Experimental infection of neonatal lambs with Samonella arizonae
by Rebecca Walker Mahurin

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Veterinary Science
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Abstract:
Pregnant ewes were vaccinated twice subcutaneously with bacterins of Salmonella arizonae, Salmonella oranienburq, or left as unvaccinated controls. Two day old lambs from these ewes were orally challenged with 10 10 viable homologous bacteria. Lambs from ewes which were vaccinated with Eu arizonae. were protected from the mild diarrhea seen in control lambs. Lambs from S. oranienburq vaccinated dams were not protected from severe diarrhea and dehydration seen in control lambs. Upon necropsy at 2 days PI both challenged groups were seen by light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to have necrotic enterocytes, loss of microvilli, PMN infiltration, mucosal and submucosal edema, edematous mitochondria and granular endoplasmic reticulum and separation of intercellular tight junctions in the duodenum, ileum, and colon. Lesions were, more severe and extensive in S. oranienburq challenged lambs. Enterotoxins produced by Eu oranienburq and S. arizonae were not neutralized in the ligated intestinal segment (LIS) test in lambs from vaccinated dams. Ultrastructurally, lesions in samples of uninoculated and inoculated loops of the LIS tested animals were indistinguishable.

EXPERIMENTAL INFECTION OF NEONATAL LAMBS WITH *SALMONELLA ARIZONAE*

by

REBECCA WALKER MAHURIN

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of the requirements for the degree

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Approved:

Lyle L. Myers
Chairman, Graduate Committee

David M. Young
Head, Major Department

Michael Malone
Graduate Dean

MONTANA STATE UNIVERSITY
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ABSTRACT

Pregnant ewes were vaccinated twice subcutaneously with bacterins of Salmonella arizonae, Salmonella oranienburg, or left as unvaccinated controls. Two day old lambs from these ewes were orally challenged with 10^{10} viable homologous bacteria. Lambs from ewes which were vaccinated with S. arizonae were protected from the mild diarrhea seen in control lambs. Lambs from S. oranienburg vaccinated dams were not protected from severe diarrhea and dehydration seen in control lambs. Upon necropsy at 2 days PI both challenged groups were seen by light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to have necrotic enterocytes, loss of microvilli, PMN infiltration, mucosal and submucosal edema, edematous mitochondria and granular endoplasmic reticulum and separation of intercellular tight junctions in the duodenum, ileum, and colon. Lesions were more severe and extensive in S. oranienburg challenged lambs. Enterotoxins produced by S. oranienburg and S. arizonae were not neutralized in the ligated intestinal segment (LIS) test in lambs from vaccinated dams. Ultrastructurally, lesions in samples of uninoculated and inoculated loops of the LIS tested animals were indistinguishable.

CHAPTER 1

INTRODUCTION

Neonatal diarrhea in lambs is an economically important disease in many areas including Idaho and Montana. Infectious agents associated with this syndrome include *Salmonella* sp., *Clostridium perfringens*, *Cryptosporidium*, *Escherichia coli*, rotavirus, and coronavirus. Management practices also influence the occurrence and severity of the disease.

Salmonella arizonae serotype 26:30 was isolated from diarrheic lambs on 6 of 12 ranches in Idaho (97). This organism was later used at this laboratory to experimentally challenge lambs from the endemic area (97). *S. arizonae* failed to produce any significant disease in that study. Other reports have indicated that *S. arizonae* may induce enteritis, abortion, and septicemia in sheep (96, 77, 141, 201, 89, 97).

The purposes of this study were:

1. To evaluate the clinical response in neonatal lambs to experimental challenge with *S. arizonae*.
2. To evaluate protection afforded by dam vaccination with a *S. arizonae* bacterin.
3. To evaluate histologically and ultrastructurally the lesions induced by *S. arizonae* and to compare these to lesions induced by *S. oranienburg*.

4. To evaluate lesions caused by *Salmonella* in the ligated intestinal segment test and to determine if colostral antibody neutralizes the enterotoxin of *S. arizonae* in this test.

CHAPTER 2

LITERATURE REVIEW

Salmonella

The genus Salmonella is in the Enterobacteriaceae family. Morphologically, Salmonella are small rods (2-3 u by 0.4 to 0.6 u), which are motile by means of peritrichous flagella. Salmonella produce acid and gas from glucose, maltose, sorbitol, and mannitol. They do not utilize lactose, salicin, or sucrose. Arginine, lysine, and ornithine decarboxylases are produced. Salmonella fails to liquefy gelatin, produce urease, or utilize malonate.

Salmonella possess H flagellar antigens and the somatic O antigen. The Vi antigens are possessed by S. typhi and S. paratyphi, but are not possessed by the species used in this study.

Salmonella grow well on enrichment media such as gram negative broth or tetrathionate.

Salmonella arizonae

The first isolation of an organism now classified as an arizona was made by Caldwell and Ryerson in 1939. The organisms identified as Salmonella sp. (Dar - es - Salaam type, variety from Arizona) were isolated from reptiles, Gila monsters, and chuckawalla. Kauffmann designated it as Salmonella arizona in 1941 (122). This classification was based on the close relationship of its H antigens to those of

Salmonella. It is also recognized to be biochemically similar to the Salmonella genus (179, 67).

The name Arizona arizonae was proposed by Kauffman and Edwards (123). Means were listed by which Salmonella and Arizona could be differentiated. The Judicial Commission of the International Committee on Nomenclature of Bacteria ruled the generic name Arizona invalid (65). Ewing (64) published under that generic name later. Subsequently the species name of arizonae was found invalid. The species name hinshawii was introduced to honor William Hinshaw who pioneered much work in the Arizona genus (66). This organism is now listed as S. arizonae in the Index Bergeyana (25).

Salmonella arizonae is a recognized etiologic agent in diarrhea of humans, poultry, and other animals (16, 17, 18, 19, 145, 54, 88). Reports have also linked S. arizonae 26:29-30 to diseases in sheep. S. arizonae has been suspected of causing abortions or still-births in ewes (96, 141, 201, 89). The time of abortion may vary from premature to full term (201). A genital discharge may accompany the vaginal infection in ewes (201). S. arizonae could be isolated from various tissues of the lamb carcass (141). One report of bovine abortions is also linked with S. arizonae (77). Ovine septicemia may be caused by S. arizonae (141, 24, 89). Gastroenteritis in sheep has also been linked to S. arizonae (48, 89, 97). Harp, et al (97)

found that S. arizonae was the most commonly isolated potential pathogen from cases of undiagnosed gastroenteritis in lambs.

S. arizonae produces enterotoxin and is invasive (97, 141). The lamb gut loop test, used to detect the production of enterotoxin, appeared to be neutralized in lambs from vaccinated mothers (97). Experimental challenge with S. arizonae failed to produce diarrhea in 22 unvaccinated lambs (97).

Salmonella arizonae 26:29-30 has been isolated from sheep abattoirs (98). It may be contracted by consuming contaminated feed as it has been isolated from oats in a feeder (96).

S. arizonae infections in humans exhibit a wide range of clinical symptoms including gastroenteritis (161, 3, 55, 181, 185), enteric fever (55, 210), septicemia (55, 113), bone and joint disease (130, 91, 74, 135, 215), hepatic abscess (55, 135), endocarditis (133), infection of atherosclerotic blood vessels (181), meningitis (56), pneumonia (113), and otitis media (3).

Humans may come in contact with S. arizonae through the consumption of contaminated food (161, 91) and association with reptiles (109).

Salmonella oranienburg

Salmonella oranienburg was first isolated from acute gastroenteritis in a children's home in Oranienburg, Germany. Kauffman

reported this isolation in 1930 (25, 52, 144). S. oranienburg isolates have been reported from chickens, turkeys, cattle, sheep, swine, horses, and reptiles (16, 17, 18, 19, 108, 50, 160, 44, 229). S. oranienburg has also been isolated from slaughter houses and feed-stuffs (16, 17, 18, 19, 50). S. oranienburg is one of the 5 most common blood isolates of Salmonella from humans (20, 203). It is the ninth most frequently isolated Salmonella species in cattle (17).

In experimentally infected lambs, S. oranienburg is invasive (97) and has been reported to cause severe diarrhea and dehydration. S. oranienburg has also been isolated from 1 ewe with naturally occurring diarrhea (78).

Salmonellosis in Sheep

Neonatal diarrhea of lambs has many etiologic agents and contributing factors. Clostridium perfringens, E. coli, coccidia, corona viruses, Cryptosporidium, and rotaviruses all have been identified as causative agents (8, 225, 211, 246, 187, 220, 189, 195, 147, 224, 250, 249, 251, 268, 168, 252, 221). Management practices may also contribute to the severity and spread of diarrhea in lambs (182).

Salmonella may cause both diarrhea and abortion in sheep. One of the first known incidents of salmonellosis in sheep was reported in Colorado in 1924. The outbreak involved a loss of 6.2% in sheep which had been shipped by rail from Montana to Fort Collins.

Clinical signs included gauntness, dullness and weak appearance. A thin watery diarrhea with an offensive odor was noted. On postmortem an inflammation of the abomasum and small intestines was observed. The organism was isolated in this outbreak and the disease reproduced in fasting lambs (167).

Diarrhea in sheep associated with Salmonella has occurred in New Zealand, Australia, India, Scotland, England, Wales, Cyprus, and Tanzania (189, 117, 172, 105, 229, 44, 45, 206, 90, 69, 42, 120). Clinical signs of the disease include diarrhea, abortion, metritis, dullness, thirst, fever, anorexia, pneumonia, and progressive weakness (6, 105, 212, 7, 111, 261, 255, 173, 22, 68, 146).

The most common Salmonella species involved in ovine disease are S. dublin, S. abortusovis, and S. typhimurium, (150, 229, 189, 44, 45, 69, 117, 105, 226). Postmortem lesions seen in salmonellosis include gastroenteritis, enteritis, lymphadenitis, and colitis, (6, 206, 105). In experimental infection of sheep with S. typhimurium, lymphadenitis is seen at 24 hours post-infection (PI); ileal and caecal inflammation is noted by 48 hours; and severe ileal duodenal inflammation is seen between 5 and 8 days PI (6).

The age at which Salmonella diarrhea may occur varies from the neonate to the adult sheep (90, 206, 105, 150, 189, 69).

The source of Salmonella in disease states may vary from contaminated water, pasture, rodent vectors, abattoirs, feces or hides of

animals, or bovine or ovine carriers (116, 243, 69, 117, 206, 218, 56, 104, 214, 219, 119, 266, 191, 230, 192). Studies indicate that as many as 4% of sheep may carry and intermittently excrete Salmonella (125, 119). The mesenteric lymph nodes are suspected of being the reservoir for Salmonella (157).

Salmonella dublin has been reported to be resistant to streptomycin and sulphonamides in sheep (260, 228, 259, 227). Use of spectinomycin and sulfadimethoxine has been effective in combating Salmonella in lambs in Idaho (214). Combinations of trimethoprim and sulphanomide and trimethoprim and sulphadiazine are also effective (263, 227).

Colostral Immunity

It has long been recognized that young animals are protected from disease by factors in the milk of dams. The first milk or colostrum is of major importance in this protection. IgA and other humoral components have received most of the credit for colostral immunity. Only recently have cellular elements of colostrum been given recognition.

In cattle and sheep the major colostral immunoglobulin is IgG rather than IgA, as in humans (138). The manner in which colostral immunoglobulins protect the young is only speculation. Antibodies may cause aggregation of microorganisms, thus preventing adherence, or act as opsonins (129, 84, 257). Finally, antibodies may neutralize

enterotoxins of gram negative bacteria and subsequently protect without acting directly on the bacteria (233).

It has been noted that mammary secretions contain a high percentage of immunoglobulins specific for intestinal pathogens. This may be a consequence of a selective migration of sensitized lymphocytes to the mammary system from the intestines (83). This could have practical application for oral immunization.

Cellular immunity in colostrum was first suspected when it was noted that children breast fed by tuberculin sensitive mothers exhibit similar sensitivity (155). Children not breast fed by their tuberculin sensitive mothers lack this sensitivity. Attention was then given to cellular elements of colostrum and observations such as enhancement of bactericidal capacity of macrophages were noted. Ability of leukocytes to migrate (155), phagocytosis and killing (100), and mitogenicity (47) indicated that cells present in colostrum were not merely dead sloughed cells, but viable, immunocompetent cells.

Colostral immunity may be assessed in a number of ways. Royal, Robinson and Nuganzich noted that fewer Salmonella organisms were excreted by calves that received colostrum than by those that did not receive colostrum (199).

Colostral immunity is the basis for dam vaccination studies. Dam vaccination against enteric diseases in neonates has met with some success. E. coli vaccines are now proving to be valuable tools

against colibacillosis (166, 165, 162, 163).

Immunization Against Salmonella

Vaccination against salmonellosis has been a controversial subject. Killed vaccines have been reported to be ineffective (21). Live vaccines cannot be utilized in combination with antibiotics (200).

Wallace and Murch described vaccination of adult sheep using a killed vaccine adjuvanted with alum (258). The bivalent vaccine produced increases in O and H antibody titers. Losses in control sheep were 100 times more than in the twice vaccinated sheep.

Beckett also prepared a killed vaccine for use in natural outbreaks of salmonellosis in sheep (12). The bivalent vaccine, administered subcutaneously, achieved protection 14 days post-vaccination. He also claimed that vaccination of a portion of the herd protected unvaccinated animals. Dewes (46) noted this occurrence in horses, cattle, and pigs. Fewer Salmonella were isolated from the environment. A killed bivalent vaccine has proven effective in sheep in New Zealand (40).

Hunter and Peck successfully utilized a killed vaccine of S. typhimurium to protect against salmonellosis in cattle (105).

A more complicated problem of Salmonella vaccination involves young animals. It has been observed that calves do not produce an active response to vaccination until about the fourth week of life. This causes a problem where neonatal immunity is necessary.

Smith (216) produced limited protection in young calves using an attenuated vaccine of S. dublin.

Porter, Kenworthy, and Thompson (186) described oral immunization with S. dublin, and S. typhimurium of young calves as a possible means of preventing salmonellosis. Preparations of both E. coli and Salmonella antigens from killed cells were mixed with milk. The calves receiving this mixture were observed to have fewer cases of diarrhea and significantly higher weight gains.

Cameron and Fuls (32) also tried vaccinating young calves against salmonellosis using a S. dublin preparation. Both live and inactivated vaccines were developed. Calves were vaccinated at approximately two weeks of age, then challenged intraduodenally two weeks post-vaccination. Calves given two doses of a Salmonella bacterin were solidly immune. No increase in titer nor serum neutralization in mice could be detected.

Husband (106) attempted another approach to protection of young animals against salmonellosis. He keyed on the observation by Pierce and Gowans (182) that in rats intestinal IgA can be effectively stimulated by a two vaccination regime involving a systemic injection of antigen followed by an oral booster. Lambs were administered a primary injection of formalin killed S. typhimuruim either subcutaneously (SC) or intraperitoneally (IP) followed by an oral booster. Challenged control lambs exhibited severe weight loss, diarrhea,

fever, and 70% mortality. Vaccinated animals showed significantly less diarrhea, bacteremia, and weight loss. These authors theorize that systemic injection may cause seeding of the Peyer's patches with sensitized precursors of IgA which actively secrete IgA following local challenge.

Husband (107) further studied neonatal immunity in lambs by prenatally priming lambs with S. typhimurium in a laparotomy exposed uterus. Oral boosting of neonates thus produced protected animals.

Rankin and Taylor (188) utilized dam vaccination with S. dublin to protect calves. Serum and colostral antibody titer exhibited a rise; however, no difference in death rate or survival time was noted. The 2 calves from dams with highest colostral titers failed to become bacteremic.

Composition of Salmonella Vaccines

Various forms of Salmonella have been utilized for vaccination purposes. Initially, killed vaccines were tested for stimulation of immunity with little success (118, 216, and 21). Botes (21) believed his work indicated that a live vaccine was superior. A comparison of these two ideas was performed in rats by Cameron and Fuls (32). A killed virulent strain of Salmonella was compared to the efficacy of a living rough mutant. The results of this work showed that a formalin killed vaccine was effective in producing protection. Live avirulent forms gave varying degrees of protection. Retention of

some virulence by the organism seemed to be necessary for production of immunity. Totally avirulent mutants were unsuccessful as vaccines. Similar findings were noted in calves (32).

An alternative to using viable cells in a Salmonella vaccine may be in hybrids of Salmonella and other organisms. Kiefer, Gransow, Schmidt, and Westphal (132) manipulated genetic transfer of Salmonella O antigens to E. coli to produce a vaccine tested in mice. Mice receiving this vaccine were protected against intraperitoneal challenge. In further studies, an avirulent S. typhi vaccine was compared to an E. coli-S. typhi hybrid (49). The hybrid vaccine was not as effective as the attenuated S. typhi. Also, it was found that addition of Vi antigens to somatic antigen in the hybrid had no added affect. Thus no role for protection could be determined for this factor.

Hohmann, Schmidt, and Rowley (101) tested an E. coli-Salmonella hybrid by oral inoculation. This route proved to be only weakly immunogenic in contrast to a similar hybrid given IP which gave good antibody responses. This strain of E. coli is not invasive, thus the hybrid strain probably did not reach the lymphoid tissues to stimulate an antibody response as does the parent Salmonella.

Another approach to vaccination involves a "ribosomal" preparation of Salmonella. Studies have shown that cellular extract rich in ribosomal material induced some protection against

challenge (253, 4). The protective factor may be protein (114, 115). Misfeldt and Johnson's study (154) indicated that the actual immunogen was ribosomal protein and the ribonucleic acid portion acted as adjuvant. Angerman and Eisenstein (4) showed that ribosomal vaccines are equivalent to acetone killed cells in immunogenicity and, in addition, that longer protection is provided. The ribosomal preparation was not pure and debate has ensued over the possibility of a contaminant actually being the immunogen.

Eisenstein (57) presented evidence that the O antigens were actually the immunogenic principle. He used isogenic bacteria differing only in one O component, noting that cross protection should occur if the protective factor was actually the ribosomal portion. Cross protection was not seen. Also, he saw that O antibodies were present in the antisera to ribosomal vaccines. Finally, a ribosomal vaccine prepared from an O deficient mutant did not protect. Other workers have also found the O antigen to be an important immunogen (142, 194, 170, 236).

Misfeldt and Johnson (153) found that endotoxin contamination could contribute to the protective effect of ribosomal vaccines.

Immune Mechanisms

The basis for immunity to Salmonella has been a controversial subject for many years. Some proclaim that cellular immunity is

the sole mechanism for protection (143, 144, 36) while others argue that humoral immunity is an integral part of the immunity produced against Salmonella (198, 112, 129).

Essential differences in vaccine preparations are known to cause different types of immunity. Adjuvanted attenuated vaccines or living cells were shown to produce both cellular and humoral responses (37, 129), whereas killed vaccines are believed to produce primarily humoral aspects of immunity (99, 235). RNA vaccines are believed to induce only cellular immunity (254).

Angerman and Eisenstein noted that agglutination titers corresponded to protection afforded by ribosomal vaccines. These authors (4) showed that ribosomal vaccines do elicit an anti-O response which can be correlated with protection. They observed that even though the antibody responses to acetone killed cells and ribosomal vaccines are similar, the protectiveness afforded by the ribosomal vaccine is superior. They suggest two possible explanations for this. First, that cellular immunity is contributing to protection or, secondly, that the ribosomal vaccines elicit the formation of different classes of antibody, slightly different antibody specificities, affinity, or avidity.

The nature of the antibody response to Salmonella is dependent on the type of antigen used (75). The antibody response to the O antigen of Salmonella yields primarily an IgM response. On the

other hand LPS induced a high level of IgG, which occurs later in the progress of antibody production. It is also noted that whole gram positive organisms are more likely to elicit IgG than are gram negative organisms.

Fisher, Martinez, Trainin and Meirom (73) studied the immunoglobulin response to salmonellosis in neonatal calves. This study noted that survival of the calf seemed to hinge on the antibody response. Calves with high antibody titers were affected by Salmonella but had a much greater chance of survival. The protection was correlated to IgM. Observations to support this include the fact that increased fecal IgM was noted in surviving calves and a depletion of IgM in dying calves.

The other main school of thought regarding immunity to Salmonella infection deals with cellular resistence. The supporters of this theory state that serum antibodies, even though produced, are not effective in protection against salmonellosis (39, 152, 143, 144). Some contend that activated macrophages are the basis of Salmonella immunity. Venneman and Berry (254) found that the level of humoral antibodies did not correlate with protection.

An attempt to measure the cellular response to Salmomella was made by Cameron and Rensburg (31). The inhibition of macrophage migration test is an accepted means of quantitating cellular immunity. This method was used to assess CMI against S. dublin

and S. typhimurium. Cross reactions were noted to the 2 organisms. However, too much individual variation was exhibited to allow definitive conclusions.

In addition, work has been done to elucidate the specific T and B lymphocyte responses in salmonellosis (76). The spleen evidenced the greatest response to Salmonella infection with an increase in both B and T lymphocytes. A few receptors for Salmonella were noted on these lymphocytes.

Recent work indicated that possibly both the cellular and humoral theories of Salmonella immunity are correct. Melendez, Gonsalez, Reid, Fuentes, and Castillo (151) supported the view that a combination of the two mechanisms were operative. They observed that only immune serum plus immune peritoneal cells protected mice against Salmonella. Thus they concluded that humoral immunity is an integral part of the overall Salmonella immunity. This is supported by Zinkernagel (269). These authors believed that opsonic properties of antibodies are one of the important contributions of the humoral factor.

As salmonellosis is a disease of the gastrointestinal tract, a third aspect of immunity, the mucosal defense system, must be considered. It is known that irradiated mice have little or no defense against infections (15). However, when comparing oral, IV, IP, and aerogenic challenges of irradiated mice with Salmonella, it was noted that mice challenged orally were more resistant to infection (38).

This difference is interpreted to reflect a local intestinal defense system which is not as severely altered as systemic defense.

Mucosal defense is largely dependent on secretory immunoglobulins. The 11 S form of IgA is locally synthesized in the gut rather than being of serum origin (248, 35). Peyer's patches have been shown to be an enriched source for IgA producing plasma cells (41). Occasionally the predominant class of immunoglobulin in secretion is either IgM or IgG (196, 2).

The mechanism by which IgA protects is only speculation. Bacteriolysis of organisms is facilitated by IgA with the help of complement and lysozyme (2). A second possibility for action of IgA is the alternate pathway of complement fixation (60). Thirdly, IgA probably is an active opsonin in the intestines (34, 262). Finally, IgA may inhibit adsorption to the mucosa (267). Bacterial toxins may be inhibited by secretory antibody (232).

Enterotoxins

The first indication that disease producing toxins existed was in 1864 when Koch suggested that cholera was not caused directly by an organism, but rather by a substance elaborated by the organism (11). This suggestion was not pursued for 75 years until De and Chatterje (11) confirmed that crude broth culture supernatants of the vibrio were capable of inducing fluid secretion in ligated ileal loops.

Bacterial enterotoxins are defined as "a form of exotoxin elaborated by intact bacteria into broth cultures - the release of enterotoxin depending on outward diffusion or transport through the bacterial cell wall and not on cell lysis" (11). Enterotoxins definitively cause fluid and electrolyte secretion into the small intestine.

E. coli enterotoxins have been the subject of many detailed studies. These enterotoxins are involved in diarrhea of children, travelers' diarrhea (197), and neonatal diarrhea of domestic animals (217). Two distinct enterotoxins have been isolated from E. coli. The heat labile toxin (LT) is known to cause porcine diarrhea. It is labile at 60°C/30 minutes; it has a molecular weight of around 35,000. This fraction is active in skin tests and causes Chinese hamster ovary cell elongation (92). It is believed to bind with GM ganglioside receptors in the intestinal tract and to activate adenyl cyclase (63).

The stable toxin (ST) of E. coli is reported to cause bovine neonatal diarrhea. This toxin has a molecular weight of between 1000-10,000 (29). It is stable to both acid and alkaline conditions (110) whereas LT is not (62). Stable toxin is not destroyed by pronase, which differs from LT (110).

Giannella investigated the possible role of enterotoxin in human S. typhimurium infections. He concluded from negative ligated

rabbit ileal loop tests that this organism lacked an enterotoxin. Further, he stated that "only strains which invade the ileal epithelium cause enteritis or fluid secretion" (82).

In 1961 Taylor and Wilkins (245) reported that some strains of Salmonella did cause fluid accumulation in ligated intestinal loops. The possible elaboration of an enterotoxin was not pursued further until 1974 when Sakajaki et al (202) discovered that culture filtrates from two strains of S. typhimurium, two strains of S. enteriditis, two strains of S. thompson and one strain each of S. heidelberg, S. derby, S. krefeld, S. sofia, and S. arizonae caused distention in the rabbit ileal loop test. These authors theorized that the pathogenesis of Salmonella gastroenteritis was similar to that caused by E. coli enterotoxins.

Koupal and Deibel (134) reported finding enterotoxin produced by S. enteriditis. This factor was assayed in both the ileal loop and Dean's suckling mouse assay. The Dean test was modified for Salmonella by shortening the four hour incubation period to 2.5 hours.

It was noted that the culture supernatant fluid consistently gave the best results in the mouse assay as compared to whole cultures or washed cells (134). The toxin is believed to be associated with the outer cell membrane and is eluted to some degree into the culture supernatant. It is characterized by a high molecular weight with

stability in both acid and alkaline conditions. Freezing seemed to decrease the toxic activity. Evidence such as sensitivity to pronase and heat lability at 80°C/30 minutes indicates that there is a protein moiety necessary for the toxic activity.

Sedlock, Koupal, and Deibel performed later studies on S. typhimurium and also isolated an enterotoxin from this organism. The stability of the toxin was studied. It was found that Salmonella enterotoxin (SE) was stable at low temperatures, but some decrease in activity occurred in the process of freezing. Lyophilization was also noted to retain stability of the toxin. The molecular weight was determined to be above 30,000 as the toxic activity was retained by a PM 30 membrane. No further conclusions regarding size were made as the toxic factor was not eluted within a narrow range by G-100 column chromatography (208). This may be similar to the heterogeneity seen with both LT and ST of E. coli.

Sedlock and Deibel devised a modification of the rabbit ligated ileal loop test for use with Salmonella species (209). The rabbit test had given variable results in the past. In attempting to find a means by which to make the test more sensitive and consistent, mucolytic wash solutions were utilized prior to inoculation of loops with toxin. This increased the consistency of the test significantly. Thus, the authors concluded that mucus in some way inhibited the action of SE on the small intestines. One possible way by which this

could occur is inhibition of activity by binding of mucin to the toxin and thus blocking the reaction with the epithelial cells. This has been reported to occur with cholera toxin (234). The other possibility is that the intestinal mucin merely acts as a barrier through which toxin, without the aid of a motile organism, cannot penetrate. This could explain why it has been reported that Salmonella does not elicit an enterotoxin but rather requires organisms which are invasive to induce fluid secretion (82). With the modification of the rabbit ileal loop test, Sedlock and Deibel reported an increased response rate from 30% to near that seen when whole cultures of Salmonella are used.

That enterotoxin production is a relatively common occurrence among Salmonella is the more recent conclusion (209). Salmonella weltevreden has also been reported to produce an enterotoxin (247). Some exceptions were noted using the modified ileal loop test. These being S. dublin, S. pullorum, S. gallinarum, and S. typhi.

Other studies have found factors which may be synonymous with the enterotoxic factor noted by Koupal and Deibel. One such work by Plant, Glynn and Wilson (184) cited a supernatant factor (SF) from S. typhimurium, with characteristics similar to the previously described SE. The SF was found to be at least partially protein in nature as the activity of the substance diminished upon treatment with pronase. Differences included the ability to pass through a

PM 30 membrane and heat stability at 100°C for 1 hour. It is unclear why the factor seems to be protein but is not inactivated by heat. Koupal and Deibel (134) noted that some enterotoxin did pass through a PM 30 membrane, but that the majority of the enterotoxin was in the retentate. Whether this fraction has enterotoxin activity is not known. The SF was used to immunize mice against challenge by homologous organisms. Protection was noted against challenge by S. typhimurium, but no protection was seen against heterologous organisms. SF elicits a delayed hypersensitivity as judged by foot pad swelling in mice.

Two permeability factors (PF) have been isolated from S. typhimurium (204). The identification of these factors had implications concerning toxin association. One PF of Salmonella acts rapidly to increase skin permeability with a maximal response time of about two hours. This rapid PF, which is heat stable at 100°C for four hours, causes no induration. The slow acting PF causes induration at approximately 18 hours after injection. This factor, on the other hand, is heat labile at 75°C for 30 minutes. Both PF's have an estimated molecular weight of near 90,000. The slow PF is not detected unless the supernatant fluid is concentrated or partially purified. This is theorized to be due to an inhibitor which is present in the culture. The slow PF cause reactions such as elongation of Chinese hamster ovary cells and alteration of skin permeability,

which cannot be distinguished from that of cholera toxin.

Sandefur and Peterson (205) further pursued the relationship of SE with cholera toxin. The delayed PF caused elongation in Chinese hamster ovary cells similar to CE. In addition, anti-cholera toxin sera neutralized the effect of delayed PF. This indicated a close relationship between the two toxins. On the other hand Donta and Smith did not find Salmonella supernatant fluid to be active in Y-1 adrenal cells (51).

Additional studies (180) have been done to further characterize the delayed PF of Salmonella. It was found that PF was inactivated at pH below 4 and above 10. It is resistant to degradation by intestinal enzymes. It is also believed to share the same binding sites as cholera toxin because procholeragenoid, a precursor of cholera toxin, blocks the binding of delayed PF. In addition, procholeragenoid eliminates the normally positive ileal loop response to PF, thus indicating that this factor is involved in the toxic process of fluid secretion in the small intestines. Finally, these authors noted that the production of this factor by Salmonella is not stable as organisms were observed to lose this ability over time.

Other outer membrane proteins which may or may not have any relation to the enterotoxin of Salmonella have been studied. Kussi et al (27) noted that a preparation of outer membrane proteins called porins were effective in inducing immunity against subsequent

challenge with S. typhimurium. These proteins were of over 30,000 molecular weight as was the toxin preparation. Another similarity is resistance to proteolytic enzymes. These proteins did not cause an increased virulence as judged by the LD_{50} of S. typhimurium.

Detection of Enterotoxins

Studies on enterotoxin hinge on the ability to detect the presence of such factors. Intact animals were first used. Oral inoculation of infant rabbits was one method used in early work. This was followed by use of the ligated ileal loop test in rabbits (27, 121) in which tied segments of intestines are inoculated with bacteria or supernatant fluid. The fluid accumulation and subsequent distention which occurs indicates enterotoxic activity. The degree of toxicity is determined by the length to fluid ratio of the segment after an 18 hour incubation period. Calf ileal loop assays have been utilized as well as pig, cat, guinea pig, and lamb (70, 217, 164).

The rabbit ileal loop technique has also been adapted for use in detection and quantitation of antitoxin (27, 121). This involves incubation of a known amount of toxin with sera for one hour followed by inoculation into rabbit ileal loops. This titration procedure may be applied to use in animal sera.

In vitro systems of enterotoxin study have also been utilized. Adrenal cells in culture become rounded and undergo steroidogenesis when exposed to LT or CT (51).

Isolated fat cells are another in vitro system similar to adrenal cell cultures. These cells obtained from digestion of rat epididymal fat pads release glycerol and free fatty acids into the medium when stimulated. A spectrophometer may be used to quantitate the response (11).

Chinese Hamster ovary cells undergo elongation when stimulated by cholera toxin (169). Increased adhesion is also a noted change in these cell cultures.

Speirs, Stavric and Konowalchuk adapted the VERO cell line for use in determining the presence of enterotoxin. This continuous cell line of green monkey kidney cells showed distinct morphological changes upon exposure to enterotoxin in that they became enlarged, thick walled, and refractile with filamentous tendrils. VERO cells have advantages over other cell cultures in that they can be quickly cultured and require little maintenance (231).

A faster and more economic test was developed for ST enterotoxin detection by Dean et al. He used gastrically inoculated infant mice and noted fluid accumulation after a four hour incubation period. Quantitation of response was performed by weighing the intestines of the mice and determining a ratio of gut to body weight. Ratios of 0.09 or greater were considered positive, 0.07-.09 questionable, and less than 0.07 negative (43). Applicability of this test for bovine and porcine strains of Escherichia coli were studied (59).

The test was consistent and sensitive in determining ST enterotoxin production.

A pooling of possible enterotoxic E. coli has been found successful in reducing number of specimens run in the Dean test (28). As many as 5 supernatant solutions may be combined and tested to save time and mice.

This test was modified by Moon et al (158) for further simplification. This involves inoculating the 3-5 day old mice orally using a blue dye with the test solution. The mice are placed on white filter paper and held 4 hours at 37°C. After 4 hours the number of blue spots on the filter paper indicate the degree of diarrhea. An average of one or more spots/mouse indicates enterotoxic activity.

Limitations to the infant mouse test have been noted by Gyles (93). He observed, as had other investigators, (26) that there are possibly 2 ST of E. coli. He found that porcine E. coli produce an enterotoxin which is not detected in either the infant mouse test or the rabbit gut loop, but only in the pig gut loop. Thus he suggests that strains suspected of producing an enterotoxin, which give negative results in the mouse test, be placed in the pig LIS.

A comparison of various enterotoxin detection tests have been accomplished (131). The most sensitive test was found to be the skin permeability test. For ST the suckling mouse test is also

sensitive. The Chinese hamster ovary elongation and rabbit ileal loop tests were found to be lacking in enterotoxin sensitivity.

Pathogenesis of Salmonella Diarrhea

E. coli and Vibrio cholerae induce diarrhea via an enterotoxin without invasion of tissues (190). With Salmonella, however, an invasive property may be a prerequisite to diarrhea (82, 86, 79). Fluid secretion has been associated only with invasive strains of Salmonella typhimurium (82). This study further categorized the pathogenesis of salmonellosis into 3 steps "(a) ability to penetrate the wall of the gastrointestinal tract; (b) ability to cause diarrhea; and (c) the ability to disseminate from the intestine and multiply and survive within the reticuloendothelial system". Salmonella attach to the villus tips and may actually invade only 5% of the villi (82). Furthermore, not all invasive Salmonella are capable of eliciting diarrhea (82). Lack of pili, flagella, nor O antigen seem to impair the invasiveness of S. typhimurium (244). HeLa cells have been used as a model for Salmonella invasiveness (81).

Giannella (79) was unable to demonstrate an enterotoxin from S. typhimurium. He did, however, note that adenyl cyclase activity was activated in a Salmonella diarrhea (85). This same system was stimulated by both cholera toxin and enterotoxin of E. coli (190). An acute inflammatory reaction may actually mediate the secretion of fluids into the intestines (80). Giannella further speculated that

prostaglandins synthesized in the inflammatory reaction, activated the adenyl cyclase system (79).

The electrolyte movement in salmonellosis is characterized by decreased sodium absorption and increased chloride secretion (79). Fluid is then secreted in the small intestine due to the new osmotic gradient. Finally, the ability of the colon to resorb this excess fluid is impaired in the disease. Thus, salmonellosis affects the function of the small and large intestine (79). In addition, a loss of potassium increases the acidosis of the blood (87). It is reported in calves that no significant increase in hematocrit nor decreases in blood volume occur in diarrhea due to Salmonella (73).

Histology and Ultrastructure

Takeuchi performed both light and scanning electron microscopical studies on S. typhimurium infection of opium injected, starved guinea pigs. He noted that at 12 hours post-infection (PI) organisms had penetrated into intestinal epithelial cells and, in some cases, had passed between epithelial cells. The junctional complexes regained integrity as the intercellular organisms moved deeper (238).

The close approach of Salmonella to the brush border triggered the degeneration of the border and terminal web. The bacterium then entered a cleft or cavity of the cell which formed ahead of it. The apical plasma membrane of the cell reformed behind the Salmonella as the organism, in its membrane bound vacuole, was surrounded by the

cell (238).

Further studies described cellular damage to guinea pig intestine by S. typhimurium (239). At 24 hour PI, the epithelium had become low columnar or cuboidal with a shortened brush border. By 48 hour PI, cellular alterations such as dilated endoplasmic reticulum, reduction of free ribosomes, and loss of microvilli were evident. Intravascular clotting and vascular endothelial cell damage were also evident by 48 hour PI. At 48 hour PI some bacteria enclosed in phagocytes in the lamina propria appeared to be intact, whereas others were degenerating.

In a similar study of salmonellosis it was noted that epithelial cell mitosis was increased and the rate of movement of enterocytes up the villi was more rapid in infected animals (1).

The histological changes which occur in sheep following dosing with S. typhimurium include early acute enteritis, infiltration of inflammatory cells into the basal lamina, typhlitis, focal necrosis of mesenteric nodes and kidney, fatty changes in the liver, and thickening of the alveolar septa in the lung (171).

Salmonella diarrhea in pigs results in shortened, broad and fused villi, loss of intestinal epithelium, and complete atrophy of villi. Lesions outside the digestive tract include septal thickening in alveolae of the lung, hepatomegaly, cardiac petechial hemorrhages, and mesenteric lymphadenitis (264, 139, 265, 9).

Lesions seen in cases of E. coli diarrhea include stunting of villi, focal degeneration of epithelial cells, with exposure of lamina propria, inflammation, and degeneration of brush border (94, 159, 178, 149, 177, 242, 13).

Lesions in swine dysentery caused by Treponema include loss of groups of intact epithelial cells, irregularity of villi, swollen mitochondria, dilated endoplasmic reticulum, and intracellular edema (127, 126). Lesions in bacillary dysentery are similar, including fusion of villi, shortening of villi, pronounced emptying of goblet cells, swollen mitochondria, dilation of endoplasmic reticulum and Golgi, and loss of epithelial cells (240). In contrast, the intestinal ultrastructure of cholera infected animals exhibited little damage. The epithelial cells and junctional complexes remained intact with primarily normal organelles (58). Damage evoked by Clostridium necretans in piglets is characterized by progressive necrosis of the small intestinal epithelium (256). Cryptosporidial infection in lambs also results in similar lesions, these include villous atrophy, villous fusion, and inflammation (5).

Viral enteric infections resulted in much the same lesions. Calves and pigs infected with coronavirus, transmissable gastroenteritis virus, rotavirus, and astrovirus all exhibited villous atrophy, villous fusion, replacement of enterocytes by cuboidal epithelium, sloughing of epithelia, and dilation of

endoplasmic reticulum and Golgi apparatus (223, 241, 222, 221, 102, 53, 148).

It has been reported that such lesions as the ones previously mentioned may be due to postmortem artifact. Samples taken while under anesthesia may be preferable to those taken postmortem (175, 174).

CHAPTER 3

MATERIALS AND METHODS

The experimental design for the sheep study is outlined in Table 1.

Vaccine Preparation and Use

Tryptic soy broth (TSB) was separately inoculated with *Salmonella arizonae* and *Salmonella oranienburg* and incubated aerobically at 37°C for 24 hours. The cells were killed by the addition of formalin to a final concentration of 0.4% followed by a similar 24 hour incubation. After chilling for 24 hours the cells were centrifuged at 8000 x g for 20 minutes. The pellet was resuspended in 1/20 the original volume of formalinized physiological saline solution (0.4% formalin). A 3% aluminum hydroxide adjuvant was added in a ratio of 1 part (by volume) adjuvant to 2 parts cells, achieving a final concentration of 15 mg of dried cells/ml of vaccine.

Ewes were separately vaccinated subcutaneously 2 and 4 weeks prior to lambing with a 2 ml dose of either *S. arizonae* or *S. oranienburg* vaccine. Some ewes were kept as unvaccinated controls.

Challenge Inoculation

An overnight growth of *S. oranienburg* or *S. arizonae* in tryptic soy broth was placed in a bulb syringe for oral inoculation of two-day-old lambs. Fifty ml of *S. arizonae* growth or 10 ml of *S. oranienburg*, both containing approximately 10^{10} colony forming units

per ml, was used as the challenge dose.

TABLE 1

EXPERIMENTAL DESIGN OF SHEEP STUDY

Pen No.	Grp. No.	No. of ewes	Treatment of ewes	Treatment of lambs
1	1	8	2 vaccinations with killed S. arizonae	Challenge with viable S. arizonae
1	2	8	Unvaccinated	Challenge with viable S. arizonae
2	3	8	2 vaccinations with killed S. oranienburg	Challenge with viable S. oranienburg
2	4	8	Unvaccinated	Challenge with viable S. oranienburg
3	5	6	2 vaccinations with killed S. arizonae	Gut loop test lambs with various Salmonellas
3	6	6	2 vaccinations with killed S. oranienburg	Gut loop test lambs with various Salmonellas
3	7	14	Unvaccinated	Gut loop 6 lambs Necropsy other lambs for controls

Isolation of Organisms

Two fecal swabs were taken from each lamb 0, 24, and 48 hours post- challenge. Later fecal swabs were taken at random. One swab was frozen for use in viral determination by electron microscopy.

The second swab was immediately streaked onto Tergitol-7 (T-7) and Salmonella arizonae (140) agar the following day. Suspected Salmonella colonies were identified biochemically using triple sugar iron agar (TSI), lysine iron agar (LIA), motility indole ornithine (MIO), urea, and ONPG. All colonies which tested as Salmonella were checked against rabbit antisera to S. arizonae and S. oranienburg.

Antisera Production

Rabbits were given 5 intravenous injections of 2.2 mg cells/ml of formalin killed S. arizonae or S. oranienburg at intervals of four days. The initial injection was 0.5 cc with the second and all subsequent injections of 1 cc. Rabbits were bled by heart puncture on the sixth day after the final injection. Blood was centrifuged at 2000 x g for 20 minutes to separate the serum. Serum was frozen for later use.

Colostral Titers

Colostrum was collected from each ewe, in most cases before the lamb nursed. Centrifugation at 100,000 x g for 3 hours separated the clear whey which was used in the plate agglutination test. Doubling dilutions of whey were made with saline and tested for O, H titers to both S. arizonae and S. oranienburg. The antigen for the agglutination test was prepared by first passing S. oranienburg and S. arizonae twice through semi-solid media to select for highly

motile strains. TSB was inoculated with the resulting organisms. Twenty-four hour aerobic cultures were formalin killed and centrifuged at 8,000 x g for 20 minutes and resuspended in 1/20 the original volume of formalinized saline (0.4%).

Lamb Gut Loop

The lamb gut loop test was performed essentially as described by Firehammer and Myers (71). Lambs 5 to 18 days of age were anesthetized using a preanethesia of 0.2 ml of Xylazine [1] followed by a 1% solution of sodium thiamylal [2]. Forty to 50 loops were tied in each lamb. Only odd numbered loops were inoculated. Even numbered loops were left uninoculated to insure that loops were not affected by adjacent ones. Three tenths ml of overnight broth cultures of viable S. oranienburg, S. arizonae, S. dublin, and S. typhimurium were individually injected into separate loops. Escherichia coli strain B41 was inoculated into the anterior and posterior loops as a positive control. Sixteen to 18 hours after surgery lambs were euthanatized using T-61 (appendix). Loops were assigned a value from 0 to 4 with 0 being no fluid accumulation and 4 indicating a tightly distended loop.

Tissue samples were taken at this time for both light and

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[1] Haver-Lockhart Laboratories, Shawnee Mission, Kansas

[2] Parke, Davis & Co., Detroit, Michigan

electron microscopy. From each lamb a sample of small intestine was taken anterior to the ligated area of intestine, from an uninoculated loop and from a loop inoculated with strain B41. Tissue samples were taken from other selected positive loops.

Necropsy

At 2 to 4 days post-challenge selected lambs were administered T-61 and necropsied. Blood samples were checked for bacterial growth. Stomach, intestines, liver, lung, colon, spleen, and mesenteric lymph node were cultured for bacteria. Tissue or contents from these areas were aspirated with a Pasteur pipette and placed onto T-7 and Salmonella arizonae agar. Colonies which resembled Salmonella were identified biochemically.

Samples from each of these tissues were also collected for light and electron microscopy. Small pieces were removed from the lambs, placed on paraffin blocks, and immediately covered with fixative. Samples were then minced with a razor blade and transferred to vials of fixative.

Light Microscopy

Tissues were placed in 10% buffered neutral formalin. Tissues were embedded in paraffin, cut at 5 um, stained with hematoxylin and eosin, and examined with a Leitz microscope and photographed using a Zeiss standard universal microscope.

Scanning Electron Microscopy (SEM)

Tissues were cut into 3 mm cubes and placed in 4% formaldehyde/1% glutaraldehyde fixative in phosphate buffer at pH 7.2. After a minimum of 24 hours the tissues were washed with distilled water and taken through a step dehydration with 30, 50, 70, 90, and 100% ethanol. Specimens were immediately placed in amyl acetate and critical point dried. Tissues were glued to a copper holder using colloidal graphite and then sputter coated with gold.

Specimens were examined using a JEOL 100 CX with an ASID scanning attachment at 40 KV.

Preparation of Salmonella for SEM

Sterile strips of filter paper were placed in tubes of TSB which were subsequently inoculated with either S. arizonae or S. oranienburg. Following overnight incubation, the filter paper was removed and placed in a test tube with 4% formaldehyde/1% glutaraldehyde fixative for 24 hours. The filter paper was air dried then treated as other specimens for SEM.

Transmission Electron Microscopy (TEM)

Tissues from lung, stomach, small intestines, colon, liver, spleen, and mesenteric lymph node of necropsied lambs were cut into 1 mm cubes and placed in 4% formaldehyde/1% glutaraldehyde fixative in phosphate buffer at pH 7.2. The tissues were washed three times, 15 minutes each, in cacodylate buffer. A two hour post-fixation

in 2% OsO_4 was followed by another wash in buffer. Stepwise dehydration in 50, 70, 95, and 100% ethanol preceded infiltration with Spurr's epoxy resin. Polymerization was accomplished at 70°C for 15 hours in size 00 BEEM capsules.

Blocks of tissue from duodenum, ileum, and colon were sectioned using glass knives on a Sorvall 5000 ultramicrotome. Sections of 50-100 nm were placed on 3 mm copper grids of 300 mesh. Sections were stained for 5 minutes in uranyl acetate and then 5 minutes in Reynold's lead citrate. A JEOL 100 CX transmission electron microscope at 100 KV was utilized to view the specimens.

Negative Staining for Virus Detection

The method of Ritchie and Fernelius (193) was used to detect viruses in lamb fecal samples. Fecal swabs were stirred in a spot well containing 1 ml of distilled water. One drop of this suspension was then placed in another spot well and diluted with 1 ml of distilled water. Two drops of 4% phosphotungstic acid and two drops of 0.3% bovine serum albumin were added. This solution was then mixed with a Pasteur pipette and placed in an all-glass nebulizer [3]. A mist of the suspension dried on a carbon coated formvar filmed grid was immediately examined by transmission microscopy at 100 KV.

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[3] Ted Pella, Inc., Tustin, California

CHAPTER 4

RESULTS

Isolation of Salmonella from Challenge Inoculated Lambs

S. arizonae was first isolated from fecal specimens of control lambs, an average of 1 day PI (Table 1). Fecal samples from 3 lambs with *S. arizonae* vaccinated dams were positive for *S. arizonae* an average of 2 days PI. *S. arizonae* was not detected in feces of 2 lambs in this group following challenge. At the last sampling, 20 days PI, 1 lamb from a control ewe had a fecal sample positive for *S. arizonae* and 1 lamb from a vaccinated ewe was positive 27 days PI.

S. oranienburg was isolated from feces of control lambs (Table 2) an average of one day, PI. Fecal samples from lambs with *S. oranienburg* vaccinated dams were positive an average of 2 days PI. At the last sampling a control lamb showed a positive sample 9 days PI and a lamb from an *S. oranienburg* vaccinated ewe was positive 11 days PI.

O, H Colostral Antibody Titers

The geometric mean (GMT) O, H colostral antibody titer to *S. arizonae* from 26 control ewes was 7 (Table 3). The GMT of O, H antibody to *S. arizonae* from 12 ewes vaccinated with *S. arizonae* was 2400. The difference was highly significant ($P < 0.01$). Individual colostral antibody titers from control and *S. arizonae*

TABLE 2. Lambs Challenged with *S. arizonae*

Lamb No.	Days PI of positive fecal sample	Clinical signs	Organs positive for *S. arizonae*
Dam vaccinated with *S. arizonae*			
5	4 - 27	None	Colon, blood spleen, duodenum
9	never positive	None	NS
10	2	Diarrhea 2 days PI	NS
11	never positive	None	NS
61	1	None	NS
69	2	None	Small intestine, stomach
Nonvaccinated dam			
16	2	Diarrhea, weak	NS
17	1 - 2	Mild diarrhea	NS
22	1	Mild diarrhea	NS
23	never positive	Mild diarrhea	NS
33	1	Mild diarrhea	NS
34	2 - 20	Mild diarrhea	NS
53	1	Diarrhea, 39.7°C	Lung, small intestine, colon
54	1	Diarrhea, 40°C	Small intestine, lung
68	3	Mild diarrhea	NS
71	1	None	NS

NS = Not Sampled

TABLE 3. Lambs Challenged with *S. oranienburg*

Lamb No.	Days PI of Positive Fecal Sample	Clinical Signs	Organ from which *S. oranienburg* was isolated
Dam vaccinated with *S. oranienburg*			
8	2 - 3	None	NS
19	3	None	NS
26	1	Diarrhea	Spleen, lung, liver, mesenteric lymph nodes
28	1 - 11	Diarrhea, 40°C	NS
32	2	Diarrhea, weak, 40.6°C	NS
38	1 - 5	Diarrhea, weak, 41°C	NS
39	1 - 2	Diarrhea, weak, 40.9°C	Liver, mesenteric lymph nodes, ileum
52	2 - 3	None	NS
Nonvaccinated Dam			
12	2 - 5	Diarrhea	NS
13	1	Diarrhea, weak	Liver, ileum, stomach, colon, spleen, blood, mesenteric lymph nodes
18	1 - 4	Diarrhea	
36	1 - 2	Diarrhea, weak, 40.5°C	Spleen, lung, ileum colon, mesenteric lymph node
37	2 - 9	Diarrhea, weak, 41°C	NS

NS = Not Sampled

TABLE 4. Geometric Means of Colostral O, H Agglutinating Antibody Titers

No. of Ewes	Treatment	O, H Titer to *S. arizonae*	O, H Titer to *S. oranienburg*
26	Unvaccinated	7	1
12	*S. arizonae* vaccinated	2400 [1]	2
10	*S. oranienburg* vaccinated	5	2692 [2]

[1] Significantly higher than O, H colostral antibody titers to *S. arizonae* in unvaccinated group. ($P < 0.01$)

[2] Significantly higher than O, H colostral antibody titers to *S. oranienburg* in unvaccinated group ($P < 0.01$)

vaccinated ewes are listed in Tables 5 and 6.

The GMT of O, H colostral antibody to *S. oranienburg* in 26 unvaccinated control ewes was 1. The GMT of colostral antibody to *S. oranienburg* in 10 *S. oranienburg* vaccinated ewes was 2692. The difference was highly significant ($p < 0.01$). Individual colostral antibody titers from control and *S. oranienburg* vaccinated ewes are listed in Tables 5 and 7.

Those sheep vaccinated with *S. oranienburg* had a GMT to *S. arizonae* of 5. The ewes vaccinated with *S. arizonae* had a GMT to *S. oranienburg* of 2. These values did not differ from the corresponding control titers ($p < 0.01$).

TABLE 5. O, H Colostral Agglutinating Antibody Titers In Unvaccinated Ewes

Ewe No.	Titer to *S. arizonae*	Titer to *S. oranienburg*
WF, NT	16	0
16	32	0
15	8	0
101	4	0
102	16	0
17	4	0
103	8	0
54	0	0
41	0	0
22	0	0
55	0	0
40	4	0
36	0	0
30	32	512
49	128	0
3	16	0
52	128	0
8	0	0
38	0	0
46	0	0
19	512	0
9	32	0
20	64	0
34	2	0
13	8	0
11	0	0

O, H Serum Antibody Titers

Blood was taken from some lambs at necropsy. The GMT to *S. arizonae* in lambs from unvaccinated mothers was 4 (Table 8). The

TABLE 6. O, H Colostral Agglutinating Antibody Titers In Ewes Vaccinated With S. arizonae

Ewe No.	Titer to S. arizonae	Titer to S. oranienburg
56	4096	0
31	4096	0
29	2048	0
23	4096	0
53	4096	0
63	2048	0
9	4096	0
5	2048	0
24	2048	64
39	4096	64
89	256	0
44	2048	0

TABLE 7. O, H Colostral Agglutinating Antibody Titers In Ewes Vaccinated With S. oranienburg

Ewe No.	Titer to S. arizonae	Titer to S. oranienburg
1	32	2048
2	8	4096
48	0	4096
42	0	1024
37	0	2048
35	16	4096
50	128	4096
21	0	2048
4	8	2048
10	4	4096

GMT of serum antibody to S. arizonae in lambs from S. arizonae vaccinated ewes was 2600.

TABLE 8. Serum O, H Agglutinating Antibody Titers In Lambs

No. of Lambs	Treatment of Ewe	Geometric Mean of O, H Titer to S. arizonae
6	Unvaccinated	4
3	S. arizonae vaccinated	2600

Significant difference ($p < 0.05$)

Serum antibody titers of lambs are compared to dam colostral titers in Table 9.

Clinical Signs and Symptoms

In lambs challenged with S. arizonae, 90% (9 of 10) from unvaccinated ewes developed diarrhea for 2 or more consecutive days (Table 10). In contrast, only 20% (1 of 5) of the lambs from vaccinated sheep develop diarrhea. The diarrheic lambs exhibited temperatures as high as 40° C (Table 2) compared to a normal of 39.1° C (283). The diarrhea which followed infection with S. arizonae seemed to have little effect on appetite or alertness of the lambs. Rather it appeared as a mild, transient diarrhea lasting an average of 3 days. The onset of diarrhea in both groups appeared

TABLE 9. Colostral and Serum Agglutinating Antibody Titers

Lamb No.	Treatment of Dam	Dam O, H Colostral Titer		Lamb O, H Serum Titer		Days of Age Serum Sample Taken
		S. arizonae	*S. oranienburg*	*S. arizonae*	*S. oranienburg*	
7	Unvaccinated	2	0	8	0	10
13	Unvaccinated	8	2	0	0	2
14	Unvaccinated	2	0	4	0	9
53	Unvaccinated	16	0	128	0	5
54	Unvaccinated	2	0	0	0	3
59	Unvaccinated	0	0	0	0	11
1	*S. arizonae* vaccinated	1028	ND	2048	0	5
62	*S. arizonae* vaccinated	2048	0	4096	0	16
69	*S. arizonae* vaccinated	2048	0	2048	64	4
63	*S. oranienburg* vaccinated	0	4096	0	0	15

O, H serum antibody titers to *S. arizonae* of lambs from *S. arizonae* vaccinated dams were significantly higher than those of lambs from unvaccinated dams. ($p < 0.05$).

ND = Not determined

approximately 2 days PI.

Diarrhea developed in 5 control lambs and in 6 of 9 lambs from vaccinated dams following challenge with S. oranienburg (Table 9).

TABLE 10. Diarrheal Incidence In Challenge Lambs

No. of Lambs	Treatment of Ewe	Occurrence of Diarrhea In S. arizonae Challenged Lambs	Occurrence of Diarrhea in S. oranienburg Challenged Lambs
9	S. oranienburg vaccinated		6/9
5	Unvaccinated controls		5/5
5	S. arizonae vaccinated	1/5	
10	Unvaccinated control	9/10	

Incidence of diarrhea due to S. oranienburg in lambs from unvaccinated controls not significantly higher than in lambs from vaccinated ewes ($p < 0.25$).

Incidence of diarrhea due to S. arizonae is significantly higher in lambs from control ewes as compared to lambs from vaccinated ewes ($p < 0.05$).

The diarrhea of S. oranienburg etiology appeared clinically to be more severe and debilitating than did the diarrhea caused by S. arizonae. Fevers as high as 40° - 41° C were common, accompanied

by anorexia and listlessness (Table 3). Five of the diarrheic lambs in this group became moribund 3 to 4 days PI and were necropsied. Thus a mortality rate cannot be determined.

At necropsy S. oranienburg was isolated from stomach, ileum, colon, mesenteric lymph node, liver, spleen, and lung in lambs from both control and vaccinated ewes (Table 3). S. arizonae also proved to be invasive as it was isolated from stomach, duodenum, colon, spleen, blood, and lung in lambs from vaccinated and control ewes (Table 2).

One unchallenged lamb, in the pen with S. oranienburg infected lambs, developed diarrhea. This lamb from an S. oranienburg vaccinated ewe exhibited a temperature of 40.6° C. Upon necropsy S. oranienburg was isolated from ileum, colon, and spleen.

One lamb from an S. arizonae vaccinated mother was challenged with S. arizonae, but never developed diarrhea. Upon necropsy the organism could only be recovered from contents of the stomach and small intestine.

Lambs in the control pen, destined to be gut loop tested, were constantly observed for signs of diarrhea. One lamb exhibited slight diarrhea for one day. On culture, no Salmonella were isolated from the fecal sample.

Viral Identification

Negatively stained fecal samples from all lambs lacked any

evidence of viruses. This included normal lambs and lambs with diarrhea.

Ligated Intestinal Loop Test (LIS)

Five lambs each from S. oranienburg vaccinated ewes, S. arizonae vaccinated ewes, and control ewes were used in the LIS test. Only loops with enough accumulation of fluid to be graded 3 or 4 were considered positive. Only 8.7% (5 of 57) of S. arizonae inoculated loops were positive in lambs from ewes vaccinated with S. arizonae (Table 11). In lambs from control ewes 24.6% (15 of 61) of the loops inoculated with S. arizonae were positive. No significant difference was observed. In lambs from ewes vaccinated with S. oranienburg, S. arizonae was positive in 16% (7 of 42) of the loops. This was not statistically different from controls.

In the LIS test in lambs from S. oranienburg vaccinated dams, 6.6% (1 of 15) of the loops inoculated with S. oranienburg were positive (Table 11). In control lambs 25% (2 of 8) of the S. oranienburg loops were positive. No significant difference was noted. In lambs from S. arizonae vaccinates 77% (7 of 9) of the loops inoculated with S. oranienburg were positive. There is a highly significant difference between this group and the S. oranienburg vaccinated group ($p < 0.005$).

S. typhimurium was positive 79% (19 of 24) of the time in all lambs. Twenty eight percent (6 of 21) of the loops inoculated with

S. dublin were positive. Only one uninoculated loop in all lambs tested gave a positive score; however, 5.8% (17 of 290) of the uninoculated loops gave results which scored either 1 or 2.

TABLE 11. Gut Loop Reactions In Lambs

No. of Lambs	Treatment of Ewe	S. arizonae inoculated loops		S. oranienburg inoculated loops	
		Positive	Negative	Positive	Negative
5	S. arizonae vaccination	5	52	7	2
5	S. oranienburg vaccination	7	35	1	14
5	Unvaccinated control	15	46	2	6

Loops rated 3 or 4 were considered positive.

Loops rated 0, 1, 2 were considered negative.

No significant difference between positive S. arizonae inoculated loops in lambs from control and S. arizonae vaccinated ewes.

No significant difference between positive S. oranienburg inoculated loops in lambs from control and S. oranienburg vaccinated ewes.

Highly significant difference between positive S. oranienburg inoculated loops in lambs from S. arizonae vaccinated and S. oranienburg vaccinated ewes ($p < 0.005$).

Histology

Three control animals appeared normal with light microscopy. The duodenal villi were intact with normal appearing Brunner's glands present (Figure 1). The ileum consistently exhibited a few

leukocytes of the PMN type in the lamina propria, but otherwise appeared normal (Figure 2). Colonic samples exhibited intact epithelial cells and normal crypts (Figure 3). One control animal had a grossly visible hemorrhagic area on the liver surface. This was speculated to be due to injury rather than a disease process.

Three diarrheic lambs, which had been challenged with S. arizonae, were necropsied. The ileum and duodenum had similar lesions which included mucosal congestion, vacuolation of tips of villi with some desquamation of epithelial cells, elevated numbers of PMN's, and prominent goblet cells (Figures 4 and 5). The lumen had mucin-entrapped bacteria and cellular debris. The colon showed only mild damage with vacuolation and some mucosal congestion (Figure 6). The lungs exhibited lesions ranging from mild interstitial pneumonia to severe, focal pneumonic consolidation. The sinuses of mesenteric nodes were filled with mononuclear cells and PMN's. The spleen, liver, and stomach appeared normal.

In 5 S. oranienburg challenged animals with diarrhea the duodenum was more severely affected. Congested mucosa, dilated capillaries, excess mucus, and necrosis at villus tips with sloughing of mucosa were seen (Figure 7). The ileum showed similar lesions with vacuolation and cell loss at villus tips, PMN infiltration in lamina propria, subserosal hemorrhage, and

excess mucus production (Figure 8). The colon was inflamed (Figure 9). The stomach, liver, spleen, and lungs appeared normal. The mesenteric nodes had purulent lymphadenitis with monocyte filled sinuses.

Histologically intestinal samples from Salmonella inoculated loops in the LIS test were indistinguishable from uninoculated loops. Both exhibited necrosis of epithelial cells, edema of mucosa, serosa, and submucosa, and inflammation. Samples taken anterior from tied off areas appeared normal.

Ultrastructure by Scanning Electron Microscopy (SEM)

Samples of duodenum, ileum, and colon from lambs of the 3 treatment groups were examined by SEM.

Samples of duodenum from control lambs showed numerous villi, all with intact epithelium (Figure 10). Microvilli were present on cells and were uniform in appearance (Figure 11).

Duodenal samples from S. arizonae infected animals showed villi which had lost their epithelium (Figure 12). In addition, in some areas the underlying connective tissue had eroded (Figure 12). Microvilli were in various stages of degeneration (Figure 13). Microvilli often did not appear individually distinct, but matted together as in Figure 26.

Duodenal samples from S. oranienburg challenged lambs appeared more severely affected. Fewer intestinal villi were present.

Most villi lacked epithelium. Occasionally, epithelial cells were present on the lower half of villi similar to those seen in Figure 12. Microvilli present on intact cells were usually matted and indistinct. Much mucus and debris were present in the lumen. Macrophages could be found on villus epithelium (Figure 14).

Ileal samples from control lambs presented numerous villi with continuous, intact epithelium (Figure 15). Microvilli were all distinct and of a uniform size and shape. The lumen was clear of debris and mucus.

Samples of ileum taken from S. arizonae infected lambs showed necrosis of villi with cavities forming into the villi (Figure 16). Some villus tips were denuded of cells with the lamina propria exposed. Other villi had intact epithelium, but very active goblet cells were observed (Figure 17). Cells along the lateral portion of the villi were separating from each other and the basal lamina (Figure 18). These separating cells had microvilli which were in various stages of degeneration. In the later stages of cell separation microvilli were matted (Figure 19).

Ileal tissue removed from S. oranienburg challenged animals exhibited distorted villi. Villus tips were eroded (Figure 20). Cleavage and loss of epithelium was noted. Epithelial cells were separated from the basal lamina (Figure 21). Microvilli were present on intact epithelium (Figure 22). Mucus was present in the lumen.

Colonic samples from control animals showed definite rugae and glandular orifices (Figure 23). Goblet cells were numerous. Microvilli appeared uniform.

Samples of the colon from S. arizonae infected animals exhibited edema which often obscured the rugae. Goblet cells were numerous. Epithelial cells, with microvilli present, were separating from one another and from the lamina propria (Figure 24). Again, the S. oranienburg challenged lambs appeared more severely affected. Edema usually obscured the rugae. Separation of cells was seen between rugae (Figure 25). Matted microvilli were present (Figure 26). Mucus and bacteria were apparent in the lumen (Figure 27).

S. arizonae and S. oranienburg were viewed by SEM following overnight incubation of cultures. These bacteria are shown to possess flagella and attach to the filter paper on which they grew (Figures 28 and 29).

Ultrastructure by Transmission Electron Microscopy (TEM)

The duodenal samples from control animals exhibited microvilli with intact plasma membrane and healthy terminal web (Figure 30). Junctional complexes were intact (Figure 31). Mitochondria appeared normal (Figure 32). Granular endoplasmic reticulum was not dilated.

Samples of duodenum from S. arizonae (Figure 33) infected animals showed decreased numbers of microvilli and shortened microvilli.

The plasma membrane was intact in areas where microvilli were absent. Junctional complexes were intact. Filaments comprising the terminal web were not as concentrated as in control samples. Both intramitochondrial and granular endoplasmic reticular edema were present. Mitochondrial cristae were degenerated. Adjacent cells, both with cytoplasmic abnormalities, varied in number and morphology of microvilli. Rarely were goblet cells from infected animals filled with mucin droplets as in controls (Figure 34).

Duodenal samples from lambs challenged with S. oranienburg (Figure 35) showed shortened and reduced numbers of microvilli. Edema was apparent in the area of the terminal web. In other areas the terminal web was congealed (Figure 36). Cells were separated at the tight junctions (Figures 35, 36, and 37). Intramitochondrial swelling as well as edema in the lumen of granular endoplasmic reticulum was apparent. Cellular debris and bacteria were present in the lumen (Figures 37 and 38).

Ileal sections from an uninfected lamb showed closely packed microvilli (Figure 39). The height of microvilli varied slightly from cell to cell. Junctional complexes were intact. Cellular organelles appeared normal.

Samples of ileum from an S. arizonae challenged lamb exhibited loss of microvilli and plasma membrane, infiltration of lymphocytes, and edema of the mucosa (Figure 40). Mitochondria and endoplasmic

reticulum were edematous. Separation of tight junctions was seen (Figure 41). Separation of the basal lamina from the epithelium (Figure 42) and edematous lamina propria were observed (Figure 43).

In ileal sections from S. oranienburg infected lambs shortening of microvilli as well as loss of microvilli occurred (Figure 44 and 45). Tight junctions were partially separated (Figure 46). Edema of mitochondria and cisterns of granular endoplasmic reticulum was evident.

Colonic samples of control lambs showed closely packed, uniform microvilli (Figure 47). Junctional complexes were intact (Figure 48). Cytoplasmic organelles appeared normal. Goblet cells were packed with mucin droplets (Figure 49).

Samples of colon taken from S. arizonae infected lambs exhibited loss of microvilli. The microvilli which remained were irregular in size and shape (Figure 50). Mucosal edema was present. Both endoplasmic reticulum and mitochondria were edematous. Tight junctions had begun to separate.

In samples of colon from S. oranienburg infected lambs microvilli were sparse, and when present were stunted. Tight junctions were separated (Figure 51). The terminal web area was edematous (Figure 52). Granular endoplasmic reticulum and mitochondria were very edematous (Figure 53). In some areas the basal lamina was bare of epithelial cells (Figure 54). The lamina propria was edematous.

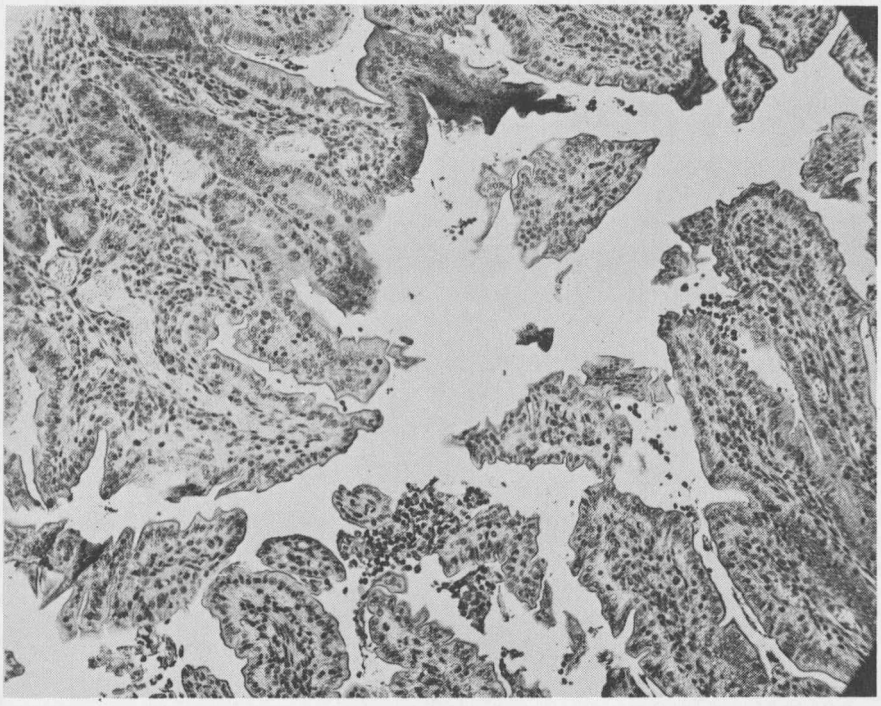

Fig. 1. LM. Normal duodenum. Villi and Brunner's glands. X100.

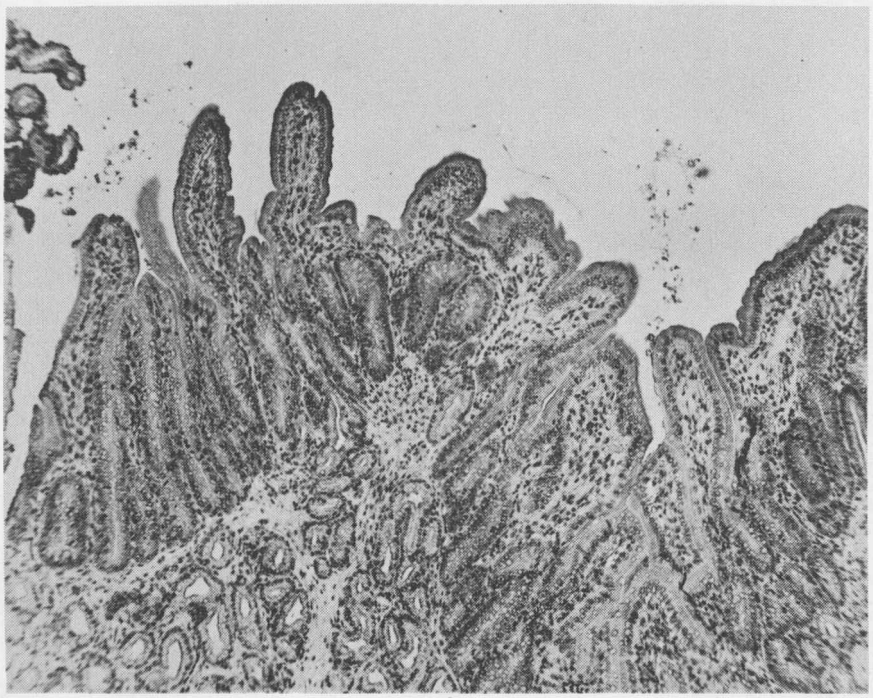

Fig. 2. LM. Duodenum of lamb challenged with S. arizonae. Irregular villi. Congestion of lamina propria. X100.

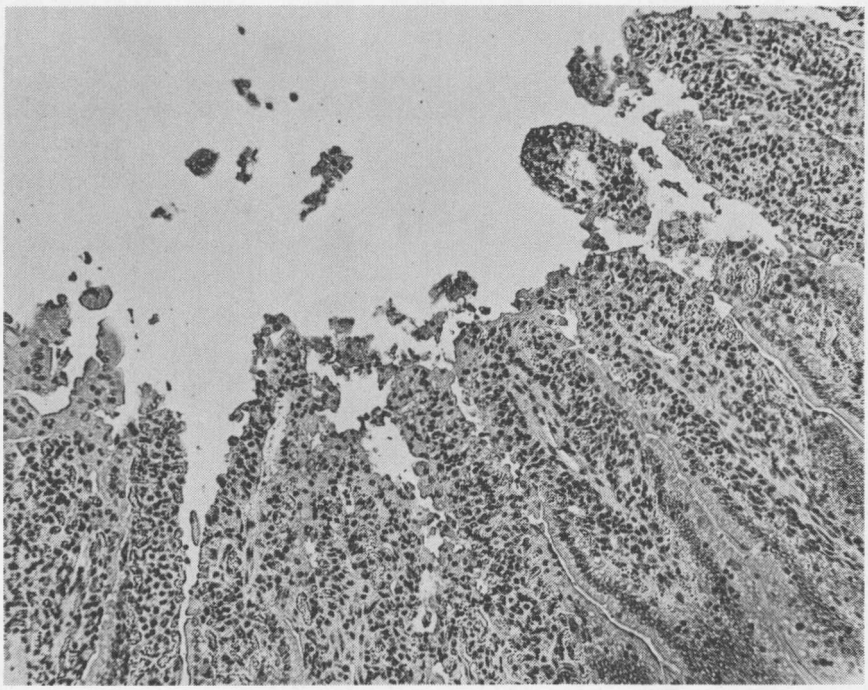

Fig. 3. LM. Ileum of lamb challenged with S. oranienburg. Loss of enterocytes. Leukocytic infiltration. X100.

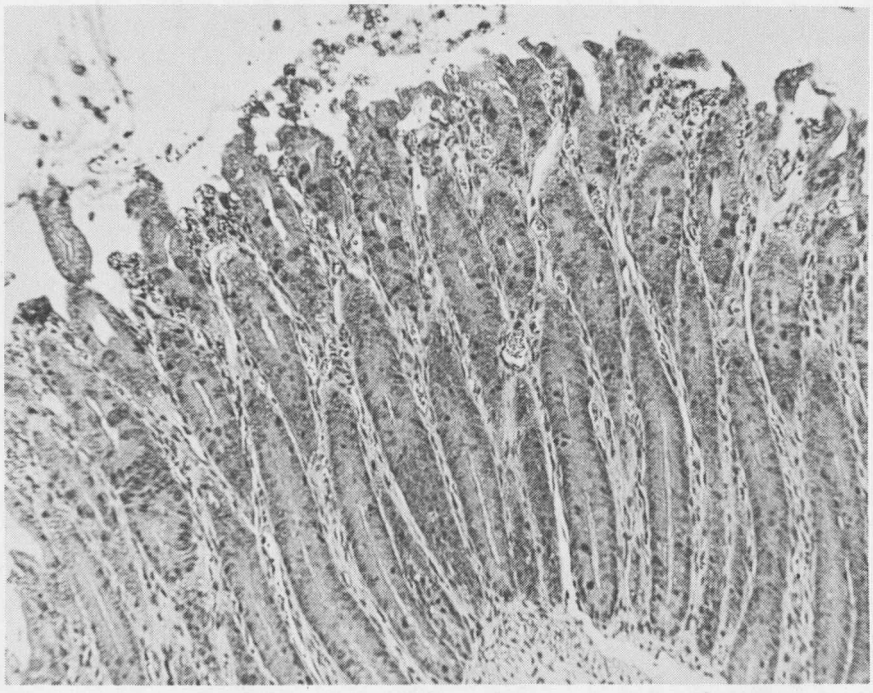

Fig. 4. LM. Normal ileum. X100.

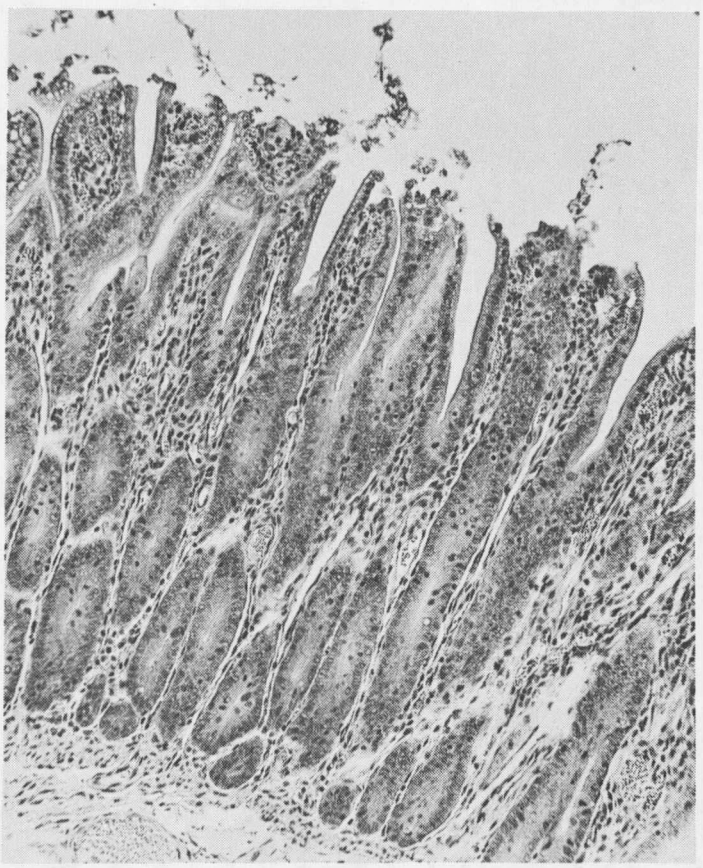

Fig. 5. LM. Ileum of lamb challenged with S. arizonae. Excessive apoptosis with exposed lamina propria at villus tip. X100.

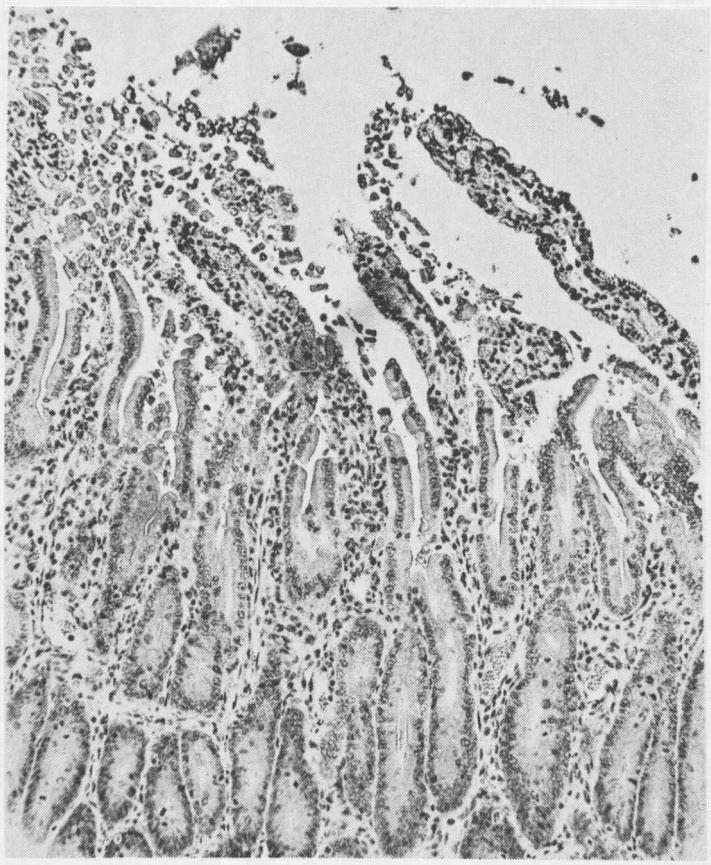

Fig. 6. LM. Ileum of lamb challenged with S. oranienburg. Loss of epithelium on villi with exposed lamina propria. X100.

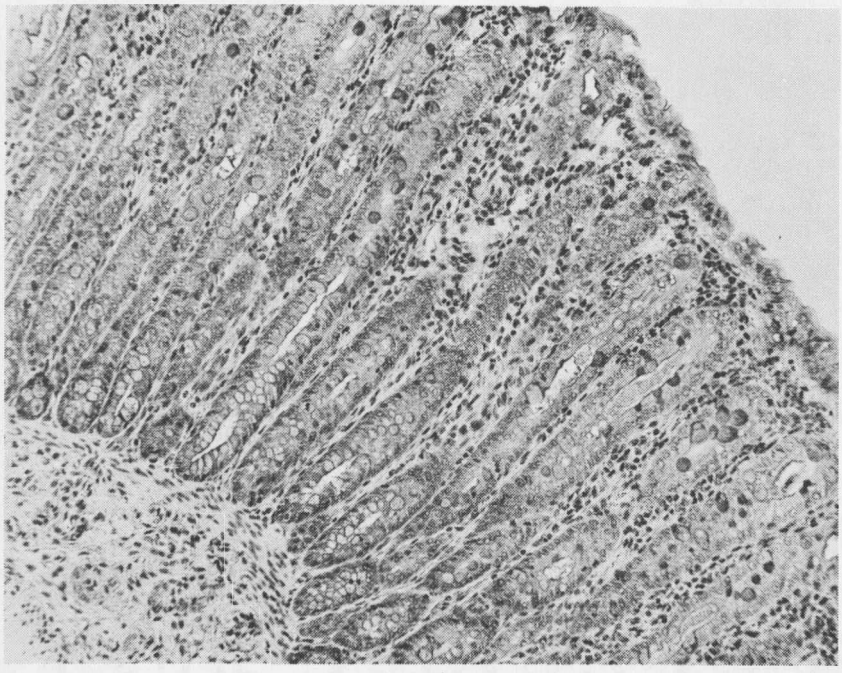

Fig. 7. LM. Normal colon. X100.