteriae infection. The MMC response in the +/+ littermates demonstrated a two-fold increase at 5 days PI, but the numbers of detectable MMC had returned to control levels by day 20 PI (Table 2).

Table 1. Comparison of T. hyodysenteriae-Induced Lesions in Mast Cell Deficient and Normal Mice

	W/W^v		+/+	
DPI[a]	lesions[b]	culture[c]	lesions	culture
5	2/3	3/3	2/3	3/3
10	8/10	10/10	5/11	9/11
15	12/12	12/12	6/12	8/12
20	6/6	6/6	7/7	7/7

[a]DPI, day post infection with 1 x 10^8 T. hyodysenteriae.

[b]Macroscopic lesions were noted as excess intraluminal mucus and/or cecal atrophy.

[c]Cecal contents were cultured on BJ blood agar media (26) for the isolation of T. hyodysenteriae.

Table 2. Determination of the Mucosal Mast Cell Response in Control and T. hyodysenteriae-Infected Mice[a]

	W/W^v		+/+	
DPI[b]	control	infected	control	infected
5	0 ± 0	0 ± 0	4.0 ± 0	10.3 ± 1.8
10	0.2 ± 0.2	0.2 ± 0.2	4.0 ± 0.5	8.0 ± 1.5
15	0 ± 0	0 ± 0	4.3 ± 0.5	9.8 ± 1.5[c]
20	0 ± 0	0 ± 0	4.3 ± 0.6	5.5 ± 2.0

[a]Number of mucosal mast cells were determined in 50 crypt-villus units as described in Materials and Methods.

[b]DPI, days post infection with 1 x 10^8 T. hyodysenteriae.

[c]At 15 DPI, the number of mucosal mast cells in the ceca of infected +/+ mice were significantly different from non-infected mice ($p<0.05$).

DISCUSSION

Treponema hyodysenteriae infection in the mouse induced cecitis and excess intraluminal mucus as reported by others (23). Lamina propria MMC numbers in the ceca of infected mice exhibited an increase at 10 days PI followed by a significant ($p<0.05$) decrease at day 20 post infection (Figure 1). Immunohistochemistry performed on cecal sections failed to show any changes in either T helper/inducer (Lyt 1+) or T suppressor/cytotoxic (Lyt 2+) cell populations. Colonization of ceca with T. hyodysenteriae remained relatively constant between 10 and 20 days PI (Fig. 1)

It has been speculated that mast cells and their degranulation products might be partially responsible for the lesions observed in swine dysentery (10) based on observations that early lamina propria changes are consistent with those resulting from a histamine-type activity (15). Although little work has been performed defining porcine MMC mediators, rat intestinal MMC have been reported to contain small amounts of histamine (29), chondroitin sulfate E (30) and a unique neutral protease (29,30). In vivo, histamine has been shown to have sundry effects, such as inducing adjacent cell proliferation (31), fine blood vessel dilation (32,33)

endothelial cell contraction (15), and directly or indirectly inducing down-regulation of immune responses (32). Similar circulatory changes (i.e., subepithelial venule endothelial cell contraction) are seen in the colonic mucosa of swine infected with T. hyodysenteriae (10).

In this study, murine cecal lesions were macroscopically evident at times when a slight MMC increase was noted (10 days PI) as well as when MMC levels decreased (20 days PI). The question of whether the decrease in the MMC population was due to a failure of differentiation or exhaustive degranulation could not be ascertained. The numbers of spirochetes colonizing the ceca and the MMC response are illustrated in Figure 2. Initially, the number of spirochetes observed was 3 x 10^8 CFU per gram of cecal tissue, and as MMC numbers decreased at 15 days PI the numbers of T. hyodysenteriae also decreased. At twenty days PI, a slight increase in the CFU of T. hyodysenteriae was observed while the numbers of MMC continued to decrease. One might speculate that the continued presence and the slight increase in the numbers of spirochetes enhanced the degranulation of the MMC and hindered their detection by light microscopy. However, these data are not sufficient to warrant a direct causal relationship between cecal MMC

degranulation and the number of T. hyodysenteriae CFU in the cecum.

Intestinal immune and cellular responses affect physiological function. Specifically, T lymphocytes have recently been implicated in regulating gut mucosal surface changes. Activated T cells (or their cytokines) have been shown to cause crypt epithelial hyperplasia in human tissue explants (34), and it has been suggested that T cell cytokines cause increased goblet cell mucus production and secretion (35). Since it appears MMC are under the proliferative control of an IL 3-like molecule (24,36), it was expected that an increase in Lyt 1+ T cells would be observed in the cecal lamina propria and epithelium. However, no significant populations shifts were observed by the techniques employed. The T cell data obtained by image analysis were computed from the area occupied by the stained cells divided by the total area in the microscopic field and then expressed as a percentage. This methodology may mask any absolute increases in T cells due to the edema which occurs following a T. hyodysenteriae infection (10,23,37). In addition, determining absolute numbers of T lymphocyte subpopulations would not address the functional activity of these cells. It could be hypothesized that T lymphocyte populations do not shift, but rather, one or

more of these populations become activated and secrete soluble factor(s) which mediate sundry cellular reactions (i.e., mast cell maturation and differentiation, and mucus secretion).

The method by which T. hyodysenteriae exerts its effect on MMC populations in the murine cecum is unclear. Since T. hyodysenteriae appears to be chemotactically attracted to and colonizes within the mucus layer of the colon (9,11), a bacterial-derived soluble toxin or mediator could diffuse between or bind to mucosal epithelium and affect lamina proprial lymphocyte populations.

The role of the murine MMC mediators in the induction of lesion formation following T. hyodysenteriae infection appears minimal based on the results we obtained by infecting mast cell-deficient W/W^v mice. Pathologic lesions (macroscopic and microscopic) and spirochetal colonization were equivalent in the W/W^v mice and its +/+ littermates. An effector function has been postulated for the MMC response following a Trichinella spiralis (38,39) infection, but the smaller response observed following a T. hyodysenteriae infection may not induce similar activities. Cecal MMC do not appear to be directly involved in T. hyodysenterale-induced lesion formation in the mouse model. However, the change

observed in cecal MMC numbers might be indicative of other cellular interactions taking place, such as T lymphocyte activation. It is evident that this bacterial infection can modulate the numbers of detectable mucosal mast cells within the murine cecum.

Figure 1. Correlation of MMC Levels with Numbers of Cecal Treponema hyodysenteriae Colony Forming Units. The numbers of MMC (open rectangles) in the ceca of T. hyodysenteriae-infected C3H/HeN mice were compared to the CFU of spirochetes (closed circles) recovered at 10, 15, and 20 days PI. Mucosal mast cells and T. hyodysenteriae CFUs were determined as described in Materials and Methods. Results are expressed as the mean ± standard error of the mean.

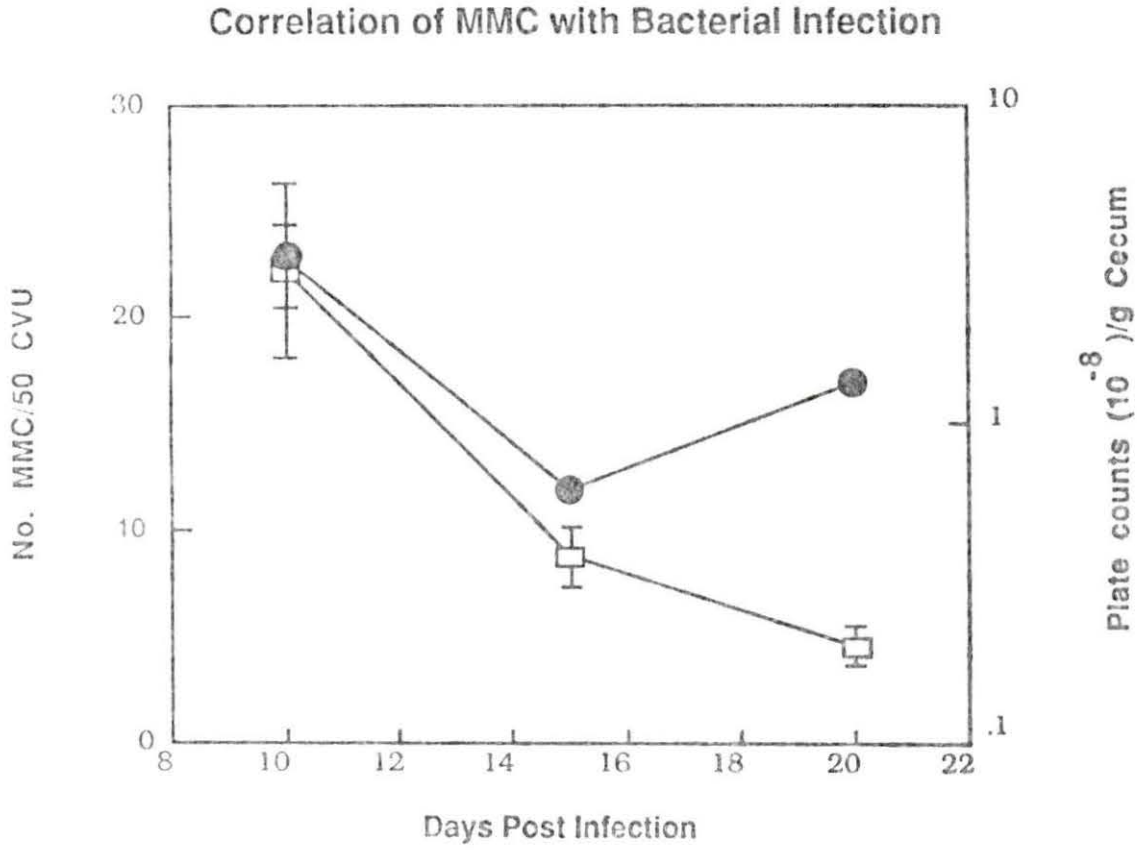
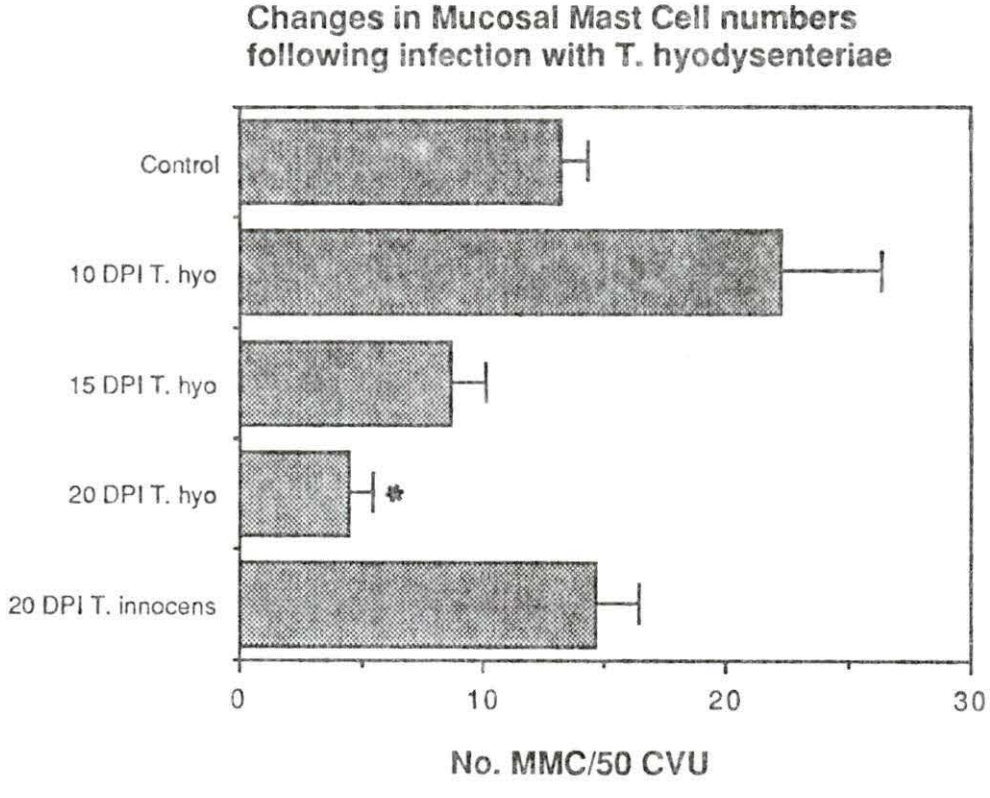

Correlation of MMC with Bacterial Infection
No. MMC/50 CVU
Plate counts (10^{-8})/g Cecum
Days Post Infection
30
20
10
0
10
1
.1
8
10
12
14
16
18
20
22

Figure 2. Changes in Mucosal Mast Cell Numbers Following Infection With T. hyodysenteriae. Mucosal mast cell numbers in 50 crypt-villus units were determined in C3H/HeN non-infected control mice (n=9), T. hyodysenteriae-infected (n=6) at 10, 15, and 20 days post-infection, and T. innocens-infected (n=3) at 20 days PI. Infected mice were challenged with 1 x 10^8 T. hyodysenteriae or T. innocens on two consecutive days. Results are expressed as the mean number of MMC ± standard error of the mean. *, $p<0.05$.


Changes in Mucosal Mast Cell numbers
following infection with T. hyodysenteriae
Control
10 DPI T. hyo
15 DPI T. hyo
20 DPI T. hyo
20 DPI T. innocens
*
0
10
20
30
No. MMC/50 CVU

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GENERAL SUMMARY

Mucosal mast cell populations in the murine cecum do undergo dynamic changes concurrent with Treponema hyodysenteriae infection. Numbers of MMCs appear to rise initially, and then decrease significantly below baseline values in an unresolving infection. T-lymphocyte subpopulations do not appear to change in the cecal lamina propria and epithelium. The increase and subsequent decrease in MMC numbers may or may not have direct physiologic action on diseased tissues or number of cecal T. hyodysenteriae via MMC granule mediators. The proper method to evaluate this hypothesis would be to perform functional assays and tests, such as directly assaying for levels of MMC mediators, and performing in vitro assays demonstrating physiologic effects of the mediators. These data would, at this point, necessarily be performed in the rat, as the granule mediators have been reasonably defined in the animal. In the murine model, MMC degranulation is not solely responsible for lesion development, as the W/W^v mice were equally susceptible to infection and development of lesions in the cecum.

The MMC data reported in this study, however, may be interpreted as evidence of other intercellular changes

which directly contribute to pathogenesis. The change in MMC numbers in the course of the infection is not a passive increase or decrease only; the numbers of MMCs increase and then decrease significantly below baseline. A potential "protective" role for the MMCs, in which their degranulation products cause rejection of the spirochete, has not been disproven by this research, but similarly has not been well supported. The W/W^{v} mice seem to have the same or greater chance of developing cecitis than their +/+ littermates, and the NFR/N mice, which we have observed to have a higher resident MMC population in the cecum, seemed to be refractory to infection by T. hyodysenteriae. However, after treatment with oral antibiotics, the NFR/N mice appeared to exhibit T. hyodysenteriae-induced lesions as other inbred strains.

We have examined many aspects of potential T. hyodysenteriae pathogenesis utilizing inbred mice of differing genotypes and phenotypes. No major effect on pathogenesis was found other than the manipulation of cecal microenvirons (presumably) with oral antibiotics. The information suggests that an important host defense is a somewhat non-specific one, which may be manipulated in all strains of mice by use of oral antibiotics. The "manipulated" factor would seem likely to be the

disruption of normal cecal microflora. Mice normally have numerous fusiform-shaped bacteria within their ceca. After cecitis is induced by infection with T. hyodysenteriae, the cecal population shifts to include predominately spirochetes, cocci, and some bacilli. If the spirochete initially requires a niche in the mucus layer for binding, then the changing of the microflora would make available transient areas where the spirochete could attach and penetrate the mucus layer to the crypts, where a soluble factor(s) may be elaborated.

If the protection was only a local bacterial exclusion, then the "immune" or refractory convalescent pig would be difficult to interpret. However, with a secretory IgA response, immune exclusion would be a valid hypothesis. More work needs to be done to further investigate what the status of protection is, how that state is achieved, and what inciting virulence determinants T. hyodysenteriae possesses to instigate pathogenesis.

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APPENDIX 1

PROTECTION STUDIES IN THE PARENTERALLY IMMUNIZED MOUSE

Purpose of the experiment. Mice (6 to 10 weeks old) were parenterally immunized with Treponema hyodysenteriae outer membrane preparations to determine if these preparations could induce protection against a virulent challenge.

Materials and Methods

Animals. C3H/HeN strain mice were obtained from Harlan Sprague Dawley, Indianapolis, Indiana. Animals were maintained on Mouse Lab Chow #5010 (Purina Mills, Inc., St. Louis, Mo.) under controlled conditions in the Laboratory Animal Resource Facility of the College of Veterinary Medicine, Iowa State University. Mice were 6 to 10 weeks of age.

Antigen. Outer membrane protein preparations of T. hyodysenteriae strain B204 (serotype 2) were obtained by Sarkosyl extraction (1).

Protocol. Five groups of mice, each group containing 5 mice, were utilized. Mice (Groups 1-4) received the indicated dose of antigen by intravenous (i.v.) injection and received an identical booster dose 15 days later. Mice in group 5 were immunized and boosted *per os*. Five days after the booster immunization, mice were challenged with virulent *T. hyodysenteriae* B204 as described below:

Group 1: 1.0 microgram B204 outer membrane protein (OMP) preparation was administered intravenously (i.v.) in a volume of 0.1 ml.

Group 2: 10.0 micrograms B204 OMP preparation was administered i.v. in a volume of 0.1 ml.

Group 3: 50.0 micrograms B204 OMP preparation was administered i.v. in a volume of 0.1 ml.

Group 4: Two doses of 0.1 ml Phosphate-Buffered Saline (PBS) were injected i.v. on days 20 and 5 pre-infection (sham injection controls).

Group 5: 50.0 micrograms B204 OMP preparation was administered *per os* by gastric intubation in a volume of 0.4 ml.

Bacteria. T. hyodysenteriae strain B204 (serotype 2) was grown anaerobically in Trypticase soy broth (BBL) supplemented with 5% horse serum (HyClone Labs) and 5% yeast extract (BBL). Overnight cultures were checked for purity by darkfield microscopy and enumerated using a Petroff-Hauser counting chamber.

Infection Procedure. Mice were infected on 2 consecutive days by intragastric inoculation of 1 ml of culture broth containing 1 x 10^8 organisms. Mice were fasted between administration of the two challenge inoculums (24 hours).

Necropsy Procedure. All mice were necropsied at 10 days post infection. Macroscopic changes in the cecum were noted as either excess intraluminal mucus and/or atrophy. The cecum was aseptically removed and weighed in a sterile WhirlPak bag (Baxter Co., Minneapolis, Minn), diluted 1:100 w/v with phosphate-buffered saline, and homogenized in a Stomacher Laboratory Blender (A. J. Seward Co., London, U. K.). The homogenate was streaked onto BJ blood agar media (4) for the isolation of T. hyodysenteriae and subjected to darkfield microscopy. In addition, serial ten-fold dilutions of the homogenate were added to molten (45 degrees C) Trypticase soy agar tubes supplemented with 5% ovine blood and antibiotics

(2) and were then pour-plated into 60 mm Petri dishes (#25010, Corning Glass Works, Corning, N.Y.) and incubated anaerobically at 37 degrees C for 96 hours. The zones of beta-hemolysis were enumerated and the number of T. hyodysenteriae colony forming units per gram of cecal tissue was calculated.

Results

Table 1. Effect of vaccination on lesion formation at 10 days post infection with T. hyodysenteriae

Group	Treatment[a]	Macroscopic Signs[b]	Dark Field[c]
1	1.0 mcg i.v.	XM, At-apex	+
1	" "	XM, At-apex	-
1	" "	NGL	-
1	" "	NGL	+
1	" "	NGL	-
2	10.0 mcg i.v.	NGL	-
2	" "	XM, At	++
2	" "	XM-apex	+
2	" "	XM, At-apex	+
2	" "	NGL	+
3	50 mcg i.v.	XM, At-apex	+
3	" "	XM, At	+
3	" "	XM, At	++
3	" "	XM, At-apex	-
3	" "	XM, At	++
3	" "	NGL	-
4	saline i.v.	XM, At	+
4	" "	XM-apex	-
4	" "	NGL	-
4	" "	XM-apex	-
4	" "	NGL	-
5	50 mcg p.o.	NGL	-
5	" "	NGL	-
5	" "	NGL	-
5	" "	NGL	-
5	" "	NGL	-

[a]Total protein of T. hyodysenteriae Sarkosyl extract (micrograms) administered per inoculation; routes given were i.v., intravenous; p.o., per os.

[b]NGL- No Gross Lesions observed; XM- Excess cecal intraluminal mucus observed; At- Cecal atrophy observed.

[c]Dark field microscopy was performed to identify large spirochetes in cecal contents.

Table 2. Effect of vaccination on culture and cecal colonization of T. hyodysenteriae 10 days post infection

Group	Culture	Organisms Recovered[a]	Group Mean
Treatment[b]-1.0 mcg i.v.			
1	+	4.8×10^7	
1	+	5.2×10^6	
1	+	1.0×10^4[c]	
1	+	1.0×10^4	
1	+	7.7×10^5	$1.1 +/- 0.9 \times 10^7$
Treatment- 10 mcg i.v.			
2	-	0.0	
2	+	3.4×10^7	
2	+	5.0×10^6	
2	+	2.5×10^7	
2	+	1.0×10^4	$1.3 +/- 0.7 \times 10^7$
Treatment- 50 mcg i.v.			
3	+	1.0×10^7	
3	+	4.3×10^6	
3	+	1.4×10^8	
3	+	2.0×10^7	
3	+	1.1×10^8	
3	+	2.7×10^6	$4.8 +/- 2.4 \times 10^7$
Treatment- PBS sham i.v. injection			
4	+	1.5×10^7	
4	+	1.0×10^4	
4	+	1.0×10^4	
4	+	8.3×10^5	
4	+	1.1×10^6	$3.4 +/- 2.9 \times 10^6$
Treatment- 50 mcg per os			
5	+	6.8×10^6	
5	+	1.9×10^7	
5	+	1.0×10^4	
5	+	9.1×10^6	
5	+	9.5×10^6	$8.9 +/- 3.0 \times 10^6$

[a]Colony forming units per gram of cecum.
[b]T. hyodysenteriae Sarkosyl extract.
[c]If culture results were positive and pour plate results negative, the value of 1×10^4 was assigned.

Discussion

The results obtained in this experiment were not anticipated. The administration of a bacterin preparation to a naive mouse appeared to increase colonization in a dose-dependent fashion, totally contrary to a protective response that one would expect. The groups 4 and 5 showed some decrease in colonization, however, the sham-injected mouse still had the lowest colonization.

Interestingly, the orally immunized mice all were culture positive, and their colonization was greater than that of the naive mice, but yet did not exhibit macroscopic lesions. The conclusions that may be drawn from this experiment should be considered preliminary; however, it is possible that *per os* administration of the antigen induced a suppressor cell population in the gut, thus allowing colonization but not macroscopic pathology. This speculation would by necessity assume that the pathologic changes involved were host-derived in nature.

These results reflect previous findings in that recovery of $> 10^7$ CFU/g cecal tissue of *T. hyodysenteriae* usually correlates with the presence of excess mucus. Excess mucus production may prove to be involved in the disease process, as *T. hyodysenteriae* has been shown in

swine to not only cause excess mucus production in vivo (3), but also to be chemotactic for mucin in vitro (4).

If the macroscopic changes and the colonization noted in murine ceca were due to the host response, the subsequent humoral immunization response might be considered detrimental. This can be observed by comparing groups 1 through 3, which exhibit in almost dose-dependent fashion the increase of macroscopic signs and colonization values as the immunization dose increased. In the i.v. immunized mice, macroscopic signs were seen concurrent with CFU > 10^7 in the infected ceca; however, the per os immunized mice all showed colonization with T. hyodysenteriae, but no pathologic changes were noted. Quite possibly two phenomena are occuring in this situation, which might show that protection from pathologic changes in the gut occur by both a secretory IgA or IgM response (antigen delivered to the gut-associated lymphoid tissue) and also suppression of a hypersensitivity response by induction of T suppressor cell populations. As to why, in the i.v. immunized mice, macroscopic changes and >10^7 CFU/g cecal tissue numbers of T. hyodysenteriae were observed, it might be postulated that released factor(s) or condition(s) were caused by the host response, such as availability of iron from heme groups of lysed

erythrocytes, increased oxygen tension in the mucus layer, or serum exudation into the cecal mucus. Also, if specific IgG or IgM is present in the lamina propria, complement might be fixed by a spirochetal antigen and inflammatory chemotaxins would be generated, increasing the inflammatory response.

Protection in the mouse model against T. hyodysenteriae challenge was not achieved by parenteral immunization with treponemal outer membrane proteins. Definitive work needs to be performed to ascertain the seemingly increased susceptibility after parenteral vaccination.

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APPENDIX 2

STUDIES OF POTENTIAL INVOLVEMENT OF MURINE H2 HAPLOTYPE ON CHALLENGE WITH *Treponema hyodysenteriae*

Purpose of the experiment. Previous work in our laboratory has shown that unstimulated Mucosal Mast Cell (MMC) numbers in the NFR/N strain of mouse were higher than the MMC levels in C3H strains of mice. NFR/N mice also exhibit an increase in cecal MMC numbers after infection with *T. hyodysenteriae*, and appear to be refractive to virulent spirochete challenge when compared with C3H strains of mice. Bell et al. (1) has classified NFR/N mice as "high responders" in response to *Trichinella spiralis* infection, and has equated this response with the rapid expulsion of the nematodes from the gut. DBA/1J mice, which are also H-2q, are also classified as a "non-responders" and do not rapidly expulse nematodes (1). BALB/c mice are also classified as "non-responders" (1). This study was designed to examine two questions: 1) the correlation of H-2 haplotype with severity of disease, and 2) the effect of the relative MMC response on the development of lesions following infection with *T. hyodysenteriae*.

Materials and Methods

Animals. NFR/N (H-2 q) mice were kindly provided by Dr. E. Jeska, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa. DBA/1J (H-2 q) and BALB/cByJ (H-2 d) mouse strains were obtained from Jackson Laboratories, Bar Harbor, Maine. Animals were maintained on Mouse Lab Chow #5010 (Purina Mills, Inc., St. Louis, Missouri) under controlled conditions in the Laboratory Animal Resource Facility of the College of Veterinary Medicine, Iowa State University. Mice were 9 to 10 weeks old.

Bacteria. T. hyodysenteriae strain B204 (18 passages in vitro) was grown anaerobically in Trypticase soy broth (BBL) supplemented with 5% horse serum (HyClone Laboratories) and 5% yeast extract (BBL). Overnight cultures were checked for purity, enumerated, and diluted as described below.

Antibiotic Treatment. Five antibiotics were utilized in this trial: Colistin, Vancomycin, Spiramycin, Rifampicin, and Spectinomycin (Sigma Chemical Company, St. Louis, Missouri). Separate stock solutions

were made in a manner similar to that described by Kunkle and Kinyon (2): Colistin: 50 mg dissolved in 100 ml deionized (di) water; Vancomyin: 48 mg dissolved in 100 ml di water; Spiramycin: 244 mg dissolved in 200 ml 20% ethanol; Rifampicin: 200 mg dissolved in 200 ml of 20% ethanol; and Spectinomycin: 1.6 g dissolved in 100 ml di water. Equal volumes of each of the five antibiotic stock solutions were mixed and added to the drinking water at a final concentration of 5% v/v (mixed antibiotics/water). Mice were maintained on this water for three days, starting one day prior to infection and concurrent with the two day infection period. Five mice from each mouse strain were treated with antibiotics, challenged as described below, and necropsied at day 10 post infection.

Protocol. Three groups of mice were utilized: DBA/1J mice, BALB/c mice, and NFR/N mice. Each group consisted of 20 challenged mice. Mice were sacrificed at three intervals post infection (PI): 5 days (PI), 10 days, and 20 days. In addition, the oral antibiotic-treated groups were necropsied at 10 days PI. Criteria used to evaluate the challenge were gross pathologic signs, culture results, colonization values, and dark field analysis of cecal contents.

Infection Procedure. Mice were infected for 2 consecutive days by intragastric inoculation of 1 ml of broth culture containing 1 x 10^8 organisms. Mice were fasted for 24 hours during the infection period.

Necropsy Procedure. Mice were sacrificed at 5, 10, and 20 days PI. Gross changes were noted in the cecum as either excess intraluminal mucus and/or atrophy. The cecum was aseptically removed and weighed in a sterile WhirlPak bag (Baxter Co., Minneapolis, Minn), diluted 1:100 w/v with phosphate-buffered saline, and homogenized in a Stomacher Laboratory Blender (A. J. Seward Co., London, U. K.). The homogenate was streaked on BJ blood agar media () for isolation of T. hyodysenteriae and subjected to darkfield microscopy. In addition, serial ten-fold dilutions of the homogenate were added to molten Trypticase soy agar tubes supplemented with 5% ovine blood and antibiotics () and were then pour-plated into 60 mm Petri dishes (#25010, Corning Glass Works, Corning, N.Y.) and incubated anaerobically at 37 degrees C for 96 hours. The zones of hemolysis were enumerated and the number of T. hyodysenteriae colony forming units per gram of cecal tissue was calculated.

Results

Table 1. Pathological and Bacteriological Findings from Mice Sacrificed 5 Days PI

Strain	Macro[a]	DF[b]	Culture	CF[c]	Group Mean
BALB/c	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	+	1.0×10^{4}[d]	
					$2.0 \pm 2.0 \times 10^{3}$
DBA/1J	XM,At	+++	+	2.0×10^{8}	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
					$4.0 \pm 4.0 \times 10^{7}$
NFR/N	XM,At	+++	+	1.4×10^{8}	
" "	NGL	-	+	1.4×10^{5}	
" "	XM,At[e]	++	+	2.5×10^{7}	
" "	NGL	-	+	0.0	
" "	NGL	-	+	1.0×10^{4}	
					$3.3 \pm 2.7 \times 10^{7}$

[a]Macroscopic cecal pathologic changes were recorded as NGL, no gross lesions, XM, excess intraluminal mucus, and At, cecal atrophy.

[b]Dark field microscopy was performed on cecal swabs and subjectively scored on an ascending scale from +; very few spirochetes observed, to +++++; predominately spirochetes observed.

[c]Colonization factor is expressed as the number of hemolytic colony forming units of T. hyodysenteriae per gram of cecum.

[d]If cecal culture results were positive and the pour plate counts were zero, an arbitrary value of 1×10^{4} was assigned.

[e]macroscopic signs noted in the cecal apex only.

Table 2. Pathological and Bacteriological Findings from Mice Sacrificed 10 days PI

Strain	Macro[a]	DF[b]	Culture	CF[c]	Group Mean
BALB/c	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
					0.0
DBA/1J	XM,At	-	+	1.6×10^7	
" "	XM,At	++	+	2.4×10^7	
" "	NGL	-	+	2.5×10^6	
" "	XM,At	+++	+	3.5×10^7	
" "	XM,At	+++	+	6.0×10^7	
					$2.8 \pm 1.0 \times 10^7$
NFR/N	XM,At	++	+	5.3×10^7	
" "	NGL	-	+	2.8×10^5	
" "	NGL	-	-	0.0	
" "	NGL	-	+	0.0	
" "	NGL	-	+	0.0	
					$1.1 \pm 1.1 \times 10^7$

[a]Macroscopic cecal pathologic changes were recorded as NGL, no gross lesions, XM, excess intraluminal mucus, and At, cecal atrophy.

[b]Dark field microscopy was performed on cecal swabs and subjectively scored on an ascending scale from +; very few spirochetes observed, to +++++; predominantly spirochetes observed.

[c]Colonization factor is expressed as the number of hemolytic colony forming units of T. hyodysenteriae per gram of cecum.

[d]If cecal culture results were positive and the pour plate counts were zero, an arbitrary value of 1×10^4 was assigned.

[e]macroscopic signs noted in the cecal apex only.

Table 3. Pathological and Bacteriological Findings from Mice Treated With Oral Antibiotics and Sacrificed 10 Days PI[a]

Strain	Macro[b]	DF[c]	Culture	CF[d]	Group Mean
BALB/c	NGL	-	+	1.0×10^{4}[e]	
" "	XM,At	++	+	7.5×10^{7}	
" "	XM,At	+++	+	2.0×10^{8}	
" "	XM,At	+++	+	1.2×10^{8}	
" "	XM,At	+++	+	2.4×10^{8}	
					$1.3 \pm 0.4 \times 10^{8}$
DBA/1J	NGL	-	-	0.0	
" "	XM,At	+	+	1.9×10^{7}	
" "	XM,At	-	+	3.9×10^{7}	
" "	NGL	-	+	1.0×10^{4}	
" "	NGL	+	+	2.7×10^{6}	
					$1.2 \pm 0.8 \times 10^{7}$
NFR/N	XM,At[f]	+	+	3.0×10^{5}	
" "	XM,At	+++	+	4.1×10^{6}	
" "	XM,At	++	+	2.4×10^{7}	
" "	XM,At[f]	++	+	6.7×10^{5}	
" "	died during trial			not applicable	
					$7.2 \pm 5.6 \times 10^{6}$

[a]Mice were maintained on oral antibiotics incorporated in the drinking water as described in Materials and Methods.

[b]Macroscopic cecal pathologic changes were recorded as NGL, no gross lesions, XM, excess intraluminal mucus, and At, cecal atrophy.

[c]Dark field microscopy was performed on cecal swabs and subjectively scored on an ascending scale from +; very few spirochetes observed, to +++++; predominantly spirochetes observed.

[d]Colonization factor is expressed as the number of hemolytic colony forming units of T. hyodysenteriae per gram of cecum.

[e]If cecal culture results were positive and the pour plate counts were zero, an arbitrary value of 1×10^{4} was assigned.

[f]Macroscopic signs noted in the cecal apex only.

Table 4. Pathological and Bacteriological Findings from Mice Sacrificed 20 Days PI

Strain	Macroa	DF[b]	Culture	CF[c]	Group Mean
BALB/c	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
					0.0
DBA/1J	NGL	+	+	2.8×10^6	
" "	NGL	-	+	1.0×10^4[d]	
" "	NGL	-	-	0.0	
" "	NGL	-	-	0.0	
" "	XM,At	+	+	1.3×10^8	
					$2.7 \pm 2.6 \times 10^7$
NFR/N	NGL	-	+	3.2×10^5	
" "	XM,At[e]	+	+	3.1×10^6	
" "	XM,At[e]	+	+	1.0×10^4	
" "	XM,At	++	+	3.0×10^7	
" "	NGL	-	+	6.9×10^6	
					$8.1 \pm 5.6 \times 10^6$

[a]Macroscopic cecal pathologic changes were recorded as NGL, no gross lesions, XM, excess intraluminal mucus, and At, cecal atrophy.

[b]Dark field microscopy was performed on cecal swabs and subjectively scored on an ascending scale from +; very few spirochetes observed, to +++++; predominately spirochetes observed.

[c]Colonization factor is expressed as the number of hemolytic colony forming units of T. hyodysenteriae per gram of cecum.

[d]If cecal culture results were positive and the pour plate counts were zero, an arbitrary value of 1×10^4 was assigned.

[e]Macroscopic signs noted in the cecal apex only.

Discussion

The results obtained in this experiment show no significant pathological or bacteriological difference between the H-2q "high responder strain" NFR/N mice and the H-2 "lower responder" DBA/1J mice following challenge with T. hyodysenteriae. The results do suggest that there was a difference between the H-2q strains and H-2d strains of mice.

The actual reason for the difference between the haplotypes cannot be ascertained from only this work. Differences in infection might relate to differences in cecal microflora/microenvironment, H-2 influenced immune response, or other reasons. This experiment demonstrates (presumably) that disrupting the microflora increases susceptibility to infection by finding that treated mice (incorporation of antibiotics in the drinking water for 3 days concurrent with the infection) renders mice of both haplotypes susceptible to infection. Antibiotic-resistant enteric pathogens may be more pathogenic after oral administration of selected antibiotics due to a decrease or shift in the enteric normal flora. In a disrupted flora situation, nutrients might be more available to the pathogen, attachment sites would be available, normal bacterial-elaborated

bacteriocins or volatile fatty acids (VFA) would be absent, and a normal pH of the lumen would not be present (3,4). It has been shown that mice are more susceptible to Salmonella typhimurium infection after oral treatment with streptomycin (3) and that this increased susceptibility may be due to changes in lumenal VFA and pH (4). Other work describing increased colonization of an enteric pathogen in a depleted cecal microflora system was performed by Lee et al. (5) in which mice were made susceptible to Campylobacter jejuni colonization by prior treatment with antibiotics per os.

The increased susceptibility of mice to T. hyodysenteriae infection after oral antibiotic treatment might be due to increased mucus binding sites or to the absence of a normal flora-produced bacteriostatic/cidal compound, such as a bacteriocin or VFA. One of the major virulence determinants of T. hyodysenteriae might be a mucus receptor, which, if the mucosal surface of the murine cecum is "cleared" of normal flora, may attach to the mucus ligand. Then, the spirochete may chemotactically traverse the mucus strands to the crypts (6), and establish disease. Those spirocheteo which do not immediately attach to the mucus are swept away by peristalsis and do not colonize the crypts. Alternatively, normally produced VFA might be absent,

thus allowing the spirochete to reproduce. Indeed, it has been shown (7) that bacterial-induced diarrhea could be inhibited by administration of VFA, and that *in vitro* growth of *T. hyodysenteriae* is inhibited by millimolar quantities of proprionic and butyric acid (8).

The most significant finding in this experiment was that pretreatment with oral antibiotics rendered all murine strains susceptible to *T. hyodysenteriae* infection. More work is warranted to determine what factor(s) in the normal cecal microenvirons are responsible for protection against *T. hyodysenteriae* infection and/or induction of pathologic lesions.

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APPENDIX 3

MOUSE STRAIN CHALLENGE STUDIES

Purpose of the Experiment. C3H/HeJ strain mice have been reported to be hyporesponsive to the physiological effects of bacterial lipopolysaccharide (LPS) (1,2). It has been suggested, since challenged C3H/HeJ mice exhibited little or no pathologic signs subsequent to T. hyodysenteriae infection, that the LPS molety of Treponema hyodysenteriae is a virulence attribute (3). Our laboratory has challenged C3H/HeJ strain mice with T. hyodysenteriae and has obtained culture results and macroscopic pathologic signs consistent with those of challenged LPS-responsive C3H/HeN mice. Therefore, this experiment was designed to investigate biological significance of LPS responsiveness and its relationship to disease following infection with T. hyodysenteriae.

Materials and Methods

Animals. C3H/HeJ, C3H/HeSn, and C3HeB/FeJ strains of mice were purchased from Jackson Laboratories, Bar Harbor, Maine. C3H/HeN and C3H/HeJ mice maintained at Iowa State University were also used. Animals were fed Mouse Lab Chow #5010 (Purina Mills, Inc., St. Louis, Missouri) and sterile water ad libitum in the Laboratory Animal Resource Facility of the College of Veterinary Medicine, Iowa State University.

Bacteria. T. hyodysenteriae strain B204 (11 passages in vitro) was grown anaerobically in Trypticase Soy Broth (BBL Microbiological Systems, Cockeysville, Maryland) supplemented with 5% horse serum (HyClone Laboratories, Logan, Utah) and 5% yeast extract (BBL). Highly motile log phase cultures were examined by dark field microscopy, enumerated (Petroff-Hauser counting chamber) and diluted to 1×10^7 organisms/ml.

Protocol. Five groups of mice were used: (1) "Domestic" C3H/HeN strain of mice (i.e., C3H/HeN mice derived from colonies maintained at the Laboratory Animal Resource Facility), (2) "Domestic" C3H/HeJ mice, (3) "New" C3H/HeJ mice (i.e., C3H/HeJ mice purchased directly

from Jackson Laboratories), (4) C3HeB/FeJ mice, and (5) C3H/HeSn mice. Mice were challenged with 1 ml of broth culture containing 1 x 10^7 T. hyodysenteriae on two consecutive days. Mice were limit fed (1 gm feed/mouse/day) during these two days. Mice were sacrificed at 10 and 15 days post infection (PI). Criteria used to evaluate challenge were macroscopic lesions in the ceca, bacteriological culture results of cecal contents, and enumeration of the number of colony forming units (CFU) of T. hyodysenteriae in the infected ceca.

Necropsy Procedure. Macroscopic cecal changes were noted as excess intraluminal mucus and/or atrophy. The ceca of each mouse strain group were pooled into two pools, weighed in a sterile WhirlPak bag (Baxter Co., Minneapolis, Minn.), diluted 1:100 w/v with phosphate-buffered saline, and homogenized in a Stomacher Laboratory Blender (A. J. Seward Co., London, U.K.). The homogenates were further diluted and added to molten (45 degrees C) Trypticase Soy Agar tubes supplemented with 5% ovine blood and antibiotics (4), and pour-plated into 60 mm Petri dishes (#25010, Corning Glass Works, Corning, N.Y.) and incubated anaerobically at 37 degrees C for 96

hours. The zones of hemolysis were enumerated and the number of T. hyodysenteriae colony forming units per gram of cecal tissue was calculated. In this manner two bacterial count values were obtained for each mouse strain (each mouse strain consisting of 4 or 5 individual mice) at each of the two necropsy dates.

Results

Table 1. Macroscopic Lesions and Numbers of *T. hyodysenteriae* Per Gram Cecum at 10 Days PI

Group	Culture	Macro[a]	CFU/g cecum[b]
Domestic C3H/HeN	+	XM	
" "	+	XM,At	3.4×10^8
" "	+	XM	
" "	+	XM,At	7.4×10^7
	group mean		$2.1 \pm 1.3 \times 10^8$
Domestic C3H/HeJ	+	XM	
" "	+	XM,At	7.9×10^6
" "	+	XM,At	
" "	+	At	9.1×10^7
	group mean		$4.9 \pm 4.2 \times 10^7$
New C3H/HeJ	+	At	
" "	+	XM,At	5.4×10^7
" "	+	At	
" "	+	XM,At	
" "	+	NGL	2.9×10^7
	group mean		$3.9 \pm 0.6 \times 10^7$
C3HeB/FeJ	+	XM,At	
" "	+	XM,At	2.4×10^8
" "	+	XM	
" "	+	NGL	
" "	+	NGL	2.6×10^7
	group mean		$1.1 \pm 0.5 \times 10^8$
C3H/HeSn	+	XM,At	
" "	+	NGL	5.4×10^6
" "	+	XM,At	
" "	+	NGL	
" "	+	XM	1.7×10^8
	group mean		$1.0 \pm 0.4 \times 10^8$

[a]Macroscopic cecal lesions were: XM, excess intraluminal mucus; At, atrophy of the cecum; and NGL, no gross lesions.

[b]*T. hyodysenteriae* colony forming units were determined as outlined in Materials and Methods.

Table 2. Macroscopic Lesions and Numbers of T. hyodysenteriae Per Gram Cecum at 15 DPI

Group	Culture	Macro[a]	CFU/g cecum[b]
Domestic C3H/HeN	+	XM,At	
" "	+	XM,At	7.0×10^7
" "	+	NGL	
" "	+	XM	
" "	+	XM,At	3.2×10^7
	group mean		$4.7 \pm 0.9 \times 10^7$
Domestic C3H/HeJ	+	XM	
" "	+	XM,At	3.4×10^7
" "	+	XM	
" "	-	NGL	
" "	-	NGL	4.6×10^6
	group mean		$1.6 \pm 0.7 \times 10^7$
New C3H/HeJ	+	NGL	
" "	+	NGL	1.0×10^4[c]
" "	+	NGL	
" "	+	XM	
" "	+	XM	5.9×10^5
	group mean		$3.5 \pm 1.4 \times 10^5$
C3Heb/FeJ	-	NGL	
" "	-	NGL	
" "	+	NGL	3.0×10^6
" "	+	XM,At	
" "	+	XM,At	3.0×10^8
	group mean		$1.2 \pm 0.7 \times 10^8$
C3H/HeSn	+	NGL	
" "	-	NGL	0
" "	-	NGL	
" "	+	XM,At	9.6×10^7
	group mean		$4.8 \pm 4.8 \times 10^7$

[a]Macroscopic lesions in the ceca were recorded as: XM, excess intraluminal mucus; At, atrophy of the cecum; and NGL, no gross lesions observed.

[b]T. hyodysenteriae colony forming units were determined as descibed in Materials and Methods.

[c]Ceca pools which were culture positive but did not exhibit plate counts were assigned a value of 1×10^4 CFU/g cecum.

Discussion

The results of this trial show that the C3H/HeJ mice are capable of exhibiting macroscopic pathologic changes subsequent to infection with T. hyodysenteriae. Similarities existed between "domestic" C3H/HeJ mice and "new" C3H/HeJ mice in terms of colonization and of macroscopic cecal changes. The C3H/HeJ mice did tend to have less cecal colonization, by slightly less than a $\log_{10}$ factor than the C3H/HeN, C3HeB/FeJ, and the C3H/HeSn inbred strains of mice.

The question as to whether treponemal LPS is involved in the pathogenesis of the disease has not been answered in this work. Other researchers (3) reported the C3H/HeJ mouse (LPS hyporesponsive) to be refractory to T. hyodysenteriae challenge. Our research indicates that C3H/HeJ mice are indeed susceptible to infection with T. hyodyseteriae. Positive results in this trial (i.e., if the C3H/HeJ mice did not exhibit lesions) would have supported the theory of the LPS being a virulence determinant; however, these results certainly do not exclude the LPS as a potential virulence determinant. Certainly, as the LPS antigen is very strain specific and is the basis for the serotyping of the organism (5), it

would be logical to assume the LPS molety might be a protective epitope.

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APPENDIX 4

INVOLVEMENT OF THE *ity* GENE IN THE PATHOGENESIS OF *Treponema hyodysenteriae* IN THE MURINE MODEL

Purpose of the Experiment. Various genes have been described in inbred mice that affect the mouse strain's ability to resist an infection by *Salmonella* spp. These genes are the *ity* gene (1,2,3), the *xid* gene (4), and the *lps* gene (4,5). The *ity* gene is a single autosomal gene which affects the murine response to *Salmonella typhimurium* infection (6) and therefore renders some inbred strains resistant to *S. typhimurium* invasion (ity^r) and some sensitive (ity^s). The inbred mouse strains characterized as ity^r are the A/J, CBA, and C3H/He (6). The strains which are ity^s are C57Bl/6J and BALB/c (6).

Treponema hyodysenteriae-induced lesions in the mouse was studied by comparing challenge susceptibility of ity^s (C57Bl/6J) and ity^r (C3H/HeN) inbred mouse strains. In previous murine challenge studies, actual invasion beneath the healthy mucosal epithelium of the mouse cecum was not observed, but invasion of goblet cells and between epithelial cells was noted (personal

observation). The purpose of this trial was two-fold: (1) to investigate the influence of the *ity* gene on pathogenesis in the mouse model after challenge with virulent *Treponema hyodysenteriae*, and (2) to titrate the minimal infective dose of *Treponema hyodysenteriae* in the murine model.

Materials and Methods

Animals. C3H/HeN strain mice were obtained from Harlan Sprague Dawley, Indianapolis, Indiana. C57Bl/6J mice were kindly provided by Dr. C.J. Warner, Department of Biochemistry, Iowa State University. Animals were maintained on Mouse Lab Chow #5010 (Purina Mills, Inc., St. Louis, Missouri) under controlled conditions in the Laboratory Animal Resource Facility of the College of Veterinary Medicine, Iowa State University. Mice were 6 to 8 weeks of age.

Protocol. Each strain of mouse was divided into four groups containing 6 to 8 mice per group. Mice in each group received two oral challenges of T. hyodysenteriae 24 hours apart at one of the following doses: 10^8, 10^7, 10^6, or 10^5 treponemes in 1.0 ml media. Mice were fasted for 36 hours during the challenge period. All mice were sacrificed at 10 days post infection (PI) to ascertain colonization and induction of lesions in the different groups.

Bacteria. Treponema hyodysenteriae strain B204 (serotype 2) was grown anaerobically in Trypticase Soy Broth (BBL Microbiological Systems, Cockeysville,

Maryland) supplemented with 5% horse serum (HyClone Laboratories, Logan, Utah) and 5% yeast extract (BBL). Highly motile cultures were examined by dark field microscopy, enumerated (Petroff-Hauser counting chamber), and diluted to the appropriate concentration in sterile culture media.

Necropsy Procedure. All mice were necropsied at 10 days post infection. Macroscopic changes in the cecum were noted as excess intraluminal mucus and/or atrophy; the ceca were aseptically removed and weighed in a sterile WhirlPak bag (Baxter Co., Minneapolis, Minn.), suspended 1:100 w/v in phophate-buffered saline, and homogenized in a Stomacher Laboratory Blender (A. J. Seward Co., London, U. K.). The homogenate was streaked onto BJ blood agar media (1) for the isolation of T. hyodysenteriae and examined by darkfield microscopy for observation of spirochetes. In addition, serial ten-fold dilutions of the homogenate were added to molten Trypticase Soy Agar tubes supplemented with 5% ovine blood and antibiotics (1) and were then pour-plated into 60 mm Petri dishes (#25010, Corning Glass Works, Corning, N. Y.) and incubated anaerobically at 37 degrees C for 96 hours. The zones of hemolysis were enumerated and the

number of T. hyodysenteriae colony forming units per gram of cecal tissue was calculated.

Table 1. Determination of the T. hyodysenteriae infective dose for C3H/HeN mice (ity^r)

Strain	Dose	Macro[a]	DF[b]	Culture	CFU[c]
C3H/HeN	10^8	XM,At	+++	+	2.3×10^8
" "	" "	XM,At	+++++	+	2.6×10^7
" "	" "	NGL	-	-	0.0
" "	" "	NGL	-	+	1.0×10^{4d}
" "	" "	XM,At	+++	+	5.8×10^7
" "	" "	XM,At	++	+	1.1×10^7
" "	" "	XM,At	++	+	2.8×10^5
				Group mean	$4.6 +/- 3.2 \times 10^7$
C3H/HeN	10^7	NGL	-	+	1.0×10^{4d}
" "	" "	NGL	-	-	0.0
" "	" "	XM,At	+++	+	4.3×10^8
" "	" "	XM,At	+++++	+	5.7×10^6
" "	" "	NGL	-	-	0.0
" "	" "	XM,At	++	+	6.9×10^7
				Group mean	$8.4 +/- 7.0 \times 10^7$
C3H/HeN	10^6	XM,At	+++	+	3.0×10^6
" "	" "	NGL	-	+	1.0×10^{4d}
" "	" "	XM,At	+++	+	1.9×10^7
" "	" "	XM,At	++	+	1.4×10^7
" "	" "	XM,At	++++	+	3.5×10^7
" "	" "	XM,At	++++	+	2.0×10^8
				Group mean	$4.5 +/- 3.1 \times 10^7$
C3H/HeN	10^5	NGL	+	+	5.6×10^7
" "	" "	NGL	-	+	1.0×10^{4d}
" "	" "	NGL	-	+	2.0×10^6
" "	" "	XM,At	+++	+	3.7×10^7
" "	" "	XM,At	+++	+	1.0×10^9
" "	" "	NGL	+	+	2.2×10^5
				Group mean	$1.8 +/- 1.6 \times 10^8$

[a]Macroscopic cecal lesions were recorded as: XM, excess mucus; At, atrophy; and NGL, no gross lesions.

[b]Dark field spirochete values (1=low, 5=high).

[c]Colony forming units per gram of cecal tissue.

[d]If the sample was culture positive but plate count negative, a value of 1×10^4 was assigned.

Table 2. Determination of the T. hyodysenteriae infective dose for C57/Bl6J mice (lty^S)

Strain	Dose	Macro[a]	DF[b]	Culture	CFU[c]
C57Bl/6J	10^8	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	XM	++	+	1.4×10^5
"	" " "	NGL	-	-	0.0
"	" " "	XM	+	+	1.0×10^{4d}
				Group mean	$2.0 +/- 2.0 \times 10^4$
C57Bl/6J	10^7	XM,At	++	+	1.3×10^8
"	" " "	NGL	-	+	1.1×10^4
"	" " "	NGL	-	+	6.1×10^5
"	" " "	XM,At	+++	+	5.0×10^7
"	" " "	NGL	-	+	1.0×10^{4d}
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
				Group mean	$2.5 +/- 1.9 \times 10^7$
C57Bl/6J	10^6	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
C57Bl/6J	10^5	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0
"	" " "	NGL	-	-	0.0

[a]Macroscopic cecal lesions were recorded as: XM, excess mucus; At, atrophy; and NGL, no gross lesions.

[b]Dark field spirochete values (1=low, 5=high).

[c]Colony forming units per gram of cecal tissue.

[d]If the sample was culture positive but plate count negative, a value of 1×10^4 was assigned.

Discussion

Two questions were investigated in this study: the maximally infective challenge dose of T. hyodysenteriae, and whether the ity gene influenced susceptibility of mice to challenge with T. hyodysenteriae. In the C3H/HeN mouse strain, a productive infection was noted with all doses examined (10^5 through 10^8 bacteria per challenge). The results indicate that, in the C3H/HeN mice, the challenge dose of 10^5 was as infective as 10^8, but less macroscopic lesions were noted with the smaller dose (Table 1). In contrast, the C57Bl/6J strain of mouse was not as susceptible to establishment of infection as the C3H/HeN mice, showing no evidence of lesions with doses of 10^6 and 10^5 spirochetes (Table 2).

A difference in susceptibility exists between these two inbred mouse strains. However, the C3H/HeN mouse is classified as ity^r, and the C57Bl/6J is ity^s, thus not supporting the involvement of the ity gene in the pathogenesis of T. hyodysenteriae in the mouse model. Many types of immune responses and pathogen susceptibilities have been shown to be based on genotype in the mouse (i.e., H-2, Ity, Lsh, and Lps). However, we have been unable to demonstrate any genetic basis for the developmment of lesions by Treponema hyodysenteriae; on

the contrary, *ity*^{s}-C57/Bl/6J mice were less susceptible to virulent challenge than the *ity*^{r} C3H/HeN mice. Inbred murine differences of *Trichinella spiralis* shedding and of H-2 haplotype (7) also have little influence on susceptibility to *Treponema hyodysenteriae* challenge.

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