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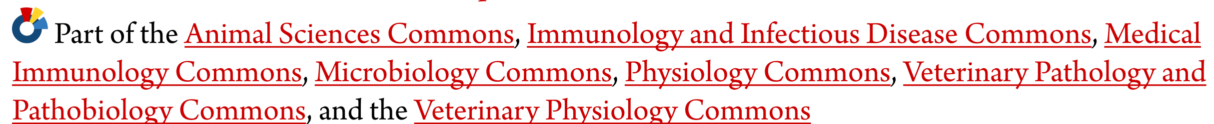
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Evidence for and effects of a factor derived from adult rat and cow small intestine capable of reducing *Cryptosporidium parvum* infection in infant rats

by

Dhuha Akili

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Physiology
Major Professors: James A. Harp and Franklin A. Ahrens

Iowa State University
Ames, Iowa
1999

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ABSTRACT

Cryptosporidium parvum is an intracellular protozoan parasite of the mammalian intestine. In rats, *C. parvum* infection is age related; infants are susceptible while adults are resistant. The transition from susceptibility to resistance usually takes place as the infant rats approach the age of weaning. In the present study, infant rats were orally inoculated with a preparation of intestinal scrapings taken from adult rats or cows. The infant rats received the scrapings from days 3 to 14 of age, and were inoculated with *C. parvum* oocysts at 9 days of age and killed at 15 days of age. Both ileal tissue and fecal samples were examined for the presence of *C. parvum*. Scores for severity of infection in the ileum, as well as the degree of oocyst shedding in the feces, were both significantly lower in the groups that received the intestinal scrapings. This suggests the presence of a protective factor in the preparation that could be transferred from adults to infants. Boiling the intestinal scrapings caused a loss in the ability to protect against infection indicating that the active component was heat labile. Infant rats that received irradiated adult intestinal scrapings were also significantly less infected than the control group, indicating that protection was not dependent on the presence of live bacteria. Attempts were made to further isolate and purify the active component(s) of the intestinal scrapings through differential centrifugation, ammonium sulfate precipitation and DEAE chromatography. The biologic activity was tested by the ability of the various isolated fractions from the purification steps to protect infant rats from *C. parvum* infection. The activity was found to be associated with the extrinsic membrane protein fraction of the intestinal scrapings with a molecular mass greater than 10 kDa. Treating the fraction with Proteinase K caused a significant loss in the ability of the fraction to protect infant rats from

infection. This indicated that the active fraction contained protein essential to its activity. The activity was found to be in the extrinsic membrane protein fraction precipitated by 40% ammonium sulfate. Separation by ion exchange resulted in four fractions, two of which were able to reduce *C. parvum* infection in infant rats.

Identification of the protective component in adult intestinal scrapings may contribute to understanding mechanisms of resistance to *C. parvum* infection.

INTRODUCTION

Cryptosporidium parvum is an intracellular protozoan parasite, which replicates within the microvillus region of epithelial cells lining the small intestine (1). It causes a zoonotic disease of mammals that produces a short-term acute diarrheal illness in immuncompetent individuals and a prolonged life threatening cholera-like disease in the immunocompromised. Young animals are generally more susceptible to infection with *C. parvum* than adults (1, 2).

In the rat and mouse, infants are susceptible up until the age of weaning; after that they become naturally resistant (3). Adults are resistant even without prior exposure to the disease. Thus, it appears that resistance is age related and acquired at the time when many changes are naturally occurring in the animal (3).

The time of weaning is a time when a more adult microflora is being acquired. Changes in the brush border enzymes and cell surface markers are also occurring at this time. In addition, the diet, enterocyte life span and makeup of mucosal secretions are changing (4). One or a number of such changes occurring within the intestine of the animal may play a role in the natural transition from being susceptible to becoming resistant to colonization with *C. parvum*. Thus, there may be a factor present in the adult intestine that protects the adult animal from infection, or induces changes making it resistant to infection. Such a factor could render the infant, normally susceptible animal, resistant if introduced orally.

It was therefore hypothesized that a factor available in the intestine of the adult animal could be transferred to the infant, rendering it resistant to infection, by orally inoculating the infant with homogenized adult intestinal scrapings. The factor might act by directly

inhibiting *C. parvum* binding and invasion of enterocytes in the infant, or it might induce a change in the infant intestine making it less susceptible to infection. In any case, the discovery, identification, and eventual purification of such a factor could shed more light on the mechanisms of invasion and binding, and the pathogenicity of *C. parvum*.

LITERATURE REVIEW

Cryptosporidium parvum

Following the initial discovery of the genus *Cryptosporidium* and its first species *Cryptosporidium muris* in mice in 1910, Ernest Edward Tyzzer identified a new species in 1912, *Cryptosporidium parvum* (1). Since then a number of other species have been identified that infect a variety of different animals in a host specific manner. *C. parvum*, first identified in mice, was later recognized to be infectious for all species of mammals including humans. This intracellular protozoan parasite has been shown to infect the mammalian intestine (Figure 1) causing a self limiting diarrheal disease in normal individuals and a prolonged and potentially life threatening condition in the young and immunocompromised (1).

This zoonotic disease is associated with atrophy of the intestinal villi, causing them to appear short and blunted, as well as crypt hyperplasia (5, 6, 7). Microscopic examination of infected small intestinal sections stained using hematoxylin and eosin reveal numerous cryptosporidia in the crypts and along the length of the microvillar border. Although the infection can be found anywhere along the length of the small intestine, it usually appears to be more localized in the distal ileum. Immune cells infiltrating the lamina propria consist of mixed mononuclear cells and neutrophils (7).

The diarrhea associated with the disease is thought to be a malabsorptive diarrhea resulting from villus atrophy, increased mucosal permeability and reduced absorptive surface area (8). Damage to the enterocytes, as a result of invading parasites, is thought to cause a reduction in the brush border hydrolase activity. Thus, carbohydrates such as lactose, part of

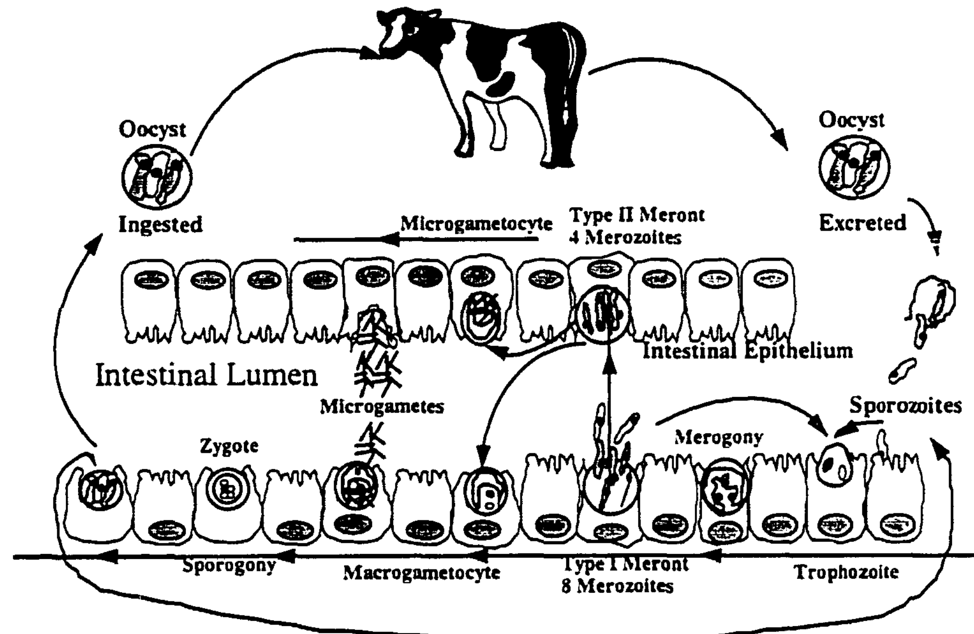

Figure 1. Life cycle of *Cryptosporidium parvum*. Transmission occurs through the fecal-oral route by ingesting the oocysts as a result of fecal contamination. After the ingestion of the oocysts, the infective stage, the sporozoites excyst in the gastrointestinal lumen of the host (4 sporozoites/oocyst). These sporozoites are then capable of invading and infecting intestinal enterocytes lining the intestinal tract. Each sporozoite will form a trophozoite that goes through the process of merogony to form a type I meront containing 8 merozoites. This meront is formed intracellularly, but extracytoplasmically in the host cell. The emerging merozoites can then either reinfect other cells to form more type I meronts (asexual cycle), or enter new cells to form type II meronts which contain only 4 merozoites which can then develop into either microgametocytes or macrogametocytes (sexual cycle). The microgametes emerge from the microgametocyte to fertilize the macrogametocytes, which then develop into zygotes. The zygotes mature (sporogony) to form either thinwalled oocysts, which can excyst to reinfect other enterocytes in the same host or thick-walled oocysts to be excreted in the feces to infect future hosts (1, 2, 9).

the infant diet, are not broken down or transported into the cells, but are retained in the intestinal lumen resulting in the osmotic movement of water into the gut.

It has also been suggested that a factor resembling that of cholera toxin may be produced by the parasite resulting in a secretory diarrhea. Like cholera toxin, it may act by altering the sodium/chloride co-transport mechanism responsible for the passive water absorption which usually takes place in the villus enterocytes, or by stimulating chloride secretion resulting in the movement of sodium and water into the lumen (8, 10). Others have suggested that the secretory diarrhea may be the result of prostanoids produced by inflammatory cells resulting in altered ion absorption and increased secretion (11).

Although *Cryptosporidium parvum* infects most mammals, it is recognized as a serious problem in humans, especially the immunocompromised, and in livestock where it is a widespread disease of economic importance (1, 2, 12). It is suspected that one source of disease in humans is due to water contaminated by infected livestock (13, 14, 15). Although the disease is acute and self-limiting in normal individuals, it is a serious chronic disease in immunocompromised individuals, such as AIDS patients (14, 16). Because a cure for the disease is yet to be found, the symptoms are usually treated with anti-diarrheal drugs to prevent dehydration (16).

Cryptosporidium parvum appears to be infectious to many mammalian species. The disease appears to naturally occur in the young and naive host (7). This disease is rarely seen in bovine calves that are older than 4 weeks of age (1). In one study the calves remained susceptible up to 3 months of age when raised in isolation (17). Reports of adult animals excreting oocysts in feces are very rare, which led to the speculation that adults are resistant

to disease either because of prior exposure, or because they have mechanisms that render them non-susceptible (1). The reasons why infants are susceptible and adults are not, may include differences in gut microflora, intestinal enterocyte maturation, brush border enzymes, maturation of the immune response or other age related differences. Studies done using mice as a disease model have shown that post-weaned mice are naturally resistant to infection even when not previously exposed to *C. parvum* (3). Similar studies have also been conducted in rats. This transition from susceptible to resistant seems to coincide with the acquisition of a more mature intestinal flora (3) and with changes in the enterocyte life span, turnover rate, and brush border hydrolases (4). All of these changes are natural occurrences that take place at the time of weaning in most mammals.

Enterocyte Growth and Turnover

The gastrointestinal mucosal growth is regulated by gastrointestinal hormones, glucocorticoids, bacterial flora, diet and the physical presence of food within the lumen of the gut (18). The life span of the enterocyte in the infant varies depending on the species of the animal, but generally speaking, the enterocyte life span is longer in the infant than it is in the adult (19, 20). In the infant rat it ranges from 7 to 10 days and in the adult it is 2 to 4 days in length (20, 21). This is referred to as the epithelial cell transit time, where transit time is defined as "the length of time that each epithelial cell is available on the villus to perform its function (22, p.163)".

The undifferentiated proliferating precursor cells reside in the crypts of Lieberkuhn. They undergo at least 2 divisions within the crypt before they migrate either upwards to form

the absorptive cells, goblet cells or enteroendocrine cells, or they migrate downwards where they become Paneth cells (4, 23).

The appearance and composition of the intestinal epithelium is different in the infant compared to the adult. In rats undergoing weaning (approximately 17 days of age), the cells differentiate and take on the appearance of those in an adult animal. In the rat and mouse, there is a marked elongation of both the crypts and villi at the time of weaning. The proliferating cells in the crypt produce cells that are more mature. The brush border membranes of the villus cells express more hydrolytic enzymes than before both in quantity and type. This transition or change continues from this point on for the rest of the animals adult life (24). Villi become longer when cell production exceeds the rate of cell loss. When the rates become steady, the villi maintain their normal adult length (4).

The enterocyte life span or proliferation rate can be affected by disease. Proliferative enteropathies associated with the bacterium *Lawsonia intracellularis* have been described in a variety of mammalian species (25). The disease is characterized by a severe watery diarrhea that when left untreated can lead to death. Histological sections reveal the presence of the intraepithelial bacteria associated with intestinal mucosal thickening and hyperplasia. The crypts often become enlarged and contain many epithelial cells undergoing mitosis (26).

In another study, intestinal epithelial cell proliferation was examined in pigs exposed to gastroenteritis virus. The disease resulted in a severe diarrhea within 48 hours and villus atrophy in the small intestine (27). The intestinal villi appeared longer in infected pigs compared to non infected pigs with crypt hyperplasia in the infected pigs. Tritiated thymidine was given to both pigs that had been exposed to the virus and others that had not

been. Autoradiography of tissue revealed an increase of more than two-fold in proliferation rates in some areas of the small intestine in the infected animals compared to those not exposed to the gastroenteritis virus (27). The increase in proliferation, as a result of tissue damage, appeared to be the result of increased activity in the dividing crypt cells to make up for losses on the villus tip.

Mice infected with *C. parvum* at a week of age were examined for changes in cell proliferation at 2 weeks of age by counting the number of proliferating cells that had incorporated bromodeoxyuridine (BrdU), a thymidine analog, in the crypts (28). There was approximately twice as much proliferation in the crypts of the infected mice compared to those of the non-infected mice (28). In another study looking at the effects of *C. parvum* infection on enterocyte proliferation in adult TCR-α-deficient mice, a strain of mice deficient in αβ T-cells (29), there was also a measurable increase in the number of proliferating enterocytes compared to that observed in their non-infected counterparts (29).

Brush Border Enzymes

In the small intestine there are 2 kinds of digestion, luminal and mucosal. The brush border enzymes responsible for mucosal digestion, located within the microvillar region play an important role in the terminal digestion of nutrients. In infant rats, the brush border lactase is present at its highest levels, but begins to decline as the animal approaches weaning (24). The level of sucrase-isomaltase, which is not expressed until rats approach weaning, increases as the rat pups begin to feed on solid food and is continually expressed all through adult life (30, 31). The decrease in the level of lactase and increase in sucrase coincides with the transition of the rats from being susceptible to *C. parvum* infection to becoming resistant.

The levels of both these enzymes were different in 2 week and 3 week old mice, that had been infected with *C. parvum* at a week of age, when compared to those in mice that had not been exposed to *C. parvum* (28). In the infected mice, the increase in sucrase and decrease in lactase was observed to take place before 2 weeks of age. In the noninfected mice, the change took place between 2 and 3 weeks of age (28). Thus, it appeared that *C. parvum* infection altered the normal time frame causing the change in enzyme patterns to take place earlier than usual.

A variety of antigens have been identified that are exclusively expressed on suckling infant rat intestinal villus cells (32). The protein antigens YBB1/89, YBB 2/29, and YBB 2/95 were found to be expressed on the villus cells of infant rat pups. The expression and distribution of these antigens was observed to change at the time of weaning (32). These antigens could not be detected in the intestinal tissue of adults. The disappearance of these antigens was found to coincide with the appearance of the enzyme sucrase-isomaltase (32). Similar antigens may play a role in the susceptibility of the intestinal tissue in the infant to infection with *C. parvum* or render an adult resistant to the disease.

It is not yet clear how sporozoites attach to enterocytes in order to invade them. It is speculated that it may involve specific receptors or a ligand type of binding between the cell and sporozoite. Sporozoite binding studies were conducted using the human intestinal epithelial cell line Caco-2 (33). Galactose and *N*-acetylgalactosamine specific surface lectins were identified on the sporozoite surface and suspected to play a role in adhesion to enterocytes (34, 35). When specific glycoprotein inhibitors to that attachment were added to the cell culture along with the sporozoites they were found to reduce attachment to the cells

compared to cultures where the inhibitors were not added (34). A 900 kDa surface glycoprotein, located on sporozoites and merozoites, both invasive forms of the life cycle stages of *C. parvum*, has also been identified (36) and antibodies against it have been shown to inhibit invasion of the sporozoites in vitro.

Intestinal Microflora

The microbial flora in the gastrointestinal tract differs depending on the diet and age of the animal. Infants acquire a greater number and variety of flora as they get older and as their diet changes from milk to solid food (37). The presence of microbial flora has been found to be beneficial to the host in many ways. Some of the bacteria produce enzymes thereby aiding in digestion and production of certain essential compounds (37). They also prevent the colonization of potentially pathogenic species by occupying potential binding sites or by steric hindrance (37). It is widely accepted that the presence of the normal flora in the gut plays an important role of continuously stimulating the immune system, there by keeping it upregulated, to prevent or eliminate colonization with pathogens (37).

The rate of epithelial cell turnover has been shown to be higher in animals with microflora compared to germ free animals (37, 38). The villi also appear to be longer thereby giving them a larger surface area compared to those in germ free animals (39). The gut associated lymphoid tissue (GALT) also appears to be more mature and defined in animals with microflora. The peristaltic movement in germ free animals has also been shown to be slower than the movement in normal animals (40). The microbial population also provides a source of polyamines, which act to stimulate mucosal growth and regulate cell renewal in the intestinal mucosa (41). The transition from susceptibility to resistance to *C. parvum* takes

place at the time that mice acquire a mature intestinal flora. The role that intestinal flora plays in the resistance of adult mice to *C. parvum* has been examined. A study conducted in germ free and antibiotic-treated adult mice, found that the germ free animals were more susceptible to infection than were normal adult mice (3). The authors suggested that the bacteria may play a role in inhibiting colonization of *C. parvum* by blocking essential receptors for binding, by producing anti-cryptosporidial agents (3) or even by increasing gut motility (3). The antibiotic-treated mice were not as susceptible as the germ free mice were to *C. parvum* infection. It was suggested that since the mice had already been exposed to the flora, prior to receiving the antibiotics, the immune system had already been stimulated or primed. The authors also speculated that the antibiotics may not have cleared all of the existing microflora. Non-culturable species may have been present and responsible for the ongoing activation of the local immune response (3).

A study was conducted to examine the role of the intestinal flora in the absence of the ability to mount an immune response (42). In that study, both severe combined immunodeficient (SCID) mice (mice which lack both T- and B-cell immunity) with and without flora were examined for susceptibility to *C. parvum* infection. The germ free SCID mice became heavily colonized with the organism within 3 weeks time, while the flora-bearing SCID mice did not show any colonization till 5 to 7 weeks post-challenge (42). Although it appeared that the presence of a specific immune response was required to overcome an infection with *C. parvum*, it became clear from the results of the study that the presence of microflora provided some protection to the flora-bearing SCID mice initially, thereby rendering them less susceptible to *C. parvum* infection than the germ free SCID mice.

A similar model was used to look at the role of interferon gamma (INF-γ) in adult flora-bearing SCID mice (43). When those mice were treated with anti-INF-γ, they became as infected as the germ free SCID mice (43). That indicated a role for and the importance of INF-γ in resisting *C. parvum* infection. In a model designed to look at the role of $CD4^+$ cells in resolving an existing *C. parvum* infection, infected SCID mice were injected with spleen cells taken from normal mice (44). The mice were able to clear the infection unlike infected SCID mice that did not get splenocytes. Other groups of infected SCID mice that were reconstituted with splenocytes received either anti-$CD4^+$ or anti-INF-γ monoclonal antibodies to examine the effects of both on clearing the infection (44). It was found that both those groups either had difficulty or could not completely eliminate the infection. These studies indicated the importance of both INF-γ and $CD4^+$ cells in overcoming a *C. parvum* infection.

Therefore, it appears that the resistance to *C. parvum* infection does not depend on the presence or activity of any one factor in particular, whether it is the presence of normal flora, functional CD4+ T cells, or INF-γ, but a combination of at least two of the factors (45).

Diet

The effect or influence of the diet on the gastrointestinal system can be seen most clearly in animals when the change in dietary intake shifts from milk to solid food at the time of weaning. The most pronounced changes that take place are the changes in gastrointestinal enzymes and brush border hydrolases (46). These changes apparently are programmed to take place even if the animal is forced to remain on a diet consisting only of milk (46). However, the level at which the enzyme is expressed is affected in animals that remain on

milk after the time where they would normally have switched to a solid diet. Sucrase-isomaltase still appears, but the levels are only half of what they would have been had the animal gone through the weaning process (46).

In a study conducted using human subjects, the effect of increased dietary carbohydrates on brush border hydrolase levels was examined (47). A diet comprised of 80% sucrose was fed to the subjects resulting in an increase in both sucrase and maltase activity 2 to 5 days after the diet began. The level of the enzymes did not increase even after the subjects remained on the diet for many more weeks. The levels decreased when the normal diets were resumed as quickly as they had increased. Because the time it took the enzyme levels to increase was about the same as the cell turnover rates, it appeared that the diet had a direct effect on the crypt cells causing them to increase the amount of enzymes expressed (47).

In a study conducted to look at the effects of prolonged suckling on the levels of lactase in rats, it was found that lactase activity was retained for a longer period of time in the pups that were prevented access to solid food than in their weaned counter parts (48). The levels of lactase did decrease following the normal pattern, but still maintained a significantly higher level of activity than those under normal conditions. Other brush border enzymes, sucrase and maltase, continued to develop at normal levels as in the controls (48). In another study designed to examine the effects of the prevention of weaning in infant rats on sucrase level, the authors reported a significant depression of sucrase in the jejunum (49).

Diet can also affect cell turnover through its physical presence in the lumen of the gut. A solid diet high in fiber can be abrasive to the one cell thick mucosal layer causing it to

continuously exfoliate (50). The rate of cell proliferation in mice that are weaned is higher than their age matched non-weaned counter parts (51). The gastrointestinal hormone gastrin has been shown to cause an increase in cell proliferation in the small intestine. The increase in gastrin levels coincides with the time the animals begin to eat solid food, as gastrin release is stimulated by the presence of food in the stomach (51).

It has been shown that changes in the diet can modify the chemical composition of mucosal glycoproteins thereby changing the ability of certain microbial organisms to bind to the mucosal layer (52).

The Mucus and the Glycocalyx Layers

The microvillar brush border on the surface of the enterocytes is covered with a layer composed of oligosaccharide chains, glycoproteins and glycolipids called the glycocalyx (53). The glycocalyx performs many functions. It serves as a sieve restricting the entry of microrganisms and macromolecules of a certain size. It serves as the base to which many of the microbial flora, viruses, toxins, antibodies, hormones, vitamins and various other elements attach. The glycocalyx plays a role in cell to cell adherence and has been described as the "intercellular cement substance between epithelial cells (53, p. 189)". It also contains the brush border hydrolases, which are important for terminal digestion. Just like cells turn over, so does the glycocalyx; this way it clears off anything attached to it on a regular basis. It binds the secretory form of IgA, thereby stabilizing it and rendering it less susceptible to proteolytic digestion (53).

Covering the glycocalyx is a gel-like viscous layer of mucous made up of glycoproteins, water, electrolytes, and dead cells (54). The glycoproteins, referred to as

mucins, are secreted by specialized mucous secreting cells, goblet cells, located both in the villi and crypts of the intestinal epithelium. The mucins are large molecular weight compounds with a protein backbone containing large amounts of carbohydrate in the form of oligosaccharides and some lipids (54). The mucins can range in size from 200 to 2000 kDa. The composition of the mucins differs in suckling rats compared to adults.

A study of the percentages of protein and carbohydrate making up the mucins in adult rats compared to that in infants revealed the total protein content was higher in the infant than in the adult (55). In the infant the levels of the amino acids threonine, alanine, and valine were higher than those in the adult. In the adult, the levels of serine, proline and lysine were the highest. In the adult the total carbohydrate content was higher than that in the infant with the levels of fucose and *N*-acetylglucosamine being the highest. The level of sulfate, however, was much higher in the infants than in the adults. The changes in the composition of mucins in the small intestine appeared to be age related and found to occur, between 14 and 21 days of age (56), at the time of weaning when the diet is changing.

The mucus layer performs many functions. Although many of the normal flora are submerged in the mucous where they are bound to the glycocalyx underneath, the mucus serves as the first protective barrier in the intestine binding potential pathogens and washing them away with gut contents. Some of the mucins can bind the surface of bacteria and agglutinate them, thereby preventing them from binding to the cell surface (54). Some mucins can act against proteolytic agents and enzymes secreted by bacteria thereby protecting the mucosal surface from bacterial attack. In some of those cases the enzymes may be produced by the normal flora to destroy invading bacteria and the mucus protects the

epithelial surface from proteolysis. The mucus contains secretory IgA that can bind to bacteria and preclude their binding to the epithelial surface or it can inactivate bacterial toxins (54, 57). It functions as a lubricant to give some protection to the intestinal lining from the abrasive gut contents. It also forms an "unstirred" layer where nutrients can diffuse through to the brush border enzymes where they can be further digested and absorbed without being washed away in the lumen (54, 57).

In a study designed to examine the effects of diet and microflora on binding patterns of intestinal mucins in normal and germ free rats, the rats were fed one of two diets. One group was fed a purified diet with cellulose as the fiber source while the other group was fed a commercial diet containing crude cereal fiber (58). The goblet cells in the rats that were fed the commercial diet were more heavily labeled with *N*-acetyl-glucosamine and sialic acid-linked D-galactose-specific lectins than goblet cells in the rats that were fed the purified diet. The small intestinal goblet cells in the germ free animals fed the commercial diet bound more lectins specific for *N*-acetyl-galactosamine than did the goblet cells in the rats with conventional flora on the same diet (58). The goblet cells of the germ free rats fed the purified diet labeled much more intensely with sialic acid specific lectins than did the ones in the rats with conventional flora on the same diet. The differences in the lectin specificity of the mucins are influenced by the presence of bacteria and may be due to the interactions between the glycoproteins on the surface of the bacteria and those on the surface of the epithelial cells (58). The effects of diet and microflora on mucin may play a role in the susceptibility or resistance to intestinal colonization with certain types of bacteria.

The changes in cell turnover, diet, microflora, brush border enzymes and mucins all occur at the time of weaning. One or a combination of these changes may be responsible for resistance to *C. parvum* infection.

MATERIALS AND METHODS

Animals

Rats

Harlan Sprague-Dawley rats (Madison, Wis.) maintained at the National Animal Disease Center (NADC), Ames, IA, were used in the experiments. The rats were given free access to food and water and were maintained in a room with alternating 12 hour periods of light and darkness. Intestinal scrapings were collected from euthanized female and male adult rats that were 2 months or older (150-250 gm). The litters used in the experiments were taken from females bred for the first time. Animals used in experiments were housed so that there were no more than 12 pups per litter per adult female.

Mice

BALB/c mice from a colony maintained at the NADC were used in some experiments. The mice were given free access to food and water and were maintained in a room with alternating 12 hour periods of light and darkness.

Cows

Small intestinal scrapings of the luminal side of the intestine were collected from Jersey or Hereford cows 8-24 months of age. The cows were all in generally good health and the collection and first processing steps were completed within 6 hours of euthanasia.

Calves

Ileal scrapings were collected from two 3 month old Jersey calves that had been naturally exposed to *C. parvum* at about 7 weeks of age. The calves recovered from the infection and were in good health when they were euthanized.

Collection and Processing of Intestinal Scrapings

Adult Sprague-Dawley rats were euthanized using a CO_2/O_2 mix. The ileum, 20 cm of the distal small intestine, was removed from each rat. It was then washed out with phosphate buffered saline (PBS). The ileum was cut lengthwise exposing the lumen and was then cut into 4 or 5 cm sections. A glass coverslip was used to scrape off all but the serosa of the sections. The volume of scrapings was measured in a graduated cylinder. Four mls of PBS / 3 mls scrapings (3 mls scrapings = 3.5 gm wet weight of tissue) were then added. This was later changed in the modified treatment schedule (Figure 3) so that 0.5 ml of PBS was added to 3 ml of intestinal scrapings. The mixture was then mixed thoroughly by aspirating and discharging through a pipette until the scrapings appeared uniform in texture. Initially, a syringe with a 22 gauge needle was used to ensure that only liquid, which could pass through that needle, would be collected. This procedure was later modified by the use of a Brinkmann polytron PT 3000 homogenizer with a PT10 probe. Samples were homogenized for 15 to 20 seconds at 14-15 x 1000 rpms and then placed on ice for two minutes. The process was repeated 4 or 5 times until thoroughly homogenized.

In initial experiments, the ileal scrapings were collected on a daily basis from adult rats just prior to administration to the infants. Later, the ileal scrapings were pooled from 30 adult rats at a time, aliquoted and frozen. They were thawed just prior to orally inoculating

the infant rats. It was later determined that the ileal scrapings taken from the third most distal part of the small intestine of an adult cow worked just as well as those taken from an adult rat. Finally, it was determined that scrapings from the entire length of the cow small intestine were able to prevent infection in pups. After that the whole length of the small intestine from cows was used in the experiments. The same methods were used to collect and process the cow intestinal scrapings.

Irradiated Ileal Scrapings

The ileal scrapings were collected and homogenized from 30 adult rats. They were placed in 25 cm^2 culture flasks and stored on ice. They were then taken to the Iowa State University linear accelerator facility where they were kept on ice and subjected to 45 KiloGrays of irradiation. To determine sterility of the ileal scrapings, samples of the irradiated scrapings were then cultured under both aerobic and anaerobic conditions on both brain heart infusion (BHI) media and trypticase soy agar with blood (TSB) media to check for bacterial viability. The irradiated scrapings were then aliquoted and stored at -20°C until used in experiments.

Boiled Ileal Scrapings

The rat ileal scrapings were placed 3 mls / tube and submerged for 3 minutes in boiling water. They were then placed on ice to cool and fed to designated rat pups. This was done on a daily basis for the duration of the experiment.

Experimental Design

Each experiment consisted of treatment groups and a control group. The treatment groups were made up of 3 litters with 10 pups each. The control group was made up of 3 litters of 12 pups each. The pups within each group were all mixed together every day and then randomly redistributed among the 3 mothers. That was done once a day for the control pups for the duration of the experiment, and up to twice a day for the pups in the treatment group with every treatment they received.

Treatment Schedule

The homogenized intestinal scrapings or products of its purification steps were fed orally starting at 3 days of age up to 14 days of age (twice/day) in the initial experiments (Figure 2). That was later modified so that they were being fed from day 7 to day 11 of age (Figure 3). The volume they received each day increased with age. A 1ml syringe with a 24 gauge feeding needle was used to administer volumes starting with 100 μl on day 3 of age, up to 1000 μl on day 14 of age. The animals in the control groups initially followed the same schedule as the treatment groups where they were inoculated with similar volumes of PBS until it was determined that PBS had no effect.

The animals in both the control and treatment groups received an oral inoculation consisting of 10^2-10^3 oocysts suspended in PBS at 9 days of age. The amount of oocysts they received was determined based on the infectivity rate of the oocyst pool assessed previously in mice. The pups in the treatment groups received the intestinal scrapings first in the morning of the day they were challenged and then one-half hour later they received the dose of oocysts. The rats in both groups were killed on day 15 of age. Fecal samples were taken

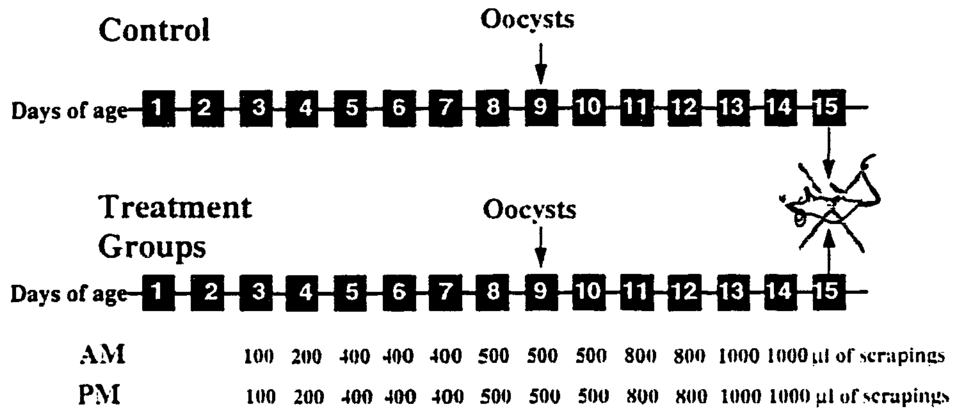

Figure 2. Treatment schedule.

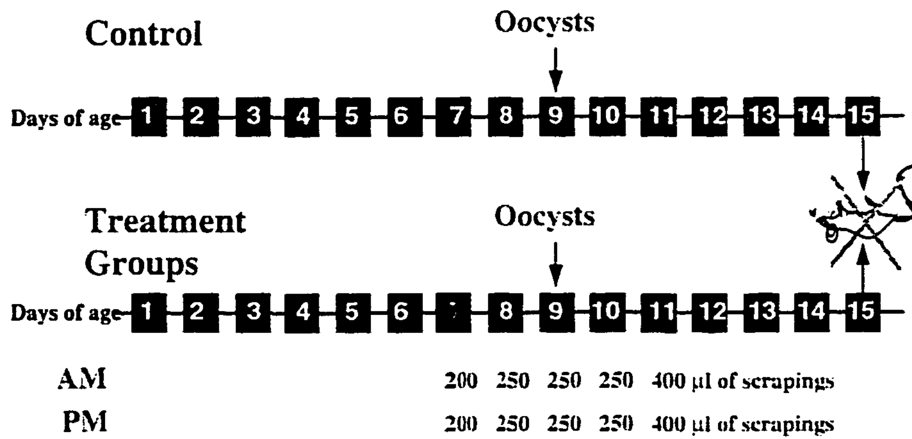

Figure 3. Modified treatment schedule.

from the distal colon. Sections of the ileum were removed approximately 2.5 cm proximal to the cecum.

Tissue Processing and Examination

The ileal sections were placed in tissue cassettes and fixed using Z-fix (buffered aqueous zinc formalin, Anatech Ltd., Battle Creek, MI) and embedded in paraffin. Thin sections (3-4 microns thick) were prepared on slides and then stained with hematoxylin and eosin. The sections were then examined for *C. parvum* using light microscopy. Each slide was scored using an arbitrary scale from 0-2 indicating the intensity of infection in the tissue section. A score of 0 was given to indicate that no *C. parvum* was present, 1 to indicate a light to moderate infection and 2 to indicate the presence of a heavy infection.

Fecal Smears and Examination

The fecal samples from each of the animals were smeared on a glass slide and stained with carbol fuchsin. The slides were examined using oil emersion (500 x) under light microscopy to determine the number of oocysts per microscopic field. Ten microscopic fields were examined per animal. Each of the fields was scored from 1 to 10 indicating the number of oocysts present in that field. The number 10 was given to indicate 10 or more oocysts were present on any given field. These numbers were used to calculate the average number of oocysts / microscopic field for each group of animals and the number of positive fields per group.

Each slide was also scored using an arbitrary scale from 0-3 as an estimation of the intensity of oocyst shedding for each animal. The score of 0 was given to slides that had no oocysts. The score 1 was given to slides that had an average of 4 or less oocysts / field. The

score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field.

Determination of Enterocyte Proliferation Using Bromodeoxyuridine (BrdU)

Bromodeoxyuridine, a thymidine analog, binds to the single-stranded DNA of dividing cells. Proliferating cells in histological sections can then be revealed by immunohistochemistry through the use of a monoclonal anti-BrdU antibody and a chromagen (Streptavidin-Peroxidase-Diaminobenzidine). This system is comparable in accuracy and easier and safer to use than the tritiated thymidine technique for labeling proliferating cells (54). Bromodeoxyuridine (Sigma chemical company, St. Louis, MO) was prepared at a concentration of 25 mg /ml and administered intraperitoneally (100 mg / Kg of body weight). The animals were euthanised one hour after the BrdU injection and the ileum was removed. The tissue samples were fixed using Z-fix and embedded in paraffin. Thin sections (3-4 microns thick) were prepared on poly-l-lysine coated slides and then stained immunohistochemicaly using a streptavidin-biotin system for BrdU staining (Zymed BrdU staining kit, Zymed Laboratories Inc., San Francisco, CA). Slides were mounted with an aqueous mounting medium, Immu-mount (Shandon, Pittsburgh, PA), to avoid section rehydration steps. The number of proliferating cells in each slide were calculated by counting the number of cells which incorporated BrdU in 15 randomly selected complete longitudinal crypts.

Purification of Cow Small Intestinal Scrapings

All the steps described below were performed at 4° C (Figure 4).

Step 1: Crude membrane and cytosol isolation.

After the cow intestinal scrapings were collected, PBS was added and then they were homogenized using a Brinkmann PT 3000 polytron homogenizer with a PT10 probe as described earlier.

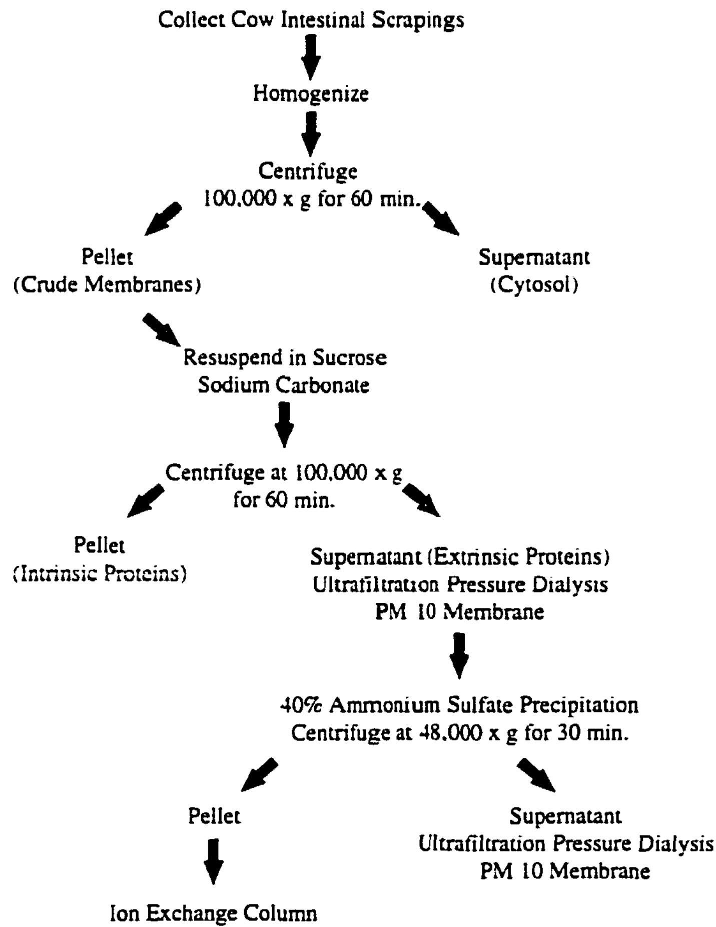

Figure 4. Cow small intestinal scrapings purification and fractionation procedure.

The original volume of the scrapings preparation was measured using a graduated cylinder. The homogenized scrapings were then placed in Beckman polycarbonate ultracentrifuge tubes (25 x 89 mm). The tubes were placed in a Beckman Ti 50.2 rotor, and spun at 100,000 x *g* for 60 minutes at 4° C in a Beckman L8-80M ultracentrifuge. The supernatant fluid contained the crude cytosol preparation and the pellet was the crude membrane preparation. The volume of the supernatant fluid when measured was the same as that in the original preparation. The pellets were resuspended in PBS. The pellet suspension was then homogenized at 12-13 x 1000 rpms for 15 - 20 seconds using the Brinkmann PT 3000 polytron homogenizer with a PT10 probe. More PBS was added in a graduated cylinder bringing the volume back up to that of the original preparation. Both the crude membrane and cytosol preparations were then aliquoted out into tubes and stored at - 20° C until they were tested for biological activity in infant rats.

Step 2: Extrinsic and intrinsic membrane protein separation.

Seven milliliters of homogenized cow intestinal scrapings were placed in each of 12 Beckman polycarbonate ultracentrifuge tubes (25 x 89 mm). The volume in each tube was then brought up to 20 mls with PBS. The tubes were then placed in a Beckman Ti 50.2 and spun at 100,000 x *g* for 60 minutes at 4° C in a Beckman L8-80M ultracentrifuge. The supernatant fluid was discarded and the pellets were resuspended in 1 ml of 0.3 M sucrose with 10 mM Tris.Cl prepared in reagent grade water and buffered to pH 7.5. The pellets were then pooled with the sucrose solution and homogenized at 12-13 x 1000 rpms for 15 - 20 seconds using the Brinkmann PT 3000 polytron homogenizer with a PT10 probe. Each of the pellet suspensions was then poured into a beaker with 21 mls of 0.1 M sodium carbonate

solution at a pH of 11.5 (252 ml sodium carbonate for 12 pellets). The beaker was then placed on ice and in a 4° C cooler for 30 minutes. The beaker contents were then spun again in the Beckman Ti 50.2 rotor at 100,000 x *g* for 60 minutes at 4° C. The tubes were then removed and the supernatants were collected (extrinsic membrane protein fraction) in a beaker and cooled on ice. The pH of the solution was adjusted to 7.5-8.0 with HCl. The solution was then placed in a 350 ml stirred cell (Amicon, Danvers, MA.) connected to a nitrogen tank to undergo ultrafiltration pressure dialysis in a cooler at 4° C. An Amicon PM10 membrane (10,000 MW cut off) that retained anything larger than 10 kDa was used. The run off was also saved to be tested later if necessary. The material was dialyzed and then washed with the same volume of PBS to ensure that all the sodium carbonate was dialyzed out. The volume was brought back to it original level and the fraction was either aliquoted and frozen at -20°C to be tested later in infant rats or used in the next purification step.

The tubes containing the pellets (crude intrinsic membrane protein fraction) were then turned over and blotted and the inside of the tubes were dried with a paper towel to remove any excess sodium carbonate or sucrose. Each of the pellets was then resuspended in PBS, to bring them back to the original volume, and they were then pooled and homogenized at 12-13 x 1000 rpms for 15 - 20 seconds using the Brinkmann PT 3000 polytron homogenizer with a PT10 probe. The solution was then aliquoted and frozen at -20°C and later tested for biological activity in infant rats.

Step 3: Ammonium sulfate precipitin of proteins in extrinsic membrane protein fraction.

Ammonium sulfate (40% solution) was added to the dialyzed extrinsic membrane protein fraction from the previous step. The mixture was placed on a magnetic stirrer for 30

minutes in a 4° C cold room. The solution was then aliquoted into centrifuge tubes and centrifuged at 4° C for 30 minutes at 48,000 x *g*. The tubes were then removed and the pellets were resuspended back to their original volume in PBS. The resuspended pellet fraction was placed in the cold room to stir for 1-2 hours before it was to be used in the next purification step or aliquoted and frozen at -20°C to be tested later for activity in infant rats.

The supernatant fraction was placed in an Amicon stirred cell connected to a nitrogen tank to undergo ultrafiltration pressure dialysis (PM 10 membrane) in a cooler at 4° C to remove all the excess ammonium sulfate. The fraction was washed 4 times with PBS to ensure that all the ammonium sulfate had been removed. PBS was then added to bring the solution back to its original volume. The fraction was then aliquoted and frozen at -20°C to be tested later for activity in infant rats.

Step 4: Ion exchange chromatography (DEAE Sephacel Column).

The ammonium sulfate precipitated pellet fraction from the previous step was prepared for ion exchange chromatography. The solution was placed into 29 x 104 mm Beckman polycarbonate centrifuge tubes and centrifuged at 4° C for 10 minutes at 12.000 x *g* to remove all the flocculent material. The flocculents (pellet) were saved to be tested for biological activity in infant rats. The remaining portion of the solution was poured onto a Diethylaminoethyl (DEAE) Sephacel column (Bio-Rad; 20 cm x 5 cm). A salt gradient was set up with concentrations from 150 mM NaCl to 1 M NaCl both in 20 mM Trizma base and 1 mM EDTA at a pH of 8.00. A peristaltic pump controlled the salt flow from the gradient to the column. The 25 ml fractions were collected as they were eluted off of the column as the salt gradient increased in concentration (Figure 5).

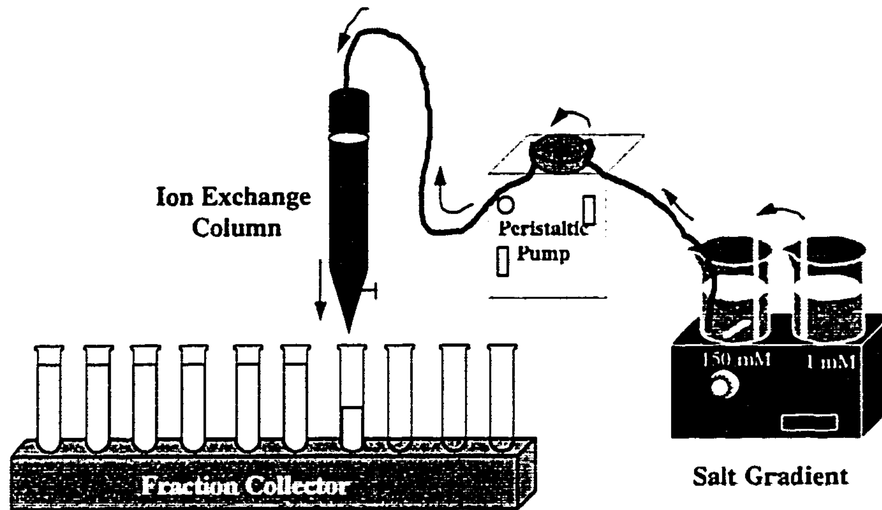

Figure 5. Ion Exchange chromatography using a salt gradient.

The protein content in each of the fractions was measured on a spectrophotometer at 280 nm. The absorbence vs. fraction number was plotted and the fractions were pooled to form 4 larger fractions that were dialyzed using ultrafiltration pressure dialysis. as mentioned in previous steps, to remove any excess NaCl. The concentrations in each of the fractions were brought down to 150 mM NaCl and the volume was adjusted back to half of that of the original volume with PBS to make up for any protein losses. The fractions were then aliquoted and stored at -20° C until the biological activity of each of the 4 fractions was assessed in infant rats.

Proteinase K

Proteinase K (Sigma, St Louis, MO.), a highly active protease of the subtilisin type that is purified from the mold *Tritirachium album* Limber, was used to inactivate proteins in the extrinsic protein faction. Proteinase K was made up at a concentration of 20 mg/ml in

PBS. It was determined in preliminary studies that incubating Proteinase K with the extrinsic protein fraction (4 mg/ml protein) for 24 hours at room temperature at a ratio of 1:10 was sufficient to inactivate protein in the preparation and eliminate any protein bands from appearing in SDS PAGE. Calcium (2 mM) was added to the preparation, bringing it to a 2 mM concentration to enhance the Proteinase K activity. The biological activity of the treated extrinsic membrane protein preparation was then tested in infant rats.

SDS / PAGE Gels

Bis-Tris NuPAGE gels (4-12%) with MOPS running buffer (NOVEX, San Diego, CA) were run according to manufacturers instructions to visualize protein bands in samples from the various purification steps. The standard used was SeeBlue prestained standard (NOVEX, San Diego, CA). Protein was quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The gels were loaded with 40 µg of protein per lane. The gels were stained using Coomassie Blue / GelCode Blue Stain Reagent (Pierce, Rockford, IL) or Silver Stain Plus Kit (Bio-Rad, Hercules, CA).

Statistical Analysis

Statistical analysis of the data representing fecal score, the average number of oocysts per microscopic field, and ileum score was done by Students t test assuming unequal variances. Data were considered statistically significant when $P \leq 0.05$.

Differences between groups of data representing the percent of infected animals and percent of positive fields was evaluated for significance by Chi-square (X^2) test of independence. Independence was accepted or rejected according to the critical value of X^2

for $\alpha = 0.05$ and the degrees of freedom (*df*) for each experiment. All values are means ± standard error of the mean (SEM).

RESULTS

Frozen, irradiated or boiled adult rat ileal scrapings were fed to infant rat pups twice a day from days 3 to 14 of age. The control pups did not receive any treatment. The pups in all of the groups received an oral dose of oocysts at 9 days of age. The rat pups were killed at 15 days of age and the feces and ileum were examined for the presence *C. parvum*. In preliminary experiments, comparing rats that received no treatment with those that received PBS, it was noted that oral inoculations with PBS alone had no effect on altering *C. parvum* infection on infants. Therefore, the controls were not given PBS twice a day as a placebo treatment after the initial trials.

Treatment of infant rats with adult rat ileal scrapings induced protection as measured by the marked reduction in the number of infant rats infected with *C. parvum* compared to those in the control group (Figure 6).

Live bacteria were not responsible for inducing this effect since irradiating the preparation at a dose sufficient to kill bacteria had no effect on altering the activity of the adult ileal preparation. Boiling the preparation, on the other hand, caused rats to become as infected as those in the control group (Figure 6). We also examined the percent of positive microscopic fields, fecal score, average number of oocysts / field, and ileum score (Figures 19, 20, 21, and 22 respectively in the appendix). All of the data analyzed followed the same trend as that shown in Figure 6.

Giving the ileal scrapings only once after incubating them with the oocysts at 37° C for one hour prior to inoculating the rats was not effective in reducing the percent of infant rats infected with *C. parvum* or the degree of infection in the ileum. The severity of oocyst

shedding however, was reduced to approximately half of that in the controls (see Figures 51-53 in the appendix).

Rats treated only once, at 9 days of age, the same day they were inoculated with *C. parvum* oocysts, were not protected compared to rats that were treated twice a day between days 3 and 14 of age (see Figures 54-58 in the appendix).

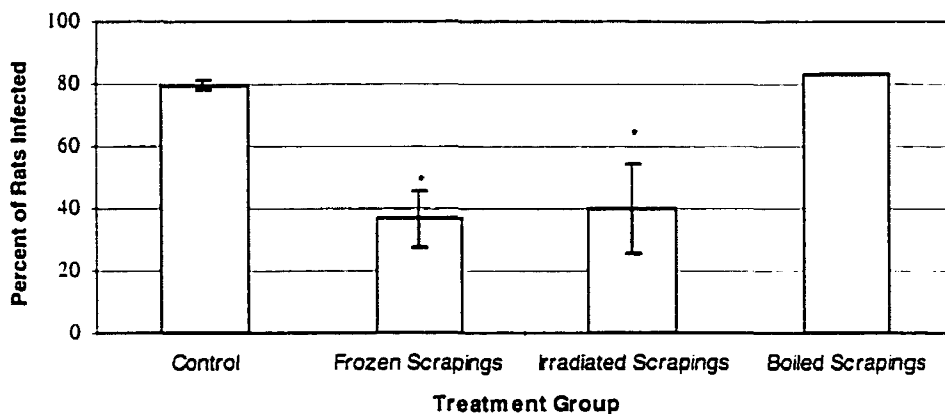

Figure 6. The effect of orally inoculating infant rats with frozen, irradiated, or boiled adult rat ileal scrapings on reducing the percent of infant rats infected with *C. parvum*. The various forms of ileal scrapings were given twice a day between 3 and 14 days of age. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values are means ± SEM, n = 2. Values with * are significantly different from the control group by X^2 analysis.

The data shown in Figure 7 shows the effects of treating the infant rat pups with scrapings from calves, that had previously recovered from an infection with *C. parvum,* and from healthy adult cows. Even though those calves were no longer susceptible to the infection themselves, their intestinal scrapings lacked the ability to reduce the percent of infected rat pups that was seen with both the adult rat and adult cow ileal scrapings. The data

from assessing other parameters (Figures 23-26 in the appendix) are consistent with the trend in Figure 7. Treating the infant rats with cow ileal scrapings only once a day on days 3, 5, 7, 9, 11, and 13 was not as effective in reducing the infection rate with *C. parvum* as treating them twice a day between days 3 and 14 of age (Figures 59, 60, 61, 62 and 63 in the appendix).

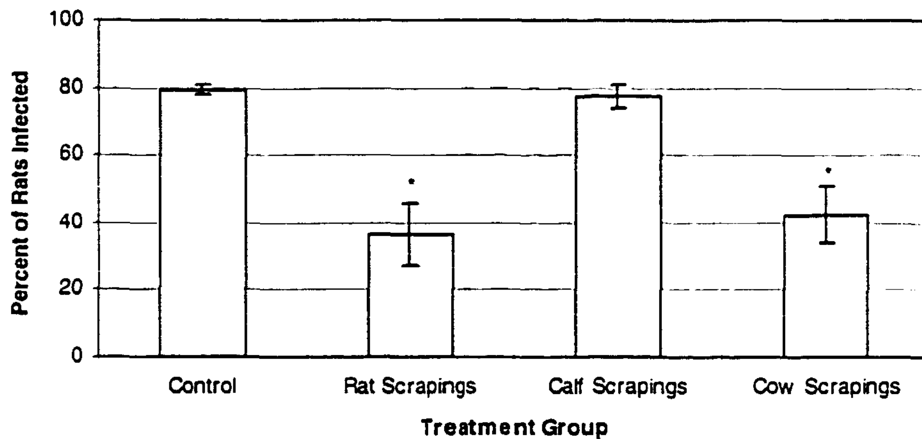

Figure 7. The effect of orally inoculating infant rats with rat, calf or cow ileal scrapings on reducing the percent of infant rats infected with *C. parvum*. The ileal scrapings from the different sources were given twice a day between 3 and 14 days of age. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values are means ± SEM, n = 2. Values with * are significantly different from the control group by X^2 analysis.

The protective activity against *C. parvum* infection in the cow intestinal scrapings collected from the ileum was also present in both the duodenum and jejunum as shown in Figure 8. Figures 27, 28, and 29 in the appendix show other relative parameters that were examined.

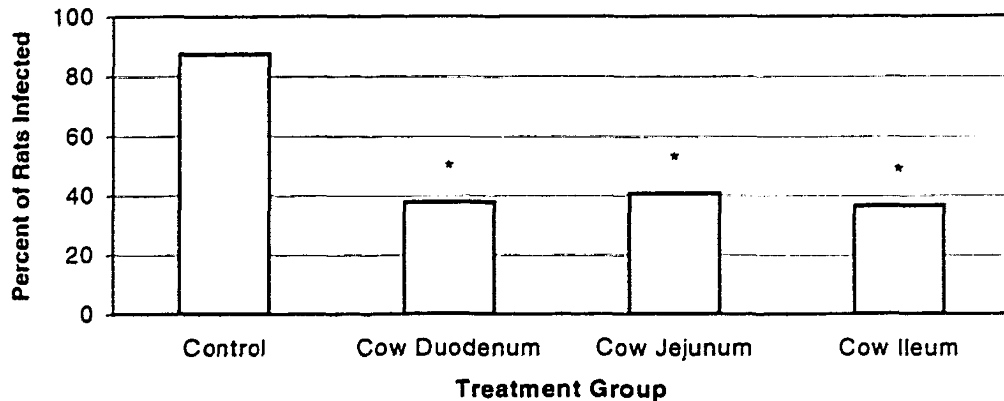

Figure 8. The effect of orally inoculating infant rats with intestinal scrapings taken from the duodenum, jejunum or ileum of adult cows on reducing the percent of infant rats infected with *C. parvum*. The intestinal scrapings from the different areas of the small intestine were given twice a day between 3 and 14 days of age. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

The cow intestinal scrapings were then centrifuged, at 100,000 x *g* for 60 minutes, to separate the crude membrane fraction from the cytosol fraction in an attempt to locate the portion of the scrapings where the majority of the activity would be found.

The activity associated with each of the fractions in reducing the percent of infant rats infected with *C. parvum* compared to the control group is shown in Figure 9. Activity was found in the membrane fraction but not in the cytosol fraction of the intestinal scrapings. See Figures 30, 31 and 32 in the appendix for data on percent of positive microscopic fields of fecal samples, fecal score, and average number of oocysts per microscopic field.

Further fractionation of the crude membrane preparation yielded an extrinsic and an intrinsic membrane protein fraction. There was activity in both of these fractions, but the extrinsic protein fraction was most effective in reducing the percent of animals infected (Figure 10). Figures 33, 34, and 35 in the appendix represent data collected on percent of positive microscopic fields of fecal samples, fecal score, and average number of oocysts per microscopic field.

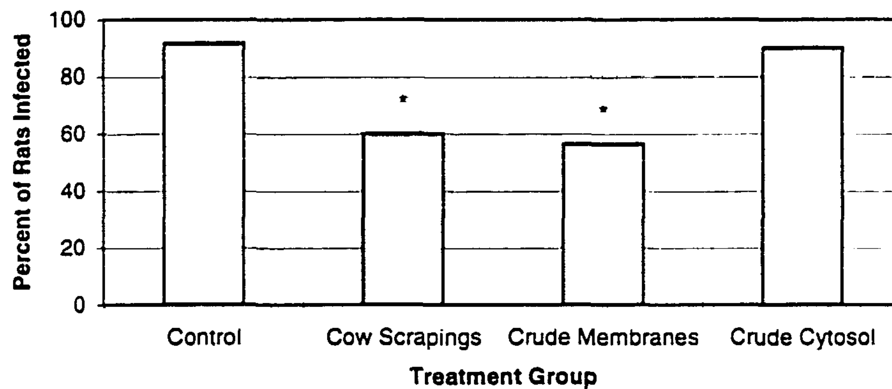

Figure 9. The effect of orally inoculating infant rats with cow intestinal scrapings, and cow intestinal scrapings divided into membrane and cytosol fractions, on reducing the percent of infant rats infected with *C. parvum*. The intestinal scrapings or their fractions were given twice a day between 3 and 14 days of age. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

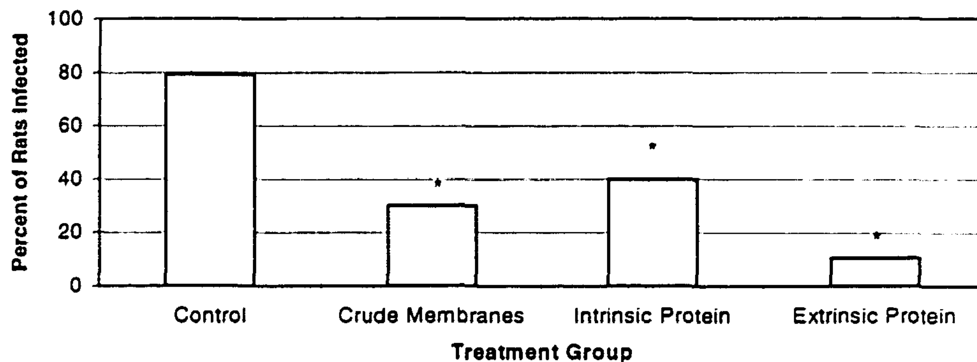

Figure 10. The effect of orally inoculating infant rats with the membrane fraction of cow intestinal scrapings, the intrinsically or the extrinsically associated membrane proteins on reducing the percent of infant rats infected with *C. parvum*. The crude membrane portion of the scrapings or its fractions were given twice a day between 3 and 14 days of age. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

Up until this point all the treatments had been given twice a day between 3 and 14 days of age. In order to determine if the treatments would still be effective if the schedule was modified, infant rats received the preparations twice a day between days 7 and 11. The percent of animals infected with *C. parvum* was similarly reduced in the animals receiving the treatment for only 5 days as it was in those receiving it for 12 days (Figure 11). The data from assessing other parameters is shown in Figures 36, 37 and 38 in the appendix and is consistent with the trend in Figure 11. In addition, the efficiency of giving the extrinsic membrane protein fraction twice a day on days 7, 8 and 9, or on days 9, 10 and 11 was compared with the effectiveness of giving it on days 7, 8, 9, 10 and 11 (Figures 64, 65, 66

and 67). Both schedules reduced the percent and rate of infection but were not as effective as the 5-day treatment.

Proteinase K, a highly active protease, was added to the extrinsic membrane protein fraction, to inactivate proteins in the preparation and to determine whether this abolished the ability of this fraction to reduce the number of rats infected with *C. parvum* (Figure 12). This treatment resulted in a loss of protective effect, suggesting that the active component in the preparation was a protein or made up partially of protein. Figures 39, 40 and 41 in the appendix show that following Proteinase K treatment there was a similar loss of effect of reducing the percent of positive microscopic fields, fecal score, and average number of oocysts per microscopic field compared to the control group.

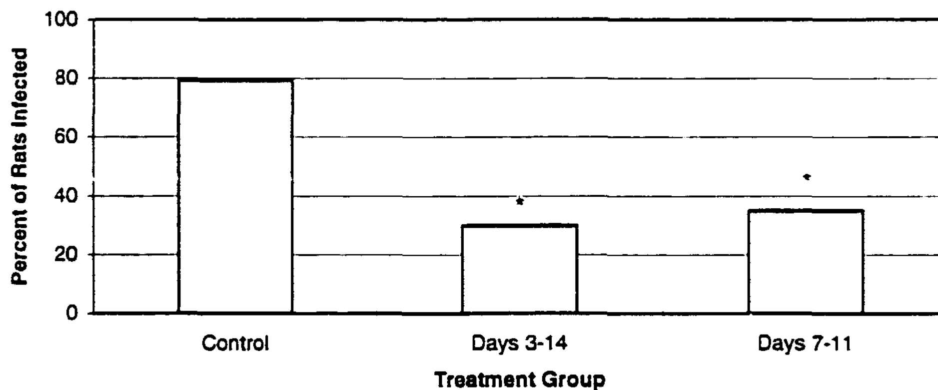

Figure 11. The effect of orally inoculating infant rats with the extrinsic membrane protein fraction of cow intestinal scrapings twice a day for 5 days compared to twice a day for 12 days on reducing the percent of infant rats infected with *C. parvum*. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

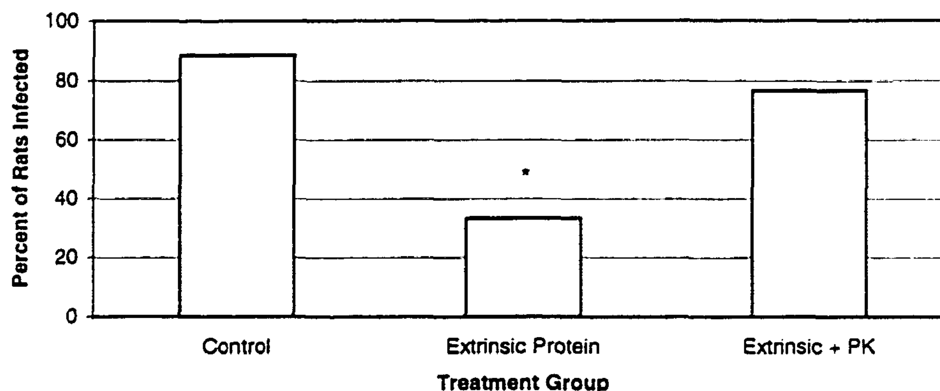

Figure 12. The effect of orally inoculating infant rats with extrinsic membrane protein with or without Proteinase K (PK) treatment twice a day from days 7 to 11 of age on reducing the percent of infant rats infected with *C. parvum*. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

Experiments using intestinal tissue preparations were then extended to examine the activity of the cow extrinsic membrane protein fraction in mice inoculated with *C. parvum*.

The preparation was effective in reducing the number of mice infected with *C. parvum* as indicated by the data in Figure 13. The percent of positive microscopic fields, fecal score, average number of oocysts / field, and ileum score were also examined (Figures 42, 43 and 44 in the appendix respectively).

The extrinsic membrane protein fraction was treated with 40% ammonium sulfate to precipitate out some of the proteins. The mixture was then centrifuged to produce a pellet made up of the ammonium sulfate precipitate and a supernatant made up of the ammonium sulfate soluble fraction. The results of testing these two fractions in infant rats is shown in

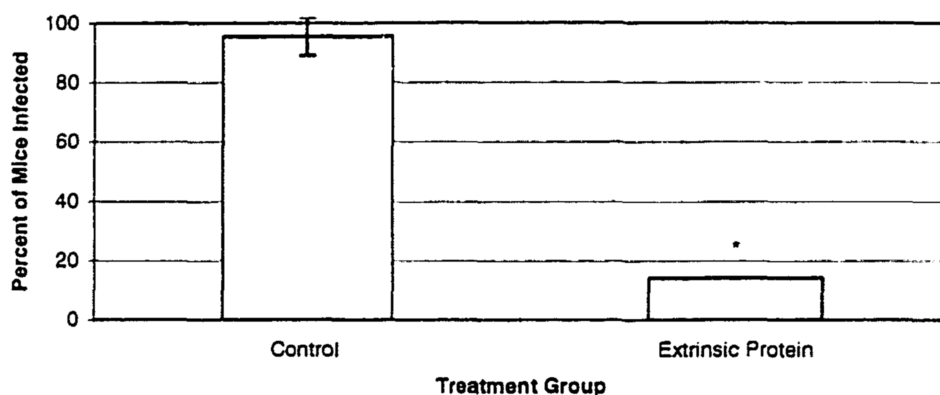

Figure 13. The effect of orally inoculating infant mice with cow intestinal extrinsic membrane protein fraction twice a day from days 7 to 11 of age on reducing the percent of infant mice infected with *C. parvum*. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values are means ± SEM, n = 2. Values with * are significantly different from the control group by X^2 analysis.

Figure 14. The fraction containing the proteins that had been precipitated out using ammonium sulfate was twice as effective at reducing the number of infant rats infected with *C. parvum* than the fraction that contained the soluble proteins. More information is available in Figures 45, 46 and 47 in the appendix.

The ammonium sulfate precipitated fraction of the extrinsic membrane proteins was further purified by ion exchange chromatography. The fractions were eluted off of the column using a sodium chloride gradient ranging from 150 mM-1 M NaCl. The 25 ml fractions were collected and measured for protein content at 280 nm using a spectrophotometer (Figure 15).

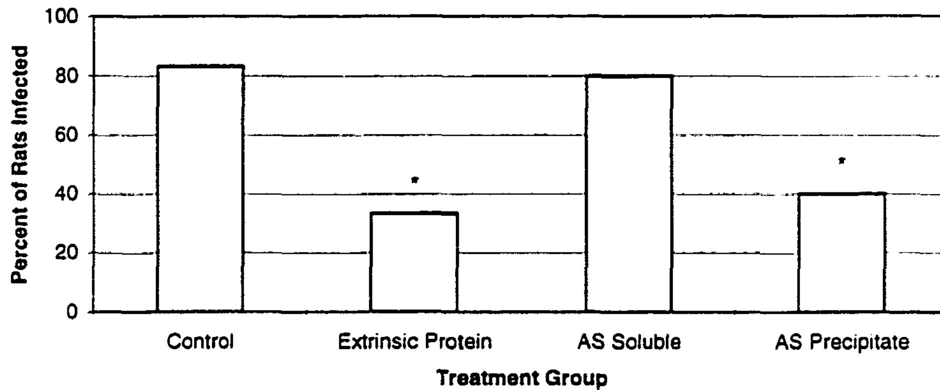

Figure 14. The effect of orally inoculating infant rats with extrinsic membrane protein, extrinsic membrane protein ammonium sulfate precipitate or soluble fractions twice a day from days 7 to 11 of age on reducing the percent of infant rats infected with *C. parvum*. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

The 25 ml fractions collected off of the DEAE column were then pooled according to peak profiles (see Figure 15) to form larger fractions that were then dialyzed using ultra filtration pressure dialysis to dialyze out the excess. The 4 fractions were then tested for their ability to reduce the number of *C. parvum* infected infant rats as shown in Figure 16. Fractions 1 and 3 were the most effective at reducing the percent of infected rats with *C. parvum*, by approximately one half compared to those in the control, while fractions 2 and 4 were not significantly different than the controls. Figures 48, 49 and 50 in the appendix show similar effects on the percent of positive microscopic fields of fecal samples, fecal score, and average number of oocysts per microscopic field.

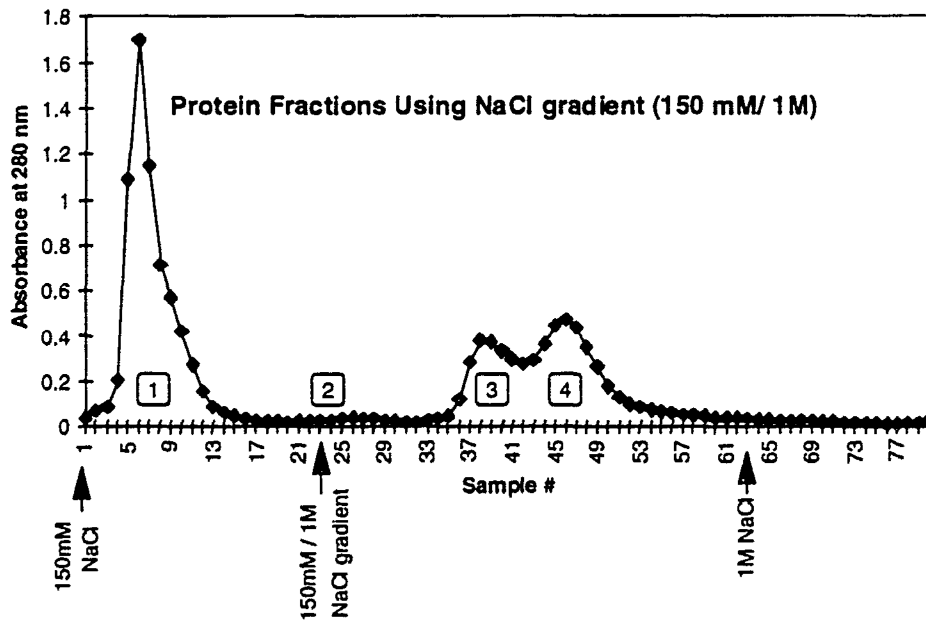

Figure 15. The absorbence readings at 280 nm for the 25 ml fractions collected off of the DEAE column using a 150 mM-1 M NaCl gradient. Numbers 1, 2, 3 and 4 represent the fractions formed by pooling samples 1-15, 16-34, 35-42, 43-53 respectively.

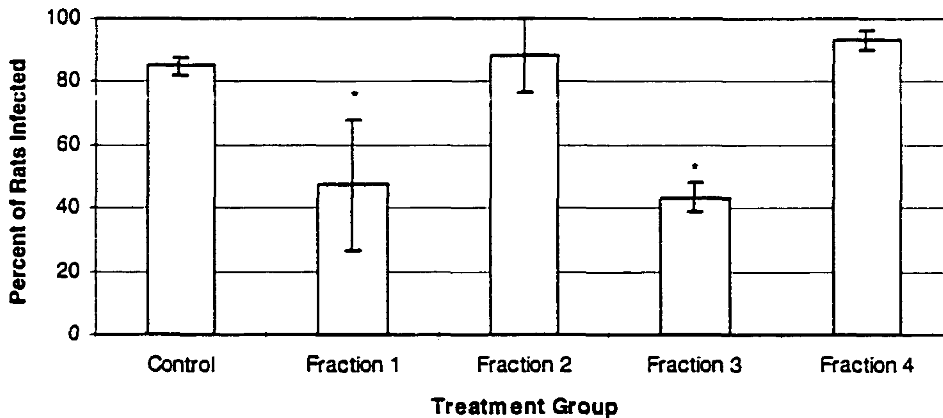

Figure 16. The effect of orally inoculating infant rats with one of the 4 fractions, collected by pooling the samples eluted off of the DEAE column, twice a day from days 7 to 11 of age on reducing the percent of infant rats infected with *C. parvum*. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values are means ± SEM, n = 3. Values with * are significantly different from the control group by X^2 analysis.

To follow the changes in protein content with the progression of purification steps, the amount of total protein in each sample was measured (Table 1) and then 2 SDS-PAGE gels were run. One was stained using Coomassie Blue and the other using a silver stain. The gels were loaded with samples, of equivalent total protein content, from the cow intestinal scrapings, crude membrane fraction, extrinsic membrane protein fraction, ammonium sulfate precipitated fraction, first DEAE-Sephacel active fraction, second DEAE-Sephacel active fraction respectively. The protein bands as they appear in the different stages of the purification steps are shown in Figure 17. The results indicate that some bands are enriched at around the 35 kDa point in the first DEAE-Sephacel active fraction and at locations 28, 45, and 60 kDa in the second DEAE Sephacel active fraction.

Table 1. Total protein content of each of the purification steps as measured using the Bio-Rad Protein Assay.

Step	Total Protein (mg/ml)
Cow Intestinal Homogenate	52.26
Crude Membrane Fraction	13.97
Extrinsic Membrane Protein Fraction	4.35
Ammonium Sulfate Precipitate	1.94
First DEAE-Sephacel Active Fraction	1.19
Second DEAE-Sephacel Active Fraction	0.1

Bromodeoxyuridine (BrdU), a thymidine analog, was used to measure the amount of enterocyte cell proliferation in the crypts of the ileum of infant rats. The data representing the difference in BrdU incorporation in enterocytes lining the crypts of normal rats, rats that received extrinsic membrane protein, rats that were infected with *C. parvum*, and rats that were infected with *C. parvum* and received doses of extrinsic membrane protein can be seen

in Figure 18. All data were collected at 15 days of age. From the data it appears that there was increased proliferation in the groups that received extrinsic membrane protein, *C. parvum*, or *C. parvum* with doses of extrinsic membrane protein compared to the group that received no treatment at all. The group that received both *C. parvum* and extrinsic membrane protein did not appear to have more proliferation than the groups that got either treatment alone.

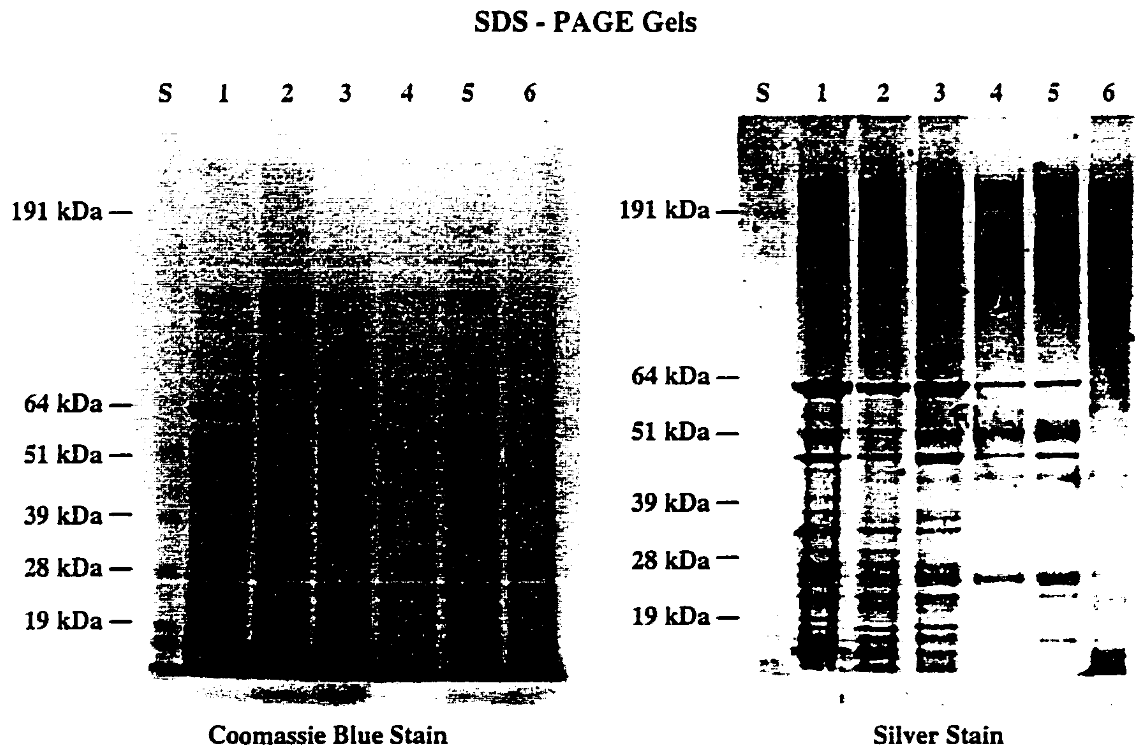

Figure 17. SDS-PAGE of the different purification steps of the cow small intestinal scrapings. The gels were stained with Coomassie Blue or Silver stain. Lane S, SeeBlue standard; lane 1, cow intestinal homogenate; lane 2, crude membrane fraction; lane 3, extrinsic membrane protein fraction; lane 4, ammonium sulfate precipitated fraction of extrinsic membrane proteins; lane 5, first DEAE-Sephacel active fraction (fraction 1 in figures 15 and 16); lane 6, second DEAE-Sephacel active fraction (fraction 3 in figures 15 and 16). Forty µg of each sample was loaded per lane.

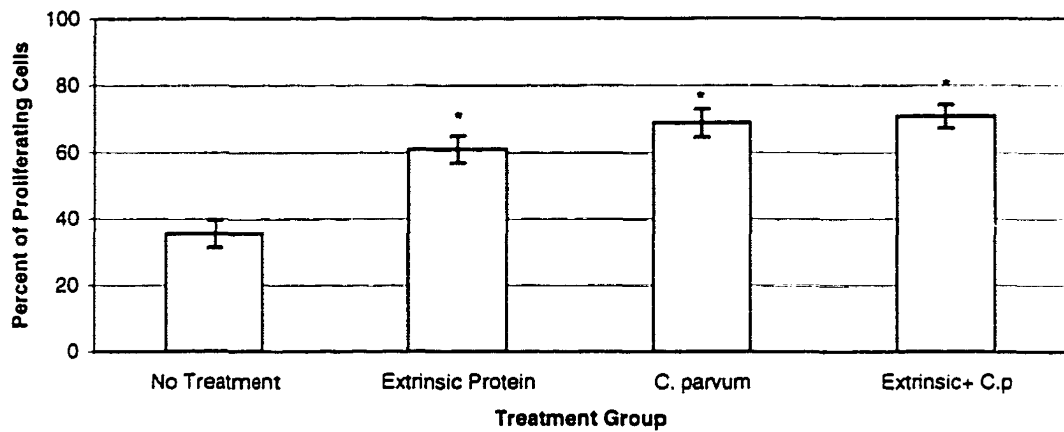

Figure 18. The effects of no treatment, treatment with extrinsic membrane protein, infection with *C. parvum*, and *C. parvum* infection and treatment with extrinsic membrane protein on cell proliferation in the crypts of the ilea of 15 day old rats as measured by BrdU incorporation. Rats that were treated with extrinsic membrane protein received it orally, twice a day from days 7 - 11 of age. Animals that were given *C. parvum* oocysts received them at 9 days of age. Values are means ± SEM, n = 20. Values with * are significantly different from the control group by X^2 analysis.

DISCUSSION

Suckling infant rats are susceptible to *C. parvum* infection while weaned juvenile and adult rats are resistant, even without prior exposure to the parasite. This indicates that a difference between the infant and adult is responsible for the susceptibility or resistance to infection.

The presence of a factor in the intestine that is protective against *C. parvum* in the adult animal may be responsible for their resistance. In that case, introducing the protective factor, taken from the adult intestine, to the infant, could result in the transfer of protection from the adult to the infant.

When adult rat ileal scrapings were fed orally to infant rats, there was a significant reduction in the percent of rats that were infected with *C. parvum*. The infection rate was half of that in the controls which received no treatment, indicating that something in the adult intestine was protecting the infants from *C. parvum* infection.

It was possible that adult gut microflora was being introduced along with the adult ileal scrapings to the infant thereby introducing bacteria that could compete with *C. parvum* for binding sites in the gut (37), thereby causing a reduction in the infection rate. Resident microflora have been shown to compete with exogenous bacteria for attachment sites, thus preventing their colonization (59). Bacterial microflora, in some cases, produce metabolic byproducts such as short chain fatty acids, which inhibit the multiplication of enteric pathogens or are bactericidal for some species such as *Shigella flexneri* (60). Acid producing bacteria, such as lactobacillus and bifidobacterium, have been shown to inhibit the growth of a variety of other bacteria by reducing the pH of the surrounding environment making it

unsuitable for bacteria such as *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* or *Clostridium perfringens* (59).

A group of researchers conducted a study to identify the bacteria involved in preventing colonization with *Clostridium botulinum* in conventional mice (61). *C. botulinum*, the cause of an age dependent illness in infants, has been shown to infect infant mice between 7 and 13 days of age (61). Adult germfree mice were also found to be susceptible to infection, while conventional adult mice were highly resistant (61, 62). Thus, it appears that the infants and germfree animals were susceptible due to the absence of the microorganisms usually present in adult mice (62, 63).

Adult gnotobiotic mice were inoculated with a limited bacterial mix or a full flora mix. They were all challenged with *C. botulinum* (61). The mice with the limited bacterial mix were not completely protected from infection but did have a significantly reduced number of deaths compared to controls, which received no bacteria at all. The gnotobiotic mice, which received the full bacterial mix, were resistant to the maximum spore challenge and showed no signs of disease. Thus, it appeared that a specific strain(s) of bacteria that was absent from the limited mix, but present in the full mix, protected some of the animals from infection (61).

Clostridium difficile is the cause of neonatal diarrhea in both germfree and conventional young hares (64), but not all hares that excrete *C. difficile* in their feces develop diarrhea. Thus, it appears that other components of the bacterial flora are capable of having protective effects against *C. difficile* (64). A study conducted to examine the effects of the microflora on infection with *C. difficile* showed that inoculating young hares with a complex

flora derived from a healthy hare protected 100% of the hares against *C. difficile* compared to the gnotobiotic hares that had no flora. Low numbers of *C. difficile* were collected from the feces of the hares that got the flora mix while high numbers were isolated from the gnotobiotic animals with diarrhea (64).

Infant mice and adult germfree mice are both susceptible to colonization with *C. parvum* while conventional adult mice are not (3, 45). It has been suggested that the flora is responsible for or plays a role in the resistance to infection with *C. parvum* (3). The microflora may exert their effects by directly binding to sites that *C. parvum* bind to in order to invade cells (3).

To make sure that the protective activity in the rat ileal scrapings was not a result of introducing adult microflora, the intestinal preparation was subjected to irradiation that would kill any live bacteria. The irradiated preparation was fed to infant rats and found to reduce the percent of rats that were infected with *C. parvum* to half of that in the control groups just as the non-irradiated adult rat intestinal preparation had. Therefore, it was concluded that live bacteria were not necessary for the decrease in the rate of infection.

Although it was determined that live bacteria were not responsible for the activity in the rat ileal scrapings, a bacterial or cell product might still be responsible for the reduction in the infection rate.

One of the properties used to characterize an active substance is to determine its resistance to heat and whether or not it will remain biologically active after being subjected to heat (65). A classical example are the two enterotoxins produced by *E. coli*. The first is a heat labile toxin, which activates adenylate cyclase in the enterocytes, impairing sodium

absorption and activating chloride secretion followed by the movement of water from the cell (66). Its mechanism of action resembles that caused by the *Vibrio cholera* heat-labile toxin (8, 67). Another example of a heat-labile toxin is that produced by *Bordetella pertussis* (68). The second toxin secreted by *E. coli* is the heat-stable toxin. This toxin induces secretion by activating guanylate cyclase causing an increase in the levels of intracellular cGMP in the enterocyte (65, 69).

In the present study, boiling the intestinal preparation resulted in a loss of the protective activity with the infection rate of treated animals similar to those in the control group, which received no treatment. This indicated that the substance responsible for the activity in the preparation was heat labile.

Experiments were conducted to determine if taking the ileal scrapings from calves that had been previously exposed to *C. parvum* and recovered, and were no longer susceptible to infection themselves, would protect the infant rats when fed orally as did the adult rat ileal scrapings. Results indicated that they did not and the animals that received the calf ileal scrapings were just as infected as those that received no treatment. However, when the infant rats were given ileal scrapings taken from more mature cows, they worked just as well as the scrapings taken from adult rats. This indicated that even though the calves were no longer susceptible to infection with *C. parvum* themselves, they had not yet developed the more mature intestinal characteristics which appear to be responsible for protecting adult animals from getting infected with *C. parvum*.

C. parvum colonization in the small intestine is generally restricted to the ileum in the infant or germ free animal. In the adult animal the ileum is no longer susceptible to

colonization by *C. parvum*. Thus it might be assumed that there could be a physiological difference in the ileum of the adult compared to that in the infant. To determine if the duodenum and jejunum also contain the same sort of protective activity against *C. parvum*, the scrapings from each of the sections from adult cows was tested. It was found that the duodenum and jejunum had a similar level of the transferable biological activity responsible for reducing the percent of *C. parvum* infected infant rat pups. Thus, it appeared that the activity was distributed evenly along the length of the small intestine and was not confined to the ileum. All of the experiments from that point on were conducted with cow intestinal scrapings from the length of the small intestine because of the large volume that could be collected at one time.

To further characterize the active portion, the cow intestinal scrapings were centrifuged at 100,000 x *g* for 60 minutes. The process resulted in 2 fractions: the pellet containing crude membranes consisting of whole cells, nuclei, plasma membranes, mitochondria, lysozomes, microsomes and small vesicles, and the supernatant containing the cytosol consisting of the cell cytoplasm and fractions not bound by organelles or membranes (70). The majority of the activity was found to be located in the crude membrane fraction.

All biological membranes contain proteins, lipids and sometimes carbohydrates. The ratios of these components depend on the membrane source, but in general, all biological membranes are found to contain proteins (71, 72). These proteins are either bound to the membrane surface (extrinsic or peripheral membrane proteins) or are more firmly bound with regions buried into the membrane (intrinsic or integral membrane proteins) (71, 72).

The extrinsic proteins are usually bound to the membrane through interactions with integral membrane proteins or lipids. These proteins can be removed by relatively mild treatments such as high salt concentrations, which disrupt the ionic bonds, or a change in pH. The intrinsic proteins, on the other hand, are more firmly bound and integrated within the membrane. They require the action of detergents or organic solvents to disrupt the hydrophobic interactions (71, 72).

The crude membrane fraction was treated with sodium carbonate (pH 11.5) which precipitated the extrinsic proteins (73). The preparation was then centrifuged to separate the membrane fraction containing the intrinsic proteins from the extrinsic proteins. After adjusting the pH and dialyzing out the sodium carbonate, the two fractions were then tested in infant rats. The volume of the preparations resulting from each purification step was adjusted using PBS to ensure that the concentration of all components remained the same as it was in the original scrapings preparation.

Both of the fractions were shown to have activity but the extrinsic membrane protein fraction was 4 times more effective than the intrinsic membrane protein fraction at reducing the percent of infant rats infected with *C. parvum*. Protein separation may not have been complete; it is possible that some of the activity retained in the membrane fraction containing the intrinsic membrane proteins was due to residual levels of extrinsic membrane proteins.

A variety of treatment schedules were designed in an attempt to verify the point in the treatment schedule where the treatment was most effective. This might also increase understanding of how the treatment was working. If the activity was seen, by treating just prior to inoculation, this could suggest that either direct binding to the cells, oocysts or

sporozoites was preventing colonization. If the treatment was found to be effective only when given for a few days prior to challenge, this could suggest that a change was occurring in the cells causing them to become less susceptible to invasion. If the treatment was found to be effective when given after the challenge dose, this could suggest that the treatment was interfering with the binding of other lifecycle stages (sporozoites, merozoites or microgametes).

When the rat ileal scrapings were given only once at 9 days of age before inoculating the rats with oocysts, the percent of infected rats was not significantly reduced from those in the control group. This suggested that the treatments needed to be given over a longer period of time in order to be effective. In another study cow ileal scrapings were given once a day on days 3, 5, 7, 9, 11 and 13. This, too, was not effective in reducing the infection rate in the infant rats. When the infant rats were orally inoculated with the extrinsic membrane proteins twice a day on days 7, 8 and 9 or twice a day on days 9, 10 and 11, the percent of infected animals in both groups was significantly lower than those of the control group. However, the group receiving twice daily treatments from days 7 to 11 had the lowest percentage of infected animals and therefore, we continued to use that schedule for the remainder of the experiments.

Treating the preparation with a protease, Proteinase K, caused a significant reduction in the activity of the extrinsic membrane fraction resulting in the inability to decrease the percent of animals infected with *C. parvum*. This indicated that the active compound was a protein or at least made up partly of protein.

The extrinsic membrane protein fraction is approximately 10 kDa or larger in size because the activity was retained in the fraction containing all molecules larger than 10 kDa after undergoing ultrafiltration pressure dialysis. This rules out small peptides found in the intestinal tissue such as the defensins, antimicrobial peptides, that are approximately 3.5 kDa and are secreted by Paneth cells located in the small intestinal crypts (74). Putrescine, a polyamine, approximately 200 Da in size is one of the polyamines which have been shown to play a role in promoting gastrointestinal mucosal growth and crypt cell proliferation (41, 75). Putrescine has been shown to provide almost complete protection against *C. parvum* infection in mice when administered orally from days 3-21 of age (76). Due to its small size, it s unlikely to be the active component in the extrinsic membrane protein fraction.

Up until this point all experiments were conducted in rats. When the preparation was tested in infant mice, it was to be just as effective as it was in infant rats at reducing the percent of infection with *C. parvum*. This suggested that the active factor that protected both the rats and mice may not be species specific. The infant rats were protected by scrapings derived from both adult rat and adult cow small intestine. Thus, it appears that the active agent is a physiologic factor related to cell maturation in the intestine of mammals.

There are a number of salts used to precipitate proteins out of solution. One of the best and most commonly used salts for this procedure is ammonium sulfate (77). By increasing the amount of salt in a solution the number of ions, competing to stay in solution by interacting with the solvent molecules, also increases. This causes the solutes to precipitate out.

A preparation of 40% ammonium sulfate was used to precipitate some of the proteins from the extrinsic membrane protein fraction. The preparation was then centrifuged yielding 2 fractions; the fraction containing soluble proteins and the fraction containing proteins that had precipitated out. Both fractions were tested in infant rats and the majority of the activity was found to reside in the ammonium sulfate precipitate fraction. It appeared to be twice as effective as the soluble fraction in reducing the percent of rats infected with *C. parvum*.

Ion exchange chromatography was then utilized to further separate the precipitate fraction. Ion exchange chromatography is useful for the purification of proteins and enzymes (78). Proteins carry both negative and positive charges with the net charge determined by the pH of the environment surrounding the protein. The technique involves the use of a stationary phase that is bound to either anions or cations. The protein solution contains many proteins that bear both positive and negative charges and these compete for binding to the stationary phase (78). The proteins bearing negative charges will bind to positively charged columns while those bearing positive charges will bind to negatively charged columns (79). The column is then washed with a buffer solution with a known pH and salt concentration. The ions in the salt solution then proceed to displace some of the proteins, which are bound to the column because of their greater affinity for the charges on the stationary phase (78). This causes the proteins, which are bound to the column to be released and wash out. As the salt concentration of the buffer added to the column increases, more proteins are released and collected in the eluate (80). The stationary phase used in our experiment was a DEAE-Sephacel gel with the anionic exchanger group diethylaminoethyl, which binds and exchanges anions. The fractions were eluted from the column and collected using an

automated fraction collector. The material eluted off of the column was measured for protein levels using a spectrophotometer at an absorbence of 280 nm. The eluted material was then pooled into 4 fractions according to the protein profiles (Figure 15). The fractions were then dialyzed and tested in infant rats. Both fractions 1 and 3 were found to have the ability to reduce the percent of rats infected with *C. parvum* compared to those in the control group (Figure 16). The 2 fractions may be related structurally or may be two separate entities altogether acting through different mechanisms.

Another method for separating proteins is electrophoresis in sodium dodecyl sulfate (SDS). This technique is based on the migration of proteins in an electric field depending on the charges they carry. This method is used to determine the molecular weight and purity of proteins (81). The SDS binds to most proteins in amounts proportional to the molecular weight of the protein (82) contributing a large net negative charge. Because of their small mass, smaller proteins migrate more rapidly towards the anode while larger proteins move more slowly. The gels containing the proteins are then removed after the completion of electrophoresis and the protein bands can be visualized by staining (83). Both Coomassie Blue, which binds proteins rich in lysine and arginine, and silver stain, which is a more sensitive stain that reacts with free amines and sulfur groups on proteins were used (83).

Gels were run to look at the variations in protein banding in each of the cow intestinal scrapings, crude membrane fraction, extrinsic membrane protein fraction, ammonium sulfate precipitated fraction, first DEAE-Sephacel active fraction, and second DEAE-Sephacel active fraction. We found that some bands appeared to be enriched at around the 35 kDa point in the first DEAE-Sephacel active fraction and at locations 28, 45, and 60 kDa in the second

DEAE-Sephacel active fraction. Further purification steps are required to get a cleaner preparation containing less protein bands.

It was proposed to look at the mechanisms through which this protective factor found in the adult rat and cow intestines could be acting to prevent or reduce infection in the susceptible infant. One mechanism could involve the increase in enterocyte proliferation, as it occurs in the adult, thereby reducing the time available for the parasite to invade and infect the cells before being sloughed off with the cells. It is also possible that other changes associated with the maturation of infant cells are taking place causing it to take on characteristics more like those in the adult, which could in turn cause an increase in the turnover rate.

Bromodeoxyuridine (BrdU) was used, which incorporates into dividing cells (84), to assess cell proliferation in rats that were given the extrinsic membrane proteins twice a day from days 7 to 11 of age with or without being challenged with *C. parvum*, rats that received only *C. parvum*, and rats that received neither treatment. There was increased proliferation in rats that got only the extrinsic membrane protein treatments, only *C. parvum*, and the rats that received both. It was found that the percent of proliferating cells in the 3 treatment groups was nearly double that of the animals that received no treatment at all.

It was shown that proliferation occurred in the group that only received the *C. parvum* challenge. It appeared that the infection was causing or triggering a change in the cells causing them to proliferate more rapidly. It may have been that the damage to the cells as a result of infection was causing them to slough off and die, resulting in an increase in

proliferation to replace the damaged and infected cells in the intestine to maintain the integrity of the intestinal epithelial cell barrier.

In a study conducted on TCR-α deficient mice, the same observations of increased enterocyte proliferation were made in mice that were infected with *C. parvum* compared to the non-infected controls (29).

This observation has been described in other diseases such as the proliferative intestinal enteropathy associated with the bacterium *Lawsonia intracellularis* (25) and that observed in pigs exposed to the gastroenteritis virus (27). The increase in cell proliferation, as a result of tissue damage, appears to be the result of increased activity in the dividing crypt cells to make up for losses on the villi tips.

A study was conducted to look at the effects of *C. parvum* infection on sucrase and lactase expression in infant mice, cell proliferation was also measured using BrdU (28). Mice infected with *C. parvum* showed increased sucrase, and decreased lactase activity, and increased cell proliferation compared to the non-infected controls at 2 weeks of age (28). Since the changes in enzyme expression coincided with the increased rate of enterocyte proliferation, the authors suggested that the two events were related. They proposed that the increase in sucrase expression and decrease in lactase levels at an age earlier than normal was due to the enterocyte proliferation. If the enterocytes are programmed to undergo a certain number of cell divisions before the changes in brush border enzymes take place, then the increased proliferation may have caused the more mature expression of enzymes to occur earlier than normal (28).

Other changes resembling those in the more mature animals may also be taking place as a result of the increase in cell turnover. Thus, if the extrinsic membrane protein is causing increased proliferation, it may also be changing some of the characteristics of the cells making them less susceptible to invasion by *C. parvum*.

Like other proteins expressed on the cell surface, the extrinsic membrane protein derived from the cow intestinal scrapings may be expressed or reconfigured during the time of weaning. A variety of antigens have been identified that are exclusively expressed only on suckling infant rat intestinal villus cells (32). The protein antigens YBB1/89, YBB 2/29, and YBB 2/95 were found to be expressed on the villus cells of infant rat pups. The expression and distribution of these antigens was observed to change at the time of weaning (32). These antigens could not be detected in the intestinal tissue of adults. The disappearance of these antigens was found to coincide with the appearance of the enzyme sucrase-isomaltase (32). Similar antigens may play a role in the susceptibility of the intestinal tissue in the infant to infection with *C. parvum* or render an adult resistant to the disease.

The active component may play a role in blocking the attachment by direct binding, or by steric hindrance of one of the life cycle stages of *C. parvum* such as the sporozoite, merozoite or microgamete stages. It is not yet clear how sporozoites attach to enterocytes in order to invade them. It is speculated that it may involve specific receptors or a ligand type of binding between the cell and sporozoite. A previous study examined sporozoite binding to the human intestinal epithelial cell line Caco-2 (33). Galactose and *N*-acetylgalactosamine specific surface lectins were identified on the sporozoite surface and suspected to play a role in adhesion to enterocytes (34, 35). When specific glycoprotein inhibitors to that attachment

were added to the cell culture along with the sporozoites they were found to reduce attachment to the cells compared to cultures where the inhibitors were not added (34). A 900 kDa surface glycoprotein, GP900, located on sporozoites and merozoites, both invasive forms of the life cycle stages of *C. parvum*, has also been identified (36). The ability of the sporozoites and merozoites to attach to the cell surfaces in cell culture through GP900 was blocked by anti GP900 antibodies.

The protective factor, which exists in the adult intestinal scrapings, may be active through a similar mechanism. It may bind directly to one of the lifecycle stages allowing them to be washed away with the other gut contents or it may bind somewhere on the host cells preventing the attachment of *C. parvum*. In the adult animal it may protect by being expressed on the cells and then released as cells slough off or secreted into the lumen where it binds the various *C. parvum* life cycle stages. It may also act by binding somewhere on the host cells thereby causing changes in the cells which in turn render them less susceptible to infection, thus acting through an indirect route to protect the cells from invasion by *C. parvum*. That may be why treating only once is not enough to reduce infection in the infant rats, but why multiple treatments before and after the inoculum are more effective.

Other proteins in the crude preparation may cause changes in the infant enterocytes that are not related to how the compound is causing its effect, thereby making it more difficult to identify how the active component is working. Further purification of the fractions collected from the ion exchange column is required in order to identify and isolate the active compound and to better understand its mechanism(s) of action. One method that may be utilized to isolate the active component is through using a technique called

representational difference analysis (RDA). Representational difference analysis is a subtractive hybridization technique used to identify and isolate a gene, gene fragment, or gene product that exists in one population or specimen but not the other (85, 86). This technique can be applied to isolate the active component(s) in the intestinal rat scrapings by taking the intestinal scrapings from infant animals and from genetically identical littermates raised to adulthood. Double stranded complementary DNA (cDNA) is derived from the original mRNA by reverse transcription and PCR is then utilized to amplify the products. The representations are then subtracted from one another by hybridization and all similar products are degraded and differences are enriched and amplified by PCR (85).

In conclusion, the identification and purification of the adult intestinal protein(s) involved in protecting the infant rats, as described in this study, may result in a better understanding of the mechanisms involved in the attachment and invasion of host cells by *C. parvum* and may ultimately result in the production of effective preventative treatments or disease therapies.

APPENDIX

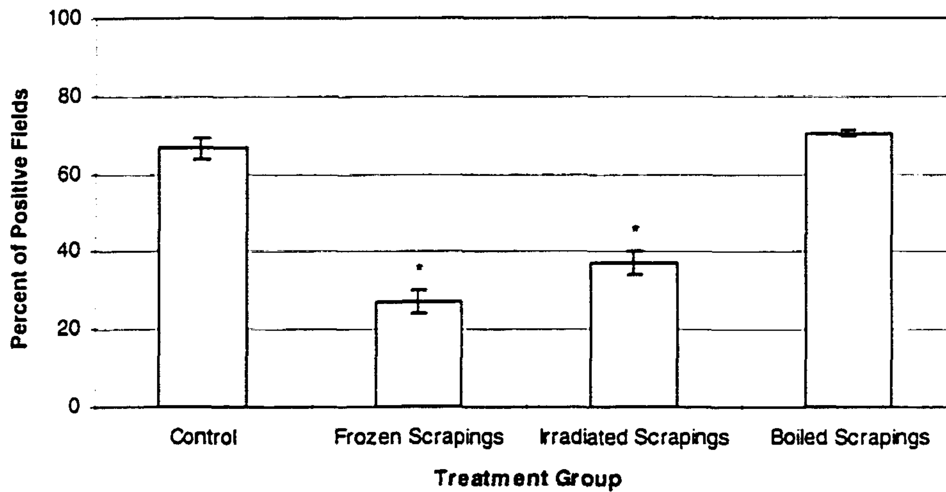

Figure 19. The effect of orally inoculating infant rats with frozen, irradiated, or boiled adult rat ileal scrapings on reducing the percent of microscopic fields positive for oocysts. The various forms of ileal scrapings were given twice a day between 3 and 14 days of age. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values are means ± SEM, n = 2. Values with * are significantly different from the control group by X^2 analysis.

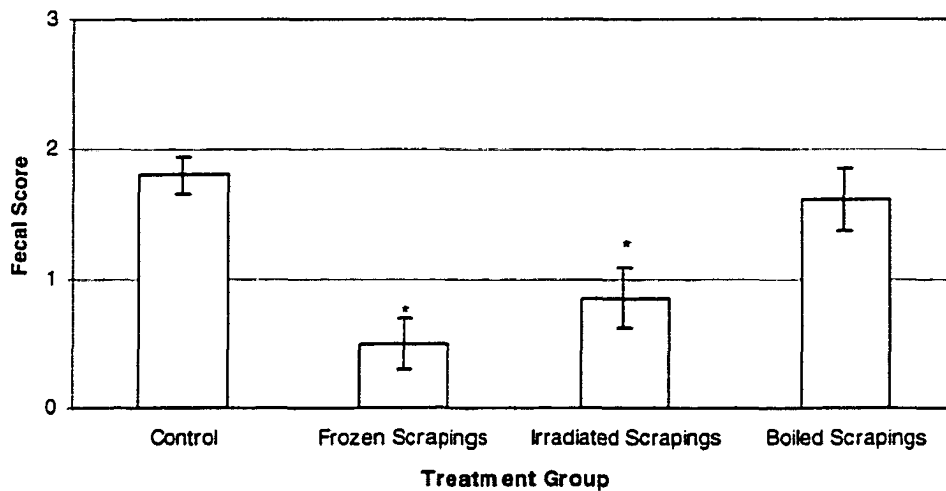

Figure 20. The effect of orally inoculating infant rats with frozen, irradiated, or boiled adult rat ileal scrapings on reducing the fecal score. The various forms of ileal scrapings were given twice a day between 3 and 14 days of age. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 72 for controls, 60 for Frozen and irradiated scrapings and 36 for boiled scrapings. Values with * are significantly different from the control group; $p \leq 0.05$.

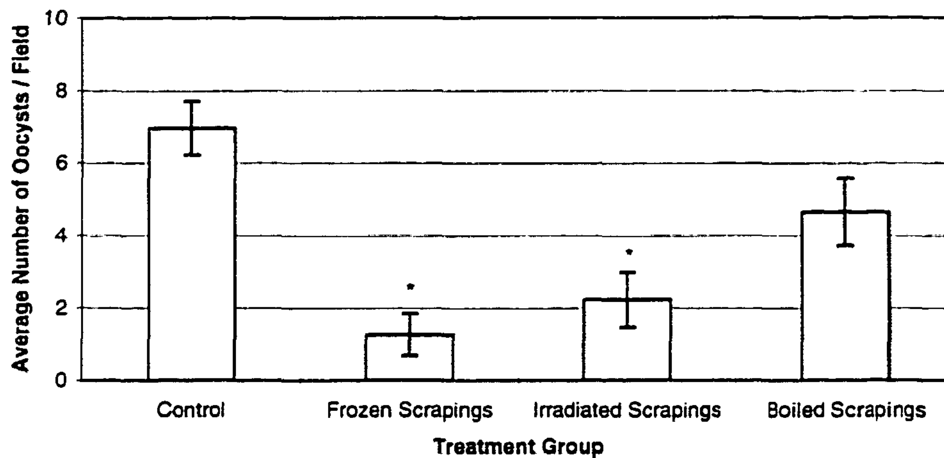

Figure 21. The effect of orally inoculating infant rats with frozen, irradiated, or boiled adult rat ileal scrapings on reducing the average number of oocysts per field. The various forms of ileal scrapings were given twice a day between 3 and 14 days of age. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 72 for controls, 60 for Frozen and irradiated scrapings and 36 for boiled scrapings. Values with * are significantly different from the control group; $p \leq 0.05$.

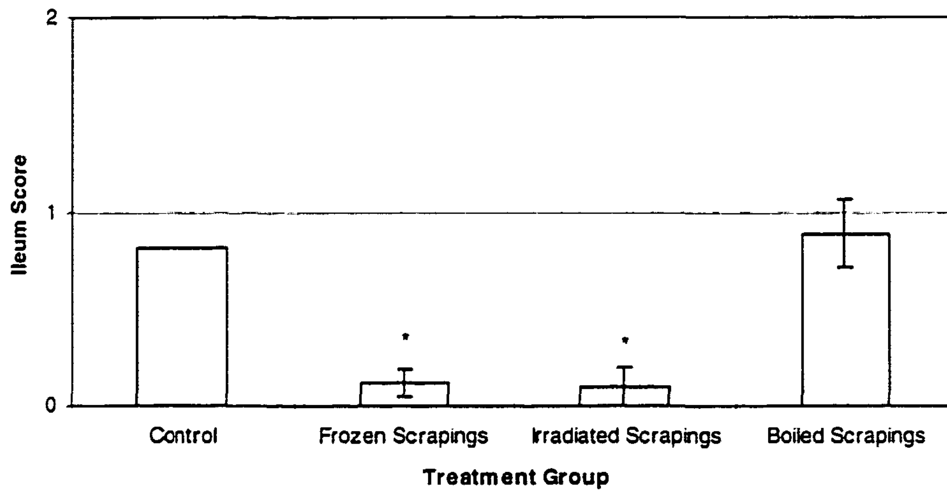

Figure 22. The effect of orally inoculating infant rats with frozen, irradiated, or boiled adult rat ileal scrapings on reducing the ileum score. The various forms of ileal scrapings were given twice a day between 3 and 14 days of age. The ileal score was calculated by assessing the intensity of *C. parvum* infection in the ileum of each animal at 15 days of age using light microscopy. A score of 0 was given to indicate that no *C. parvum* was present, 1 to indicate a light to moderate infection and 2 to indicate the presence of a heavy infection. Values are means ± SEM, n = 72 for controls, 60 for Frozen and irradiated scrapings and 36 for boiled scrapings. Values with * are significantly different from the control group; $p \leq 0.05$.

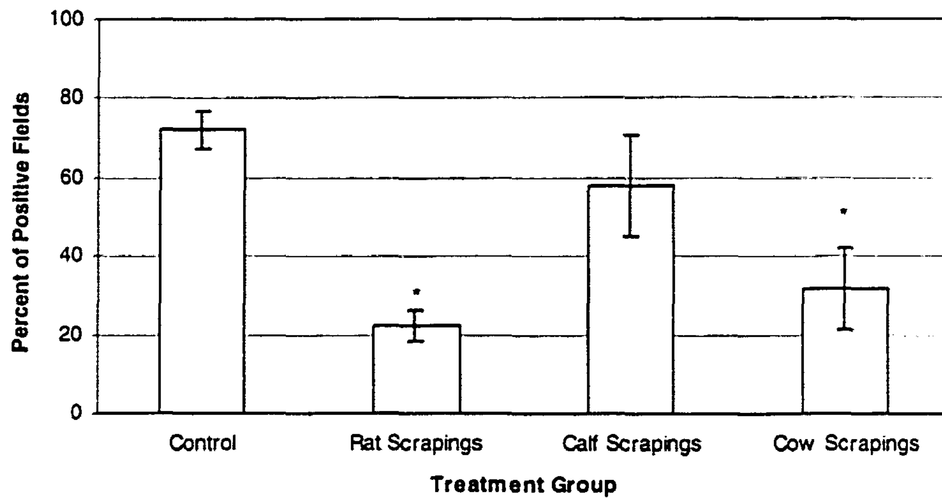

Figure 23. The effect of orally inoculating infant rats with rat, calf, or cow ileal scrapings on reducing the percent of microscopic fields positive for oocysts. The ileal scrapings from the different sources were given twice a day between 3 and 14 days of age. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values are means ± SEM, n = 2. Values with * are significantly different from the control group by X^2 analysis.

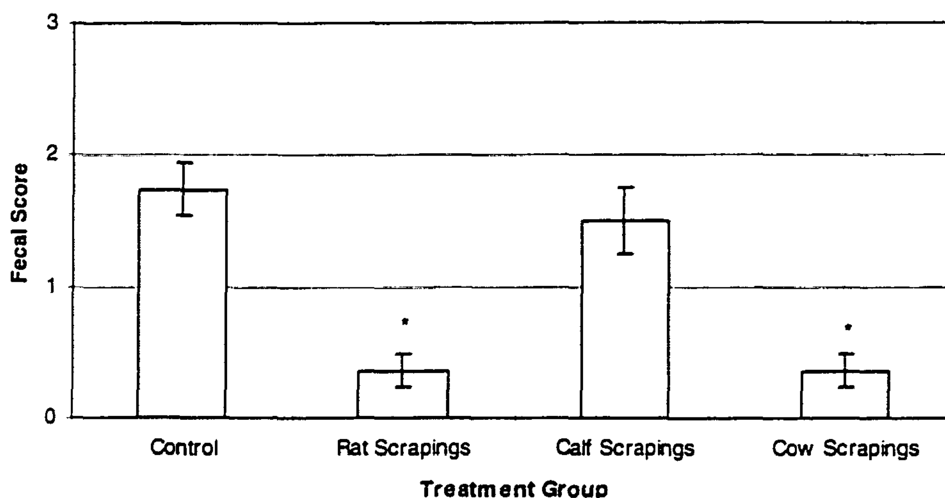

Figure 24. The effect of orally inoculating infant rats with rat, calf, or cow ileal scrapings on reducing the fecal score. The various forms of ileal scrapings were given twice a day between 3 and 14 days of age. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 72 for controls, and 60 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

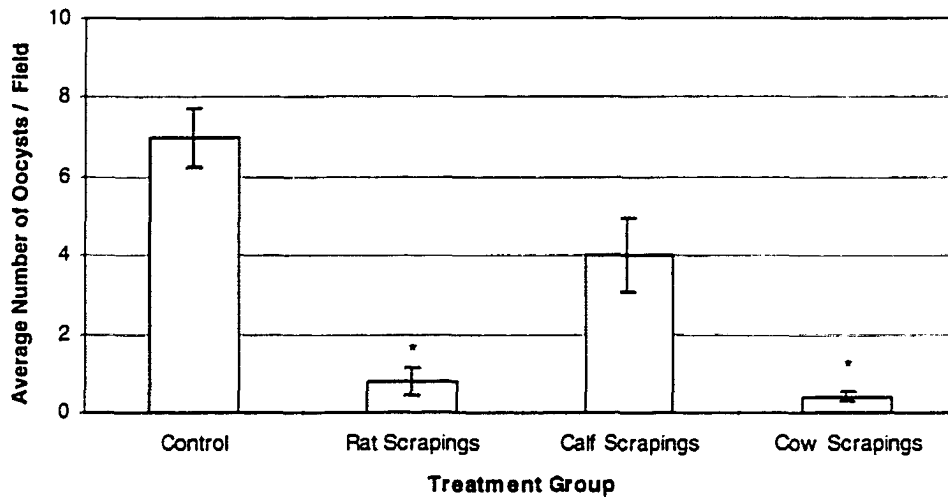

Figure 25. The effect of orally inoculating infant rats with rat, calf, or cow ileal scrapings on reducing the average number of oocysts per microscopic field. The various forms of ileal scrapings were given twice a day between 3 and 14 days of age. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 72 for controls, and 60 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

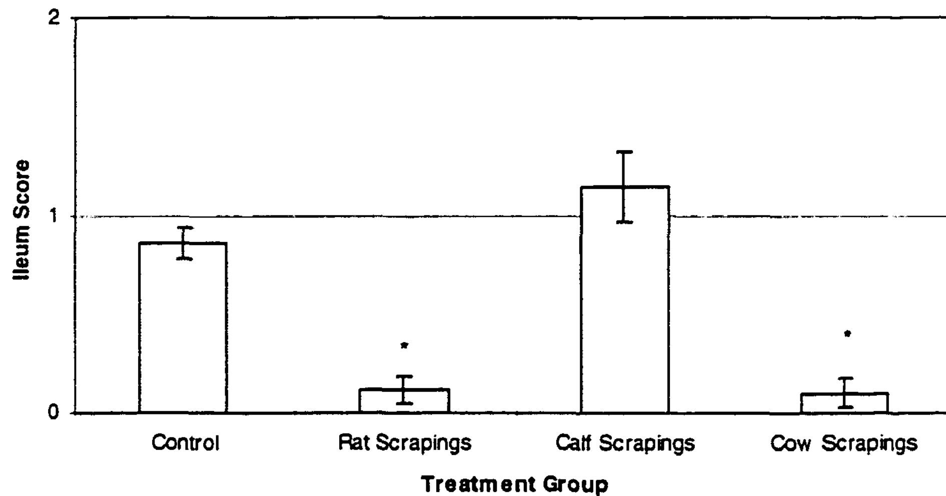

Figure 26. The effect of orally inoculating infant rats with rat, calf, or cow ileal scrapings on reducing the ileum score. The various forms of ileal scrapings were given twice a day between 3 and 14 days of age. The ileal score was calculated by assessing the intensity of *C. parvum* infection in the ileum of each animal at 15 days of age using light microscopy. A score of 0 was given to indicate that no *C. parvum* was present, 1 to indicate a light to moderate infection and 2 to indicate the presence of a heavy infection. Values are means ± SEM, n = 72 for controls, and 60 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

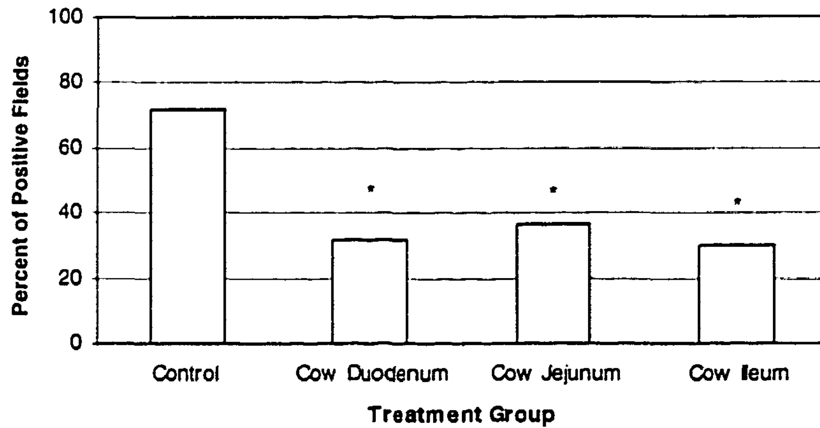

Figure 27. The effect of orally inoculating infant rats with intestinal scrapings taken from the duodenum, jejunum or ileum of adult cows on reducing the percent of microscopic fields positive for oocysts. The intestinal scrapings from the different parts of the small intestine were given twice a day between 3 and 14 days of age. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

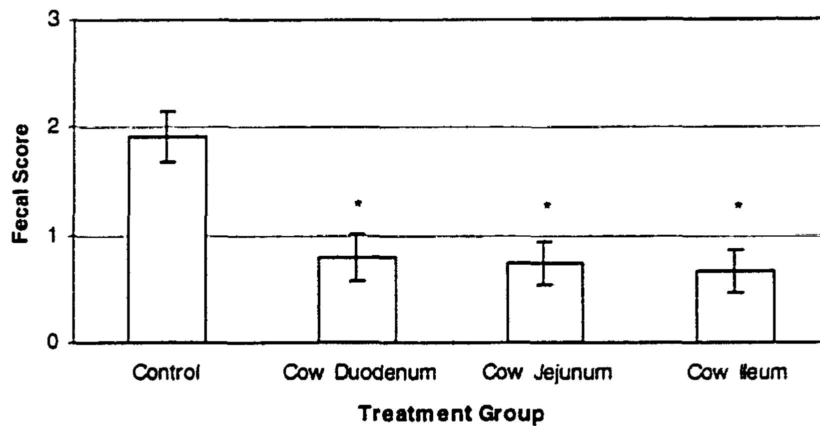

Figure 28. The effect of orally inoculating infant rats with intestinal scrapings taken from the duodenum, jejunum or ileum of adult cows on reducing the fecal score. The various sources of small intestinal cow scrapings were given twice a day between 3 and 14 days of age. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

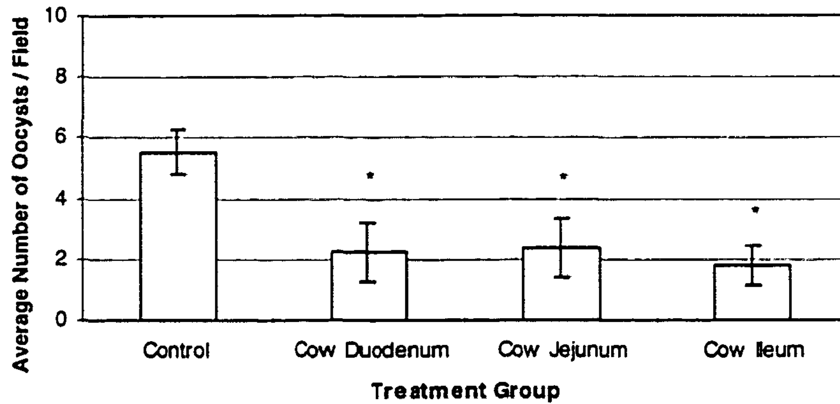

Figure 29. The effect of orally inoculating infant rats with intestinal scrapings taken from the duodenum, jejunum or ileum of adult cows on reducing the average number of oocysts per microscopic field. The various parts of the small intestinal cow scrapings were given twice a day between 3 and 14 days of age. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

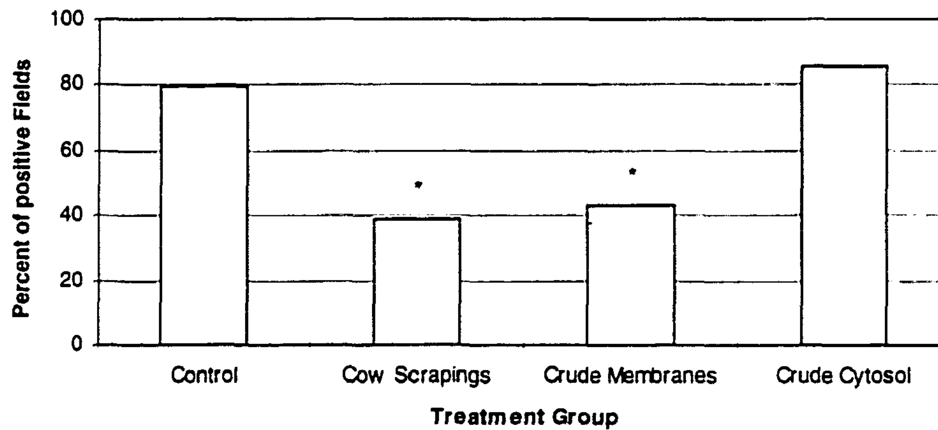

Figure 30. The effect of orally inoculating infant rats with cow intestinal scrapings, and cow intestinal scrapings divided into membrane and cytosol fractions, on reducing the percent of microscopic fields positive for oocysts. The intestinal scrapings or their fractions were given twice a day between 3 and 14 days of age. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

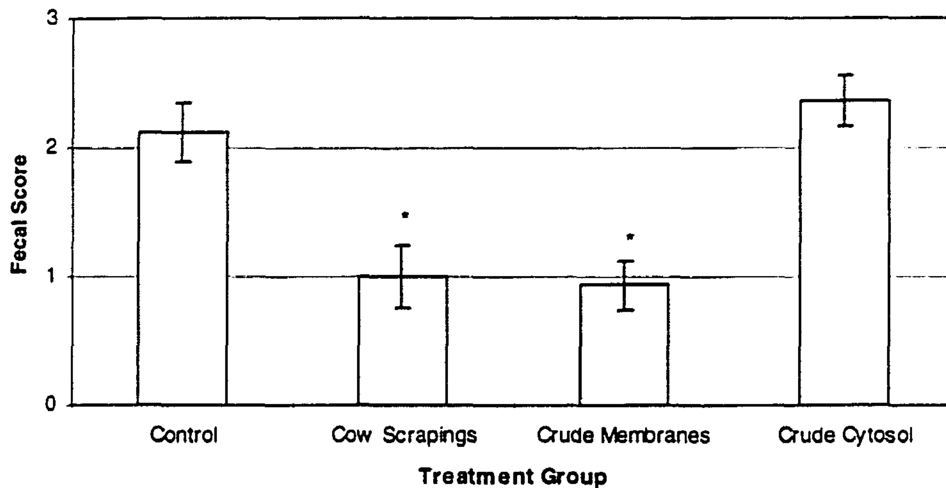

Figure 31. The effect of orally inoculating infant rats with cow intestinal scrapings, and cow intestinal scrapings divided into membrane and cytosol fractions, on reducing the fecal score. The intestinal scrapings or their fractions were given twice a day between 3 and 14 days of age. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

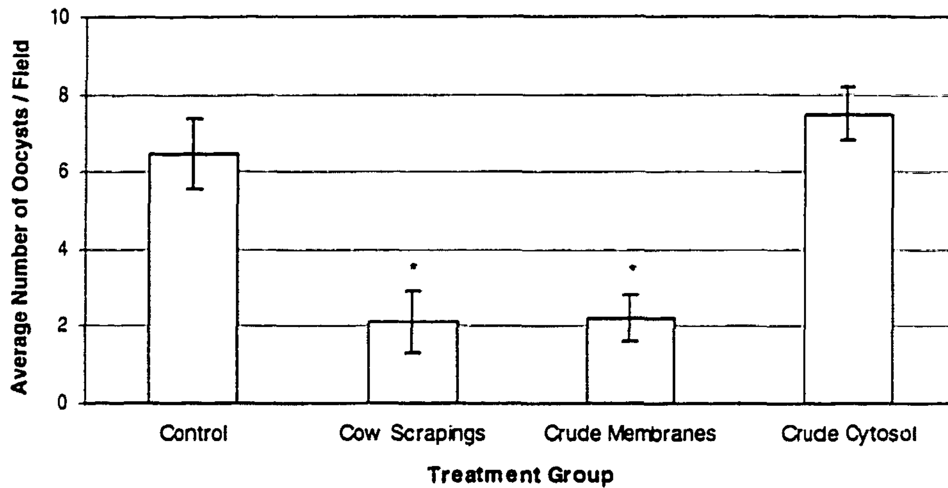

Figure 32. The effect of orally inoculating infant rats with cow intestinal scrapings, and cow intestinal scrapings divided into membrane and cytosol fractions, on reducing the average number of oocysts per microscopic field. The intestinal scrapings or their fractions were given twice a day between 3 and 14 days of age. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

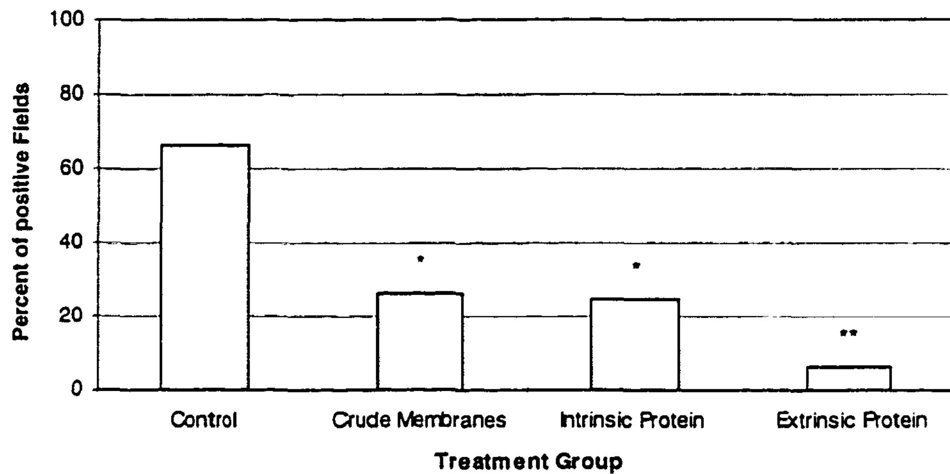

Figure 33. The effect of orally inoculating infant rats with the membrane fraction of cow intestinal scrapings, the intrinsically or the extrinsically associated membrane proteins, on reducing the percent of microscopic fields positive for oocysts. The crude membrane portion of the scrapings or its fractions were given twice a day between 3 and 14 days of age. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group and values with ** are significantly different from all groups by X^2 analysis.

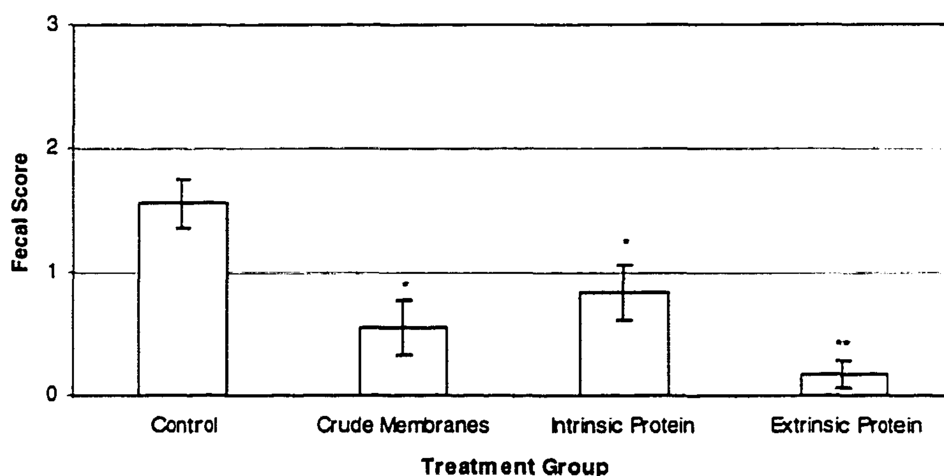

Figure 34. The effect of orally inoculating infant rats with the membrane fraction of cow intestinal scrapings, the intrinsically or the extrinsically associated membrane proteins, on reducing the fecal score. The crude membrane portion of the scrapings or its fractions were given twice a day between 3 and 14 days of age. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group and values with ** are significantly different from the intrinsic membrane protein group; $p \leq 0.05$.

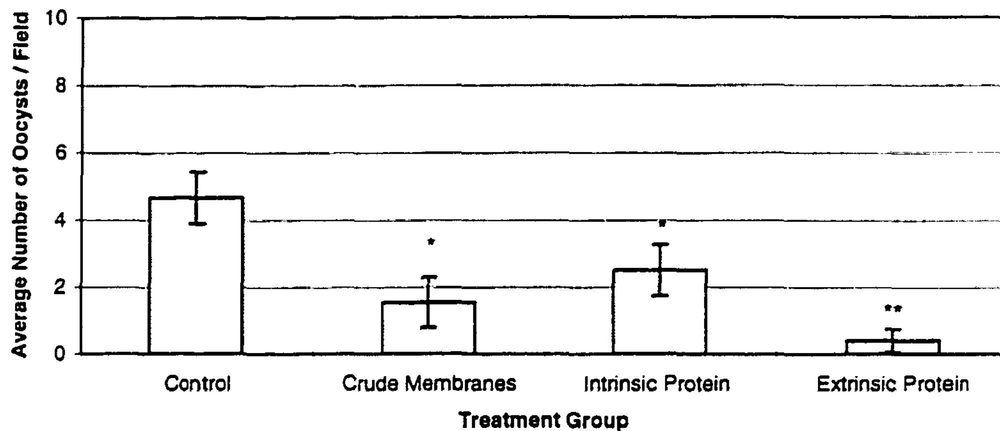

Figure 35. The effect of orally inoculating infant rats with the membrane fraction of cow intestinal scrapings, the intrinsically or the extrinsically associated membrane proteins, on reducing the average number of oocysts per field. The crude membrane portion of the scrapings or its fractions were given twice a day between 3 and 14 days of age. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group and values with ** are significantly different from the intrinsic membrane protein group; $p \leq 0.05$.

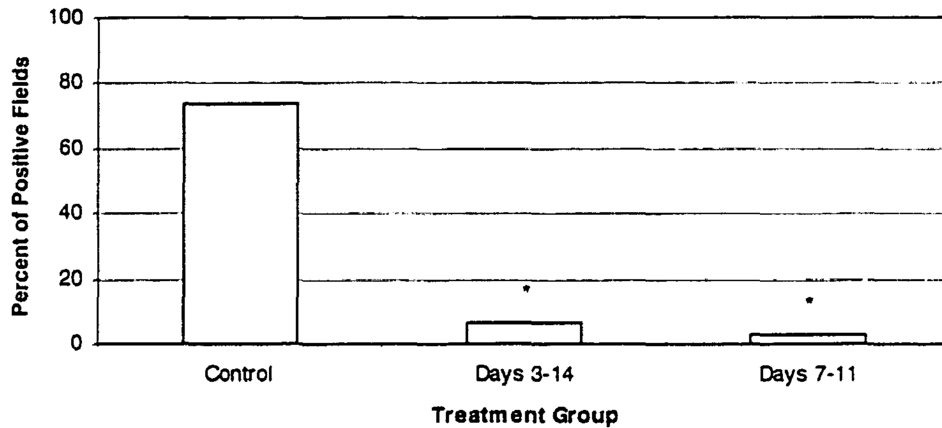

Figure 36. The effect of orally inoculating infant rats with the extrinsic membrane protein fraction of cow intestinal scrapings twice a day for 5 days compared to twice a day for 12 days on reducing the percent of microscopic fields positive for oocysts. The extrinsic membrane protein portion of the cow intestinal scrapings was given twice a day between 3 and 14 days of age or between 7 and 11 days of age. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

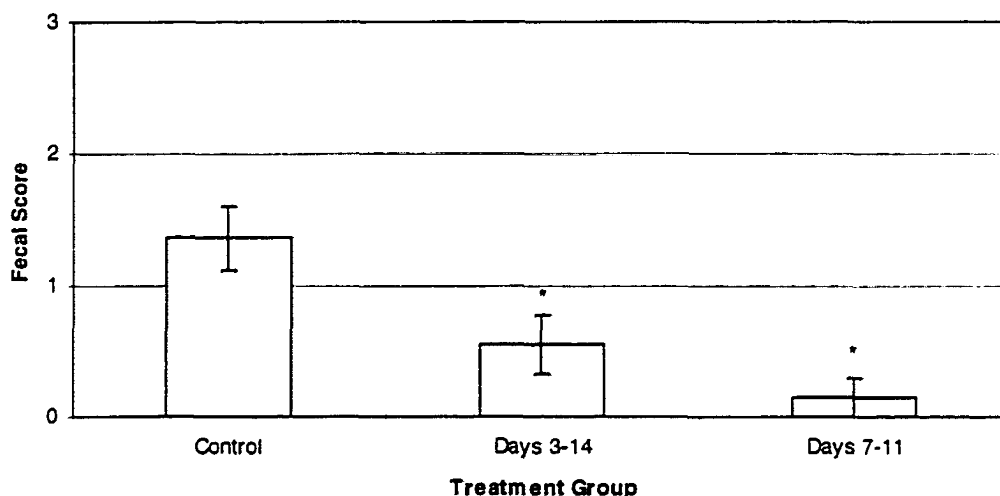

Figure 37. The effect of orally inoculating infant rats with the extrinsic membrane protein fraction of cow intestinal scrapings twice a day for 5 days compared to twice a day for 12 days on reducing the fecal score. The extrinsic membrane protein portion of the cow intestinal scrapings was given twice a day between 3 and 14 days of age or between 7 and 11 days of age. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

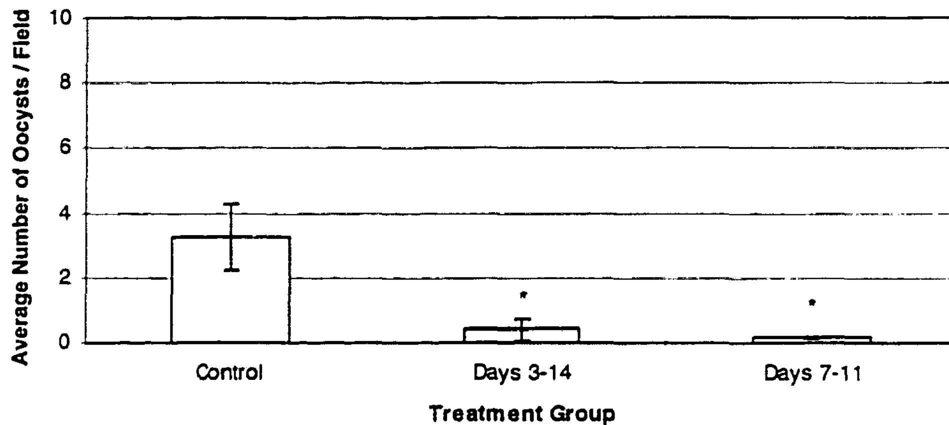

Figure 38. The effect of orally inoculating infant rats with the extrinsic membrane protein fraction of cow intestinal scrapings twice a day for 5 days compared to twice a day for 12 days on reducing the average number of oocysts per field. The extrinsic membrane protein portion of the cow intestinal scrapings was given twice a day between 3 and 14 days of age or between 7 and 11 days of age. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

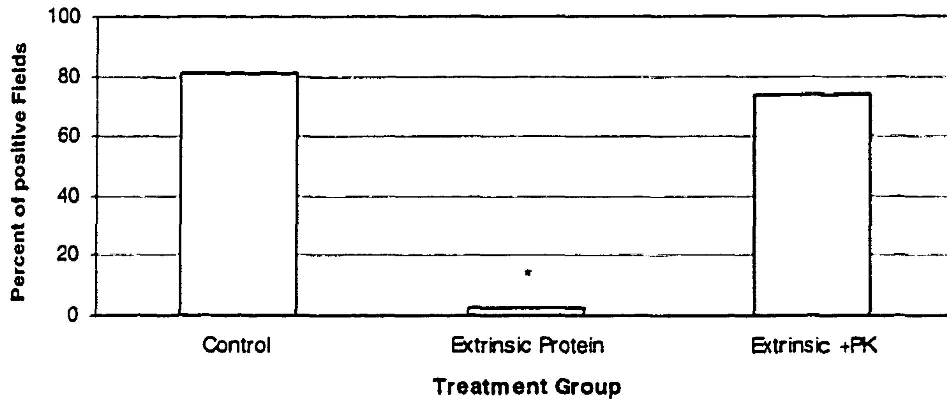

Figure 39. The effect of orally inoculating infant rats with the extrinsic membrane protein with or without Proteinase K (PK) treatment twice a day from days 7 to 11 of age on reducing the percent of microscopic fields positive for oocysts. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

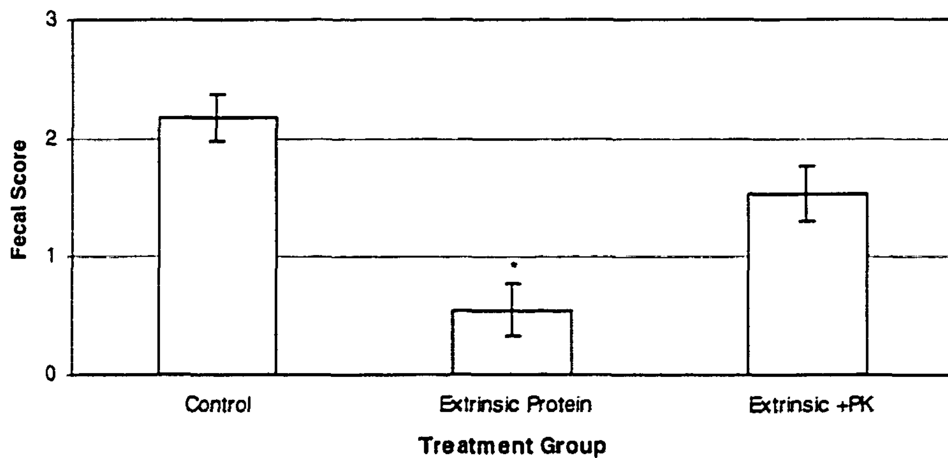

Figure 40. The effect of orally inoculating infant rats with the extrinsic membrane protein with or without Proteinase K (PK) treatment twice a day from days 7 to 11 of age on reducing the fecal score. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

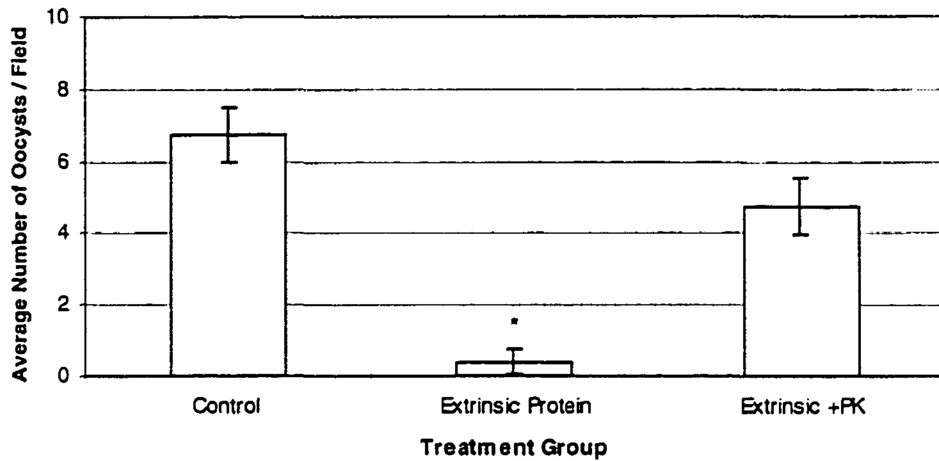

Figure 41. The effect of orally inoculating infant rats with the extrinsic membrane protein with or without Proteinase K (PK) treatment twice a day from days 7 to 11 of age on reducing the average number of oocysts per microscopic field. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

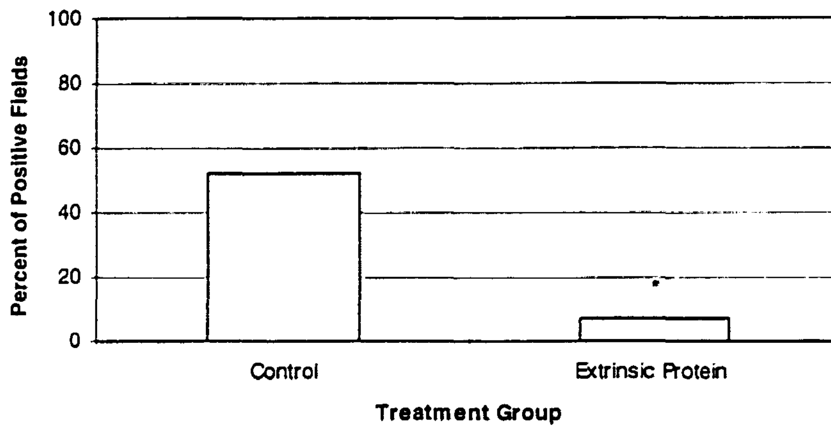

Figure 42. The effect of orally inoculating infant mice with cow intestinal extrinsic membrane protein fraction twice a day from days 7 to 11 of age on reducing the percent of microscopic fields positive for oocysts. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

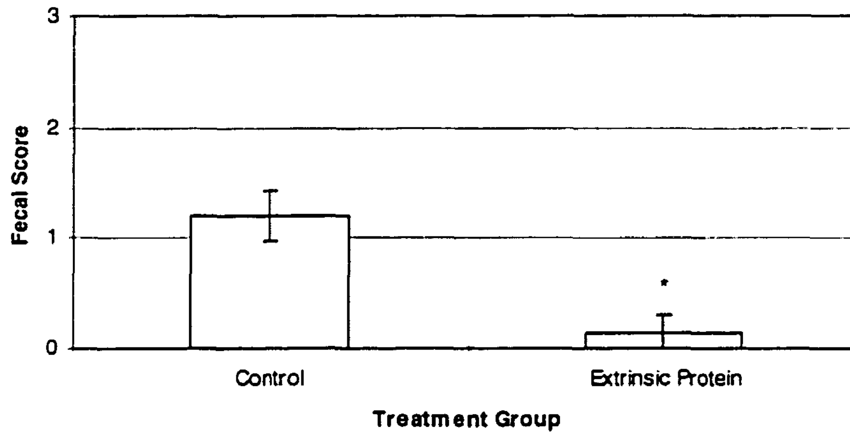

Figure 43. The effect of orally inoculating infant mice with cow intestinal extrinsic membrane protein fraction twice a day from days 7 to 11 of age on reducing the fecal score. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for the extrinsic membrane protein group. Values with * are significantly different from the control group; $p \leq 0.05$.

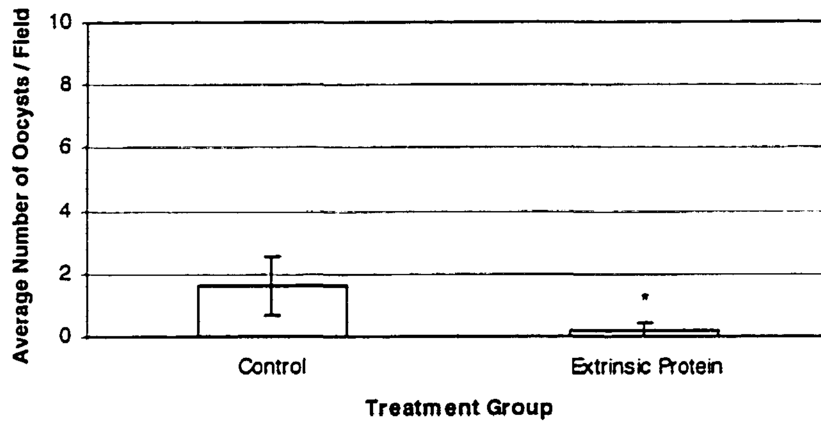

Figure 44. The effect of orally inoculating infant mice with cow intestinal extrinsic membrane protein fraction twice a day from days 7 to 11 of age on reducing the average number of oocysts per field. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

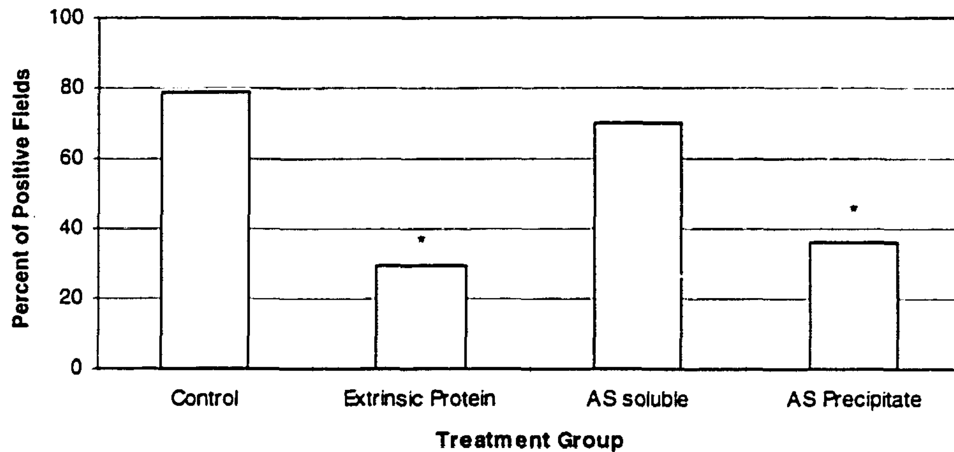

Figure 45. The effect of orally inoculating infant rats with extrinsic membrane protein, extrinsic membrane protein ammonium sulfate precipitate or soluble fractions twice a day from days 7 to 11 of age on reducing the percent of microscopic fields positive for oocysts. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

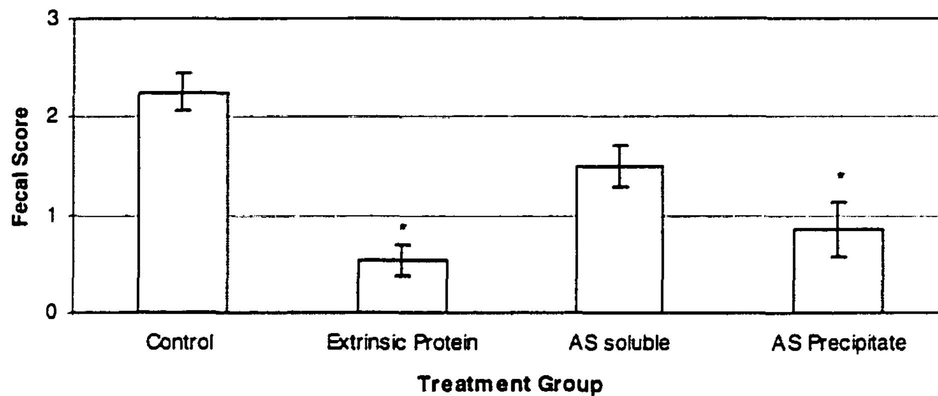

Figure 46. The effect of orally inoculating infant rats with extrinsic membrane protein, extrinsic membrane protein ammonium sulfate precipitate or soluble fractions twice a day from days 7 to 11 of age on reducing the fecal score. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

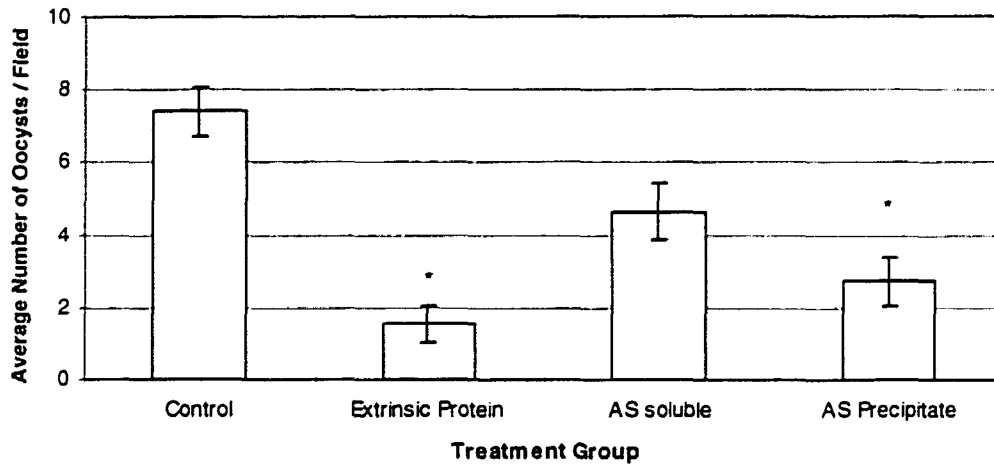

Figure 47. The effect of orally inoculating infant rats with extrinsic membrane protein, extrinsic membrane protein ammonium sulfate precipitate or soluble fractions twice a day from days 7 to 11 of age on reducing the average number of oocysts per microscopic field. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

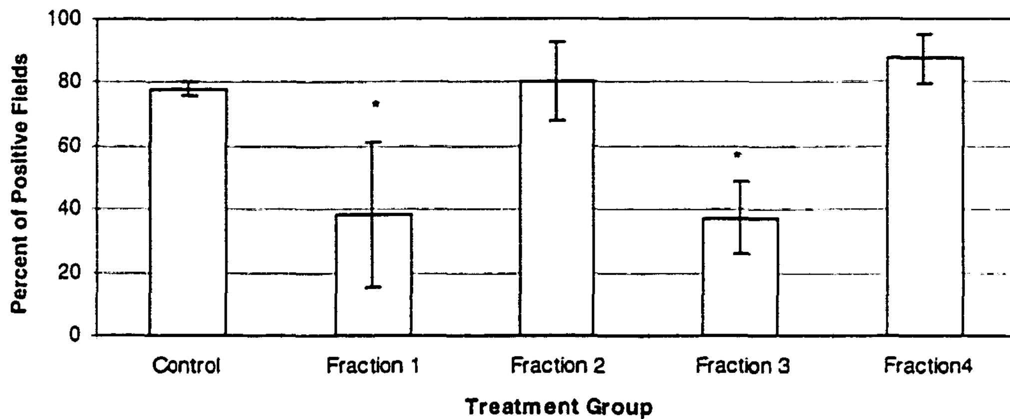

Figure 48. The effect of orally inoculating infant rats with one of the 4 fractions, collected by pooling the samples eluted off of the DEAE column, twice a day from days 7 to 11 of age on reducing the percent of microscopic fields positive for oocysts. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values are means ± SEM, n = 3. Values with * are significantly different from the control group by X^2 analysis.

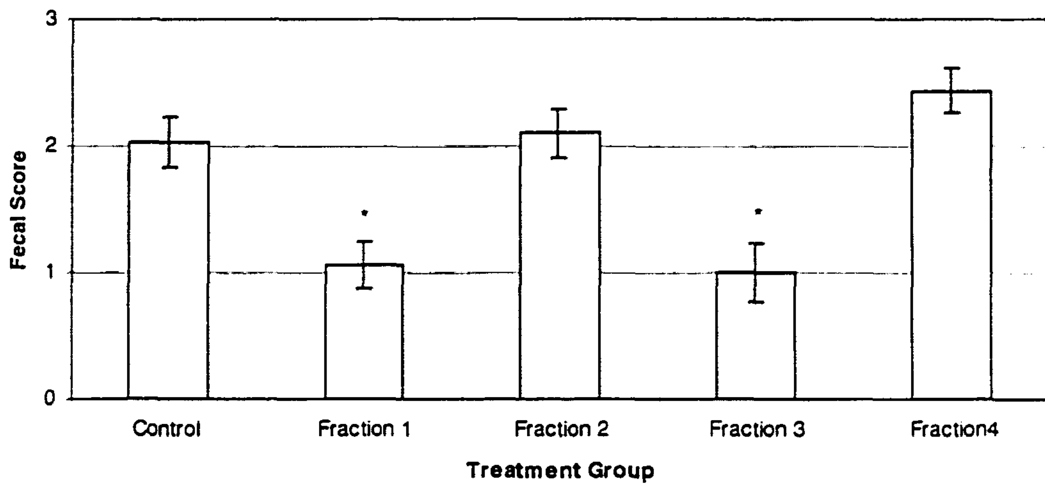

Figure 49. The effect of orally inoculating infant rats with one of the 4 fractions, collected by pooling the samples eluted off of the DEAE column, twice a day from days 7 to 11 of age on reducing the fecal score. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 108 for controls, and 90 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

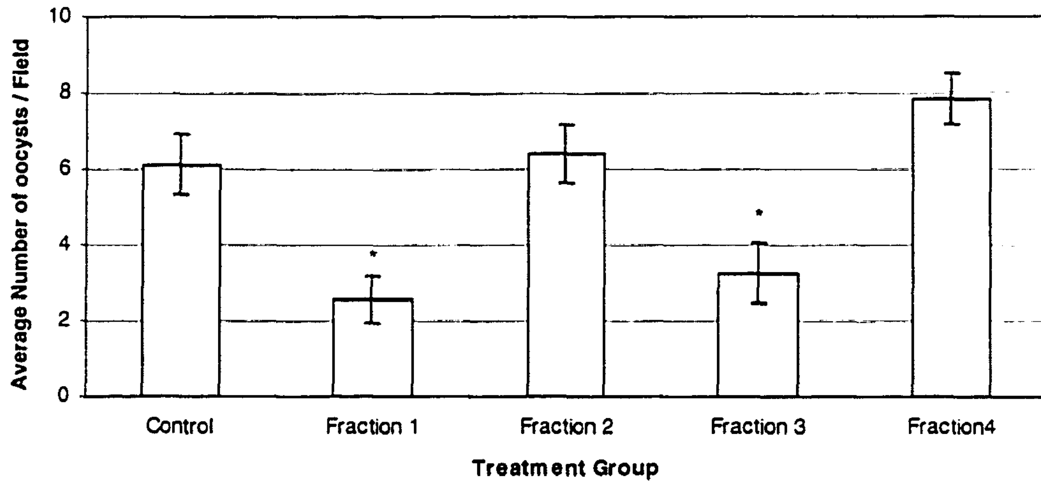

Figure 50. The effect of orally inoculating infant rats one of the 4 fractions, collected by pooling the samples eluted off of the DEAE column, twice a day from days 7 to 11 of age on reducing the average number of oocysts per microscopic field. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 108 for controls, and 90 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

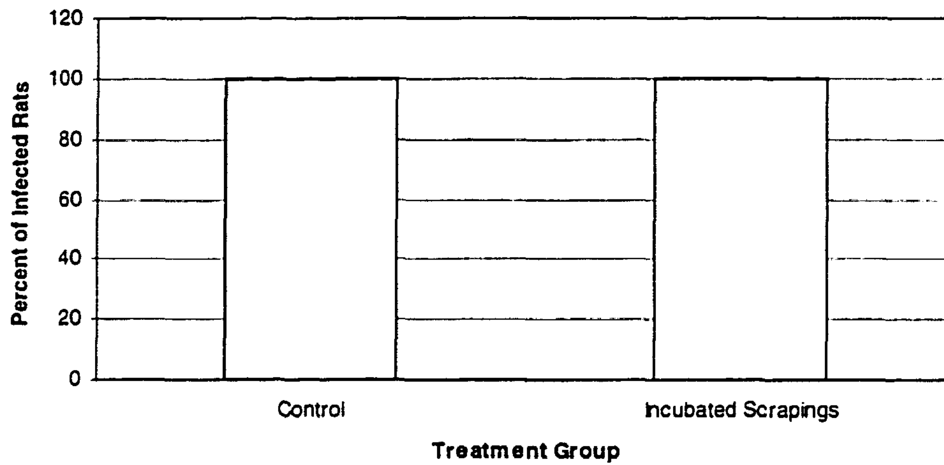

Figure 51. The effect of orally inoculating infant rats with adult rat ileal scrapings that had been incubated with 10^3 oocysts at 37° C for one hour once at 7 days of age. The control group received 10^3 oocysts at that had been incubated alone at 37° C for one hour once at 7 days of age. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 14 days of age. Values with * are significantly different from the control group by X^2 analysis.

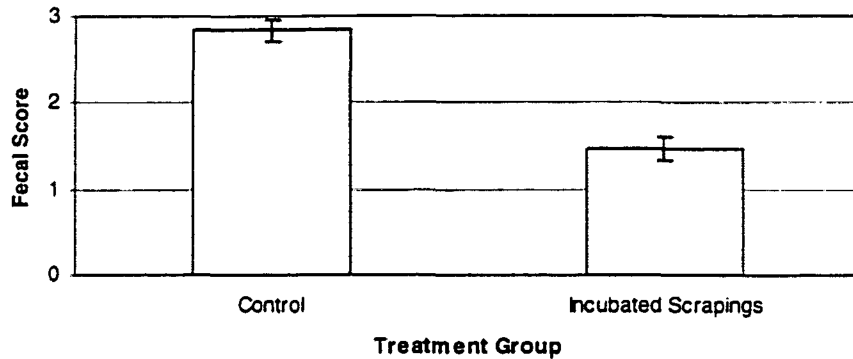

Figure 52. The effect of orally inoculating infant rats with adult rat ileal scrapings that had been incubated with 10^3 oocysts at 37° C for one hour once at 7 days of age on reducing the fecal score. The control group received 10^3 oocysts at that had been incubated alone at 37° C for one hour once at 7 days of age. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 14 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for incubated scrapings group. Values with * are significantly different from the control group; $p \leq 0.05$.

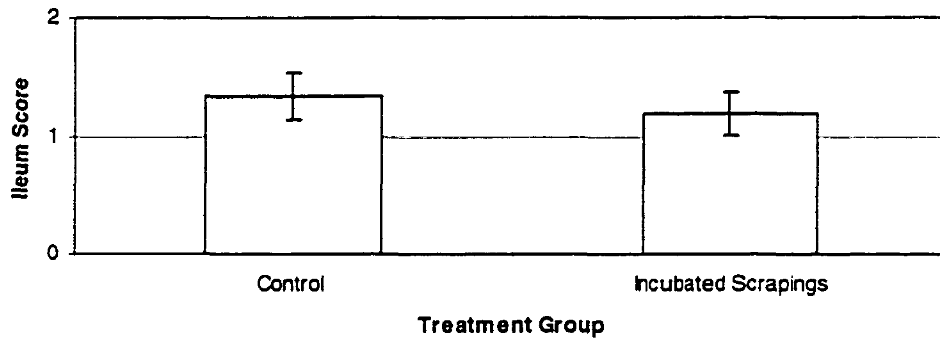

Figure 53. The effect of orally inoculating infant rats with adult rat ileal scrapings that had been incubated with 10^3 oocysts at 37° C for one hour once at 7 days of age on ileum score. The control group received 10^3 oocysts at that had been incubated alone at 37° C for one hour once at 7 days of age. The ileal score was calculated by assessing the intensity of *C. parvum* infection in the ileum of each animal at 14 days of age using light microscopy. A score of 0 was given to indicate that no *C. parvum* was present, 1 to indicate a light to moderate infection and 2 to indicate the presence of a heavy infection. Values are means ± SEM, n = 36 for controls, and 30 for the incubated scrapings group. Values with * are significantly different from the control group; $p \leq 0.05$.

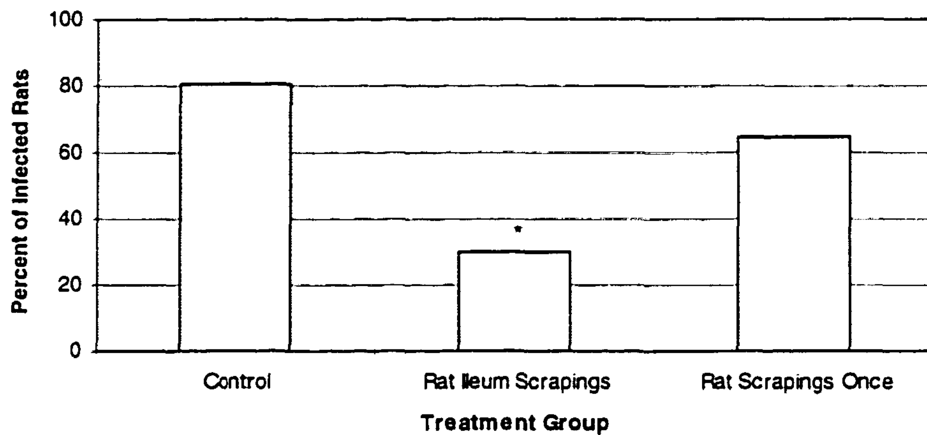

Figure 54. The effect of orally inoculating infant rats with adult rat ileal scrapings only once at 9 days of age, the same day they were inoculated with *C. parvum* oocysts, on reducing the percent of infant rats infected with *C. parvum*. The rat ileal scrapings were given to another group twice a day between 3 and 14 days of age. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

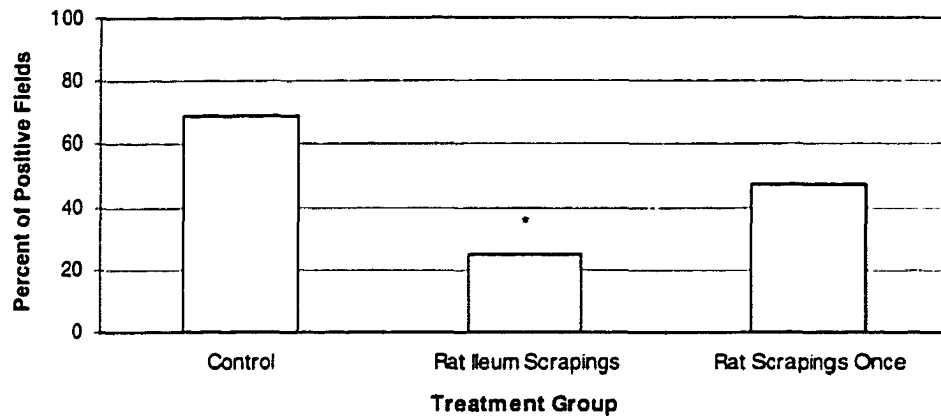

Figure 55. The effect of orally inoculating infant rats with adult rat ileal scrapings only once at 9 days of age, the same day they were inoculated with *C. parvum* oocysts, on reducing the percent of microscopic fields positive for oocysts. The rat ileal scrapings were given to another group twice a day between 3 and 14 days of age. The rat ileal scrapings were given twice a day between 3 and 14 days of age. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

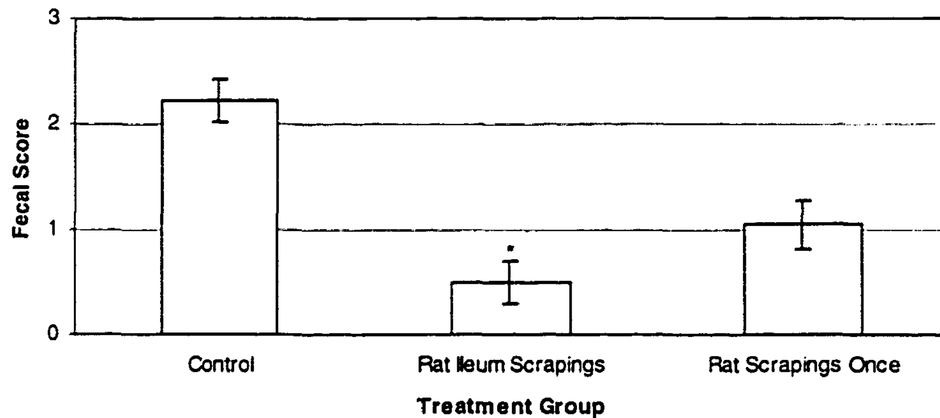

Figure 56. The effect of orally inoculating infant rats with adult rat ileal scrapings only once at 9 days of age, the same day they were inoculated with *C. parvum* oocysts, on reducing the fecal score. The rat ileal scrapings were given to another group twice a day between 3 and 14 days of age. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

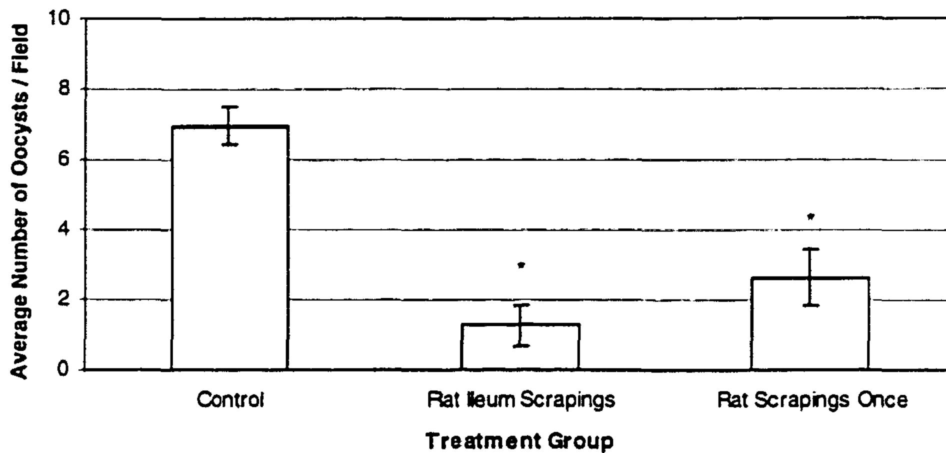

Figure 57. The effect of orally inoculating infant rats with adult rat ileal scrapings only once at 9 days of age, the same day they were inoculated with *C. parvum* oocysts, on reducing the average number of oocysts per field. The rat ileal scrapings were given to another group twice a day between 3 and 14 days of age. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n =36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

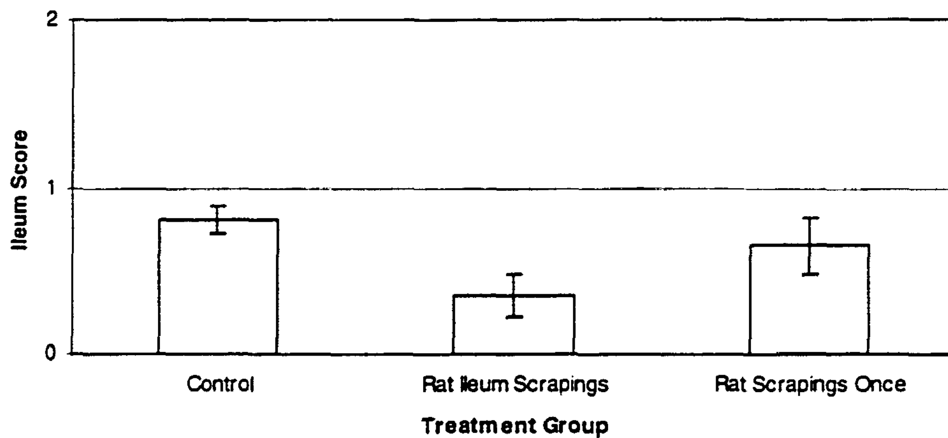

Figure 58. The effect of orally inoculating infant rats with adult rat ileal scrapings only once at 9 days of age, the same day they were inoculated with *C. parvum* oocysts, on reducing the ileum score. The rat ileal scrapings were given to another group twice a day between 3 and 14 days of age. The ileal score was calculated by assessing the intensity of *C. parvum* infection in the ileum of each animal at 15 days of age using light microscopy. A score of 0 was given to indicate that no *C. parvum* was present, 1 to indicate a light to moderate infection and 2 to indicate the presence of a heavy infection. Values are means ± SEM, n =36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

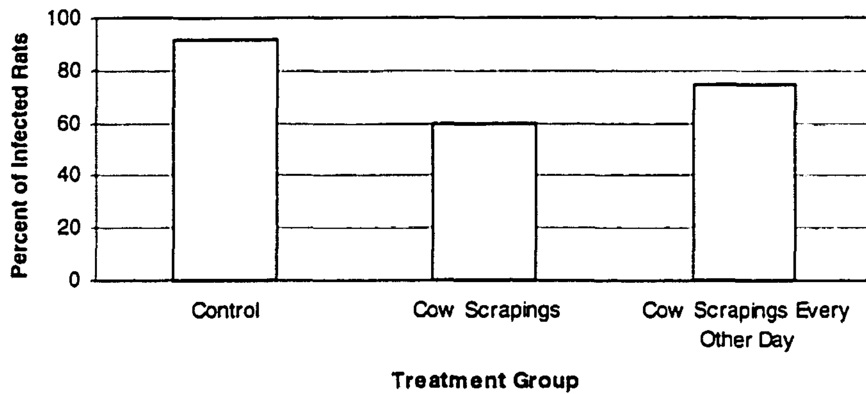

Figure 59. The effect of orally inoculating infant rats with cow ileal scrapings on reducing the percent of infant rats infected with *C. parvum*. The cow ileal scrapings were given twice a day between 3 and 14 days of age or once every other day between 3 and 14 days of age (3, 5, 7, 9, 11, and 13). The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

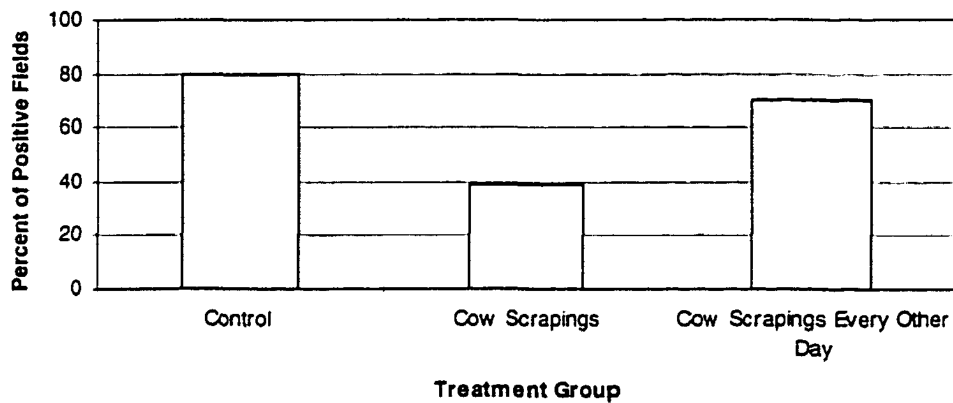

Figure 60. The effect of orally inoculating infant rats with cow ileal scrapings on reducing the percent of microscopic fields positive for oocysts. The cow ileal scrapings were given twice a day between 3 and 14 days of age or once every other day between 3 and 14 days of age (3, 5. 7, 9, 11, and 13). The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

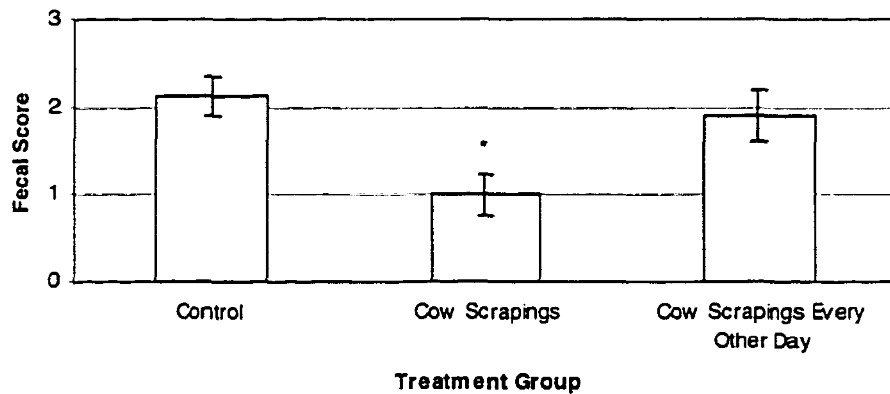

Figure 61. The effect of orally inoculating infant rats with cow ileal scrapings on reducing the fecal score. The cow ileal scrapings were given twice a day between 3 and 14 days of age or once every other day between 3 and 14 days of age (3, 5, 7, 9, 11, and 13). The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

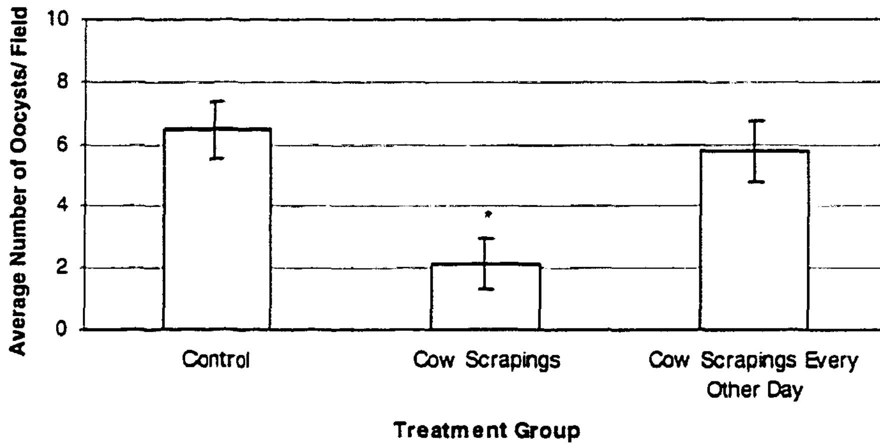

Figure 62. The effect of orally inoculating infant rats with cow ileal scrapings on reducing the average number of oocysts per field. The cow ileal scrapings were given twice a day between 3 and 14 days of age or once every other day between 3 and 14 days of age (3, 5, 7, 9, 11, and 13). The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

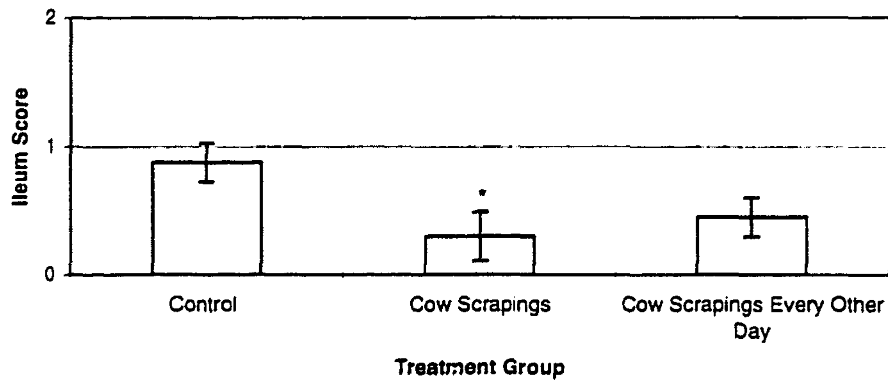

Figure 63. The effect of orally inoculating infant rats with cow ileal scrapings on reducing the ileum score. The cow ileal scrapings were given twice a day between 3 and 14 days of age or once every other day between 3 and 14 days of age (3, 5, 7, 9, 11, and 13). The ileal score was calculated by assessing the intensity of *C. parvum* infection in the ileum of each animal at 15 days of age using light microscopy. A score of 0 was given to indicate that no *C. parvum* was present, 1 to indicate a light to moderate infection and 2 to indicate the presence of a heavy infection. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

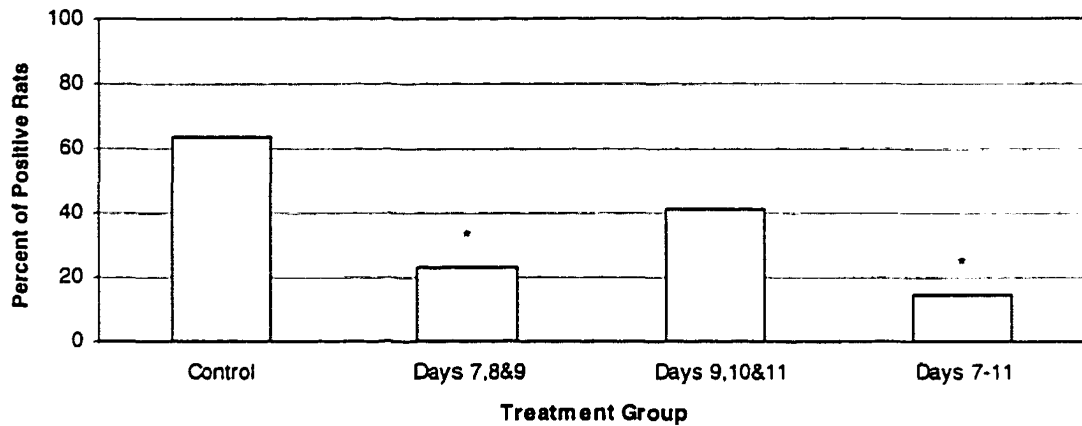

Figure 64. The effect of orally inoculating infant rats with the extrinsic membrane protein fraction of cow intestinal scrapings twice a day from days 7-9, days 9-11 or day 7-11 of age on reducing the percent of infant rats infected with *C. parvum*. The percent of infected animals was calculated by noting the presence of *C. parvum* in the ileum or feces of the animals at 15 days of age. Values with * are significantly different from the control group by X^2 analysis.

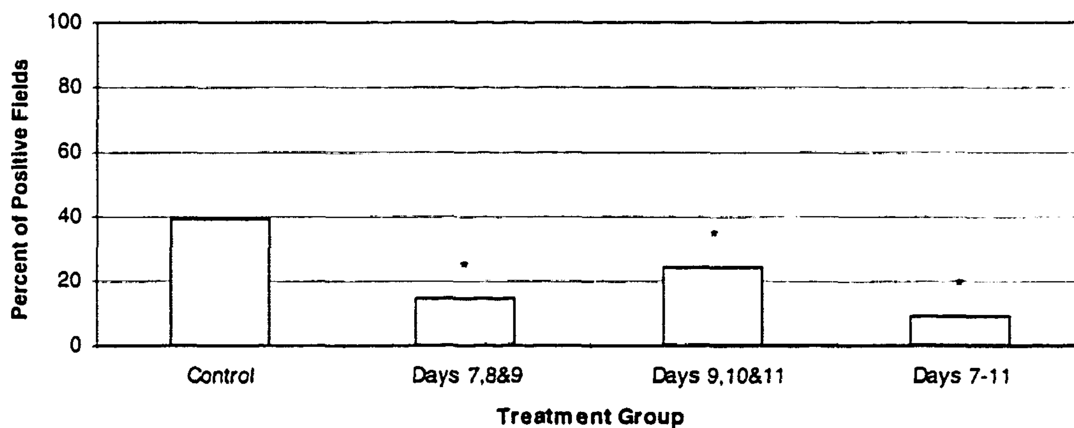

Figure 65. The effect of orally inoculating infant rats with the extrinsic membrane protein fraction of cow intestinal scrapings twice a day from days 7-9, days 9-11 or day 7-11 of age on reducing the percent of microscopic fields positive for oocysts. The percent of positive fields was calculated by counting the number of microscopic fields out of a total of 10 per fecal sample per animal that contained *C. parvum* oocysts at 15 days of age using light microscopy. Values with * are significantly different from the control group by X^2 analysis.

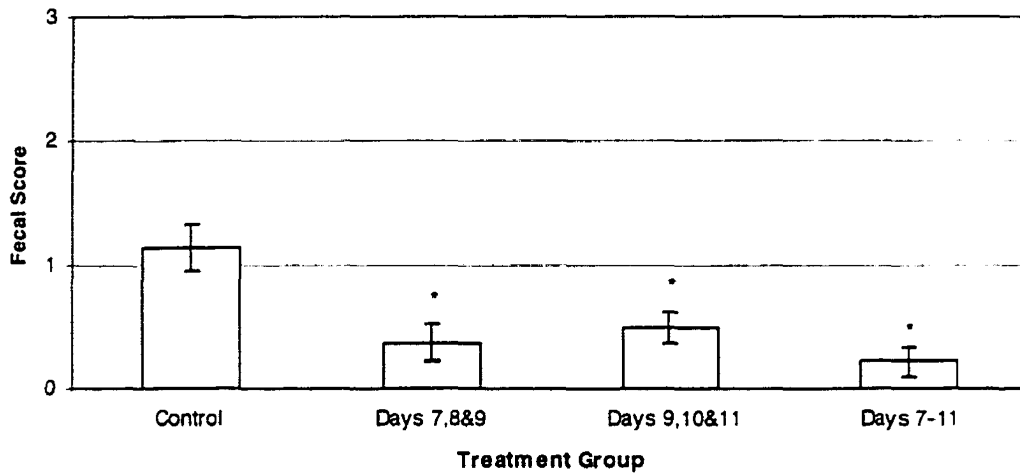

Figure 66. The effect of orally inoculating infant rats with the extrinsic membrane protein fraction of cow intestinal scrapings twice a day from days 7-9, days 9-11 or day 7-11 of age on reducing the fecal score. The fecal score was calculated by counting the number of *C. parvum* oocysts in the fecal smear of each animal at 15 days of age using light microscopy. The score 1 was given to slides that had an average of 4 or less oocysts / field. The score of 2 was given to slides that had an average of 5 to 9 oocysts / field. The score of 3 was given to slides that had 10 or more oocysts / field. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

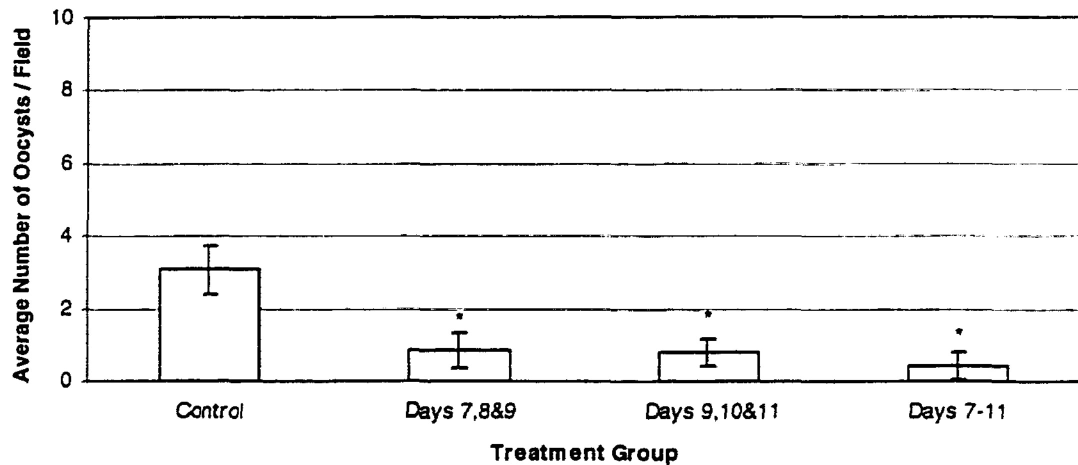

Figure 67. The effect of orally inoculating infant rats with the extrinsic membrane protein fraction of cow intestinal scrapings twice a day from days 7-9, days 9-11 or day 7-11 of age on reducing the average number of oocysts per microscopic field. The average number of oocysts per field was calculated by counting the number of *C. parvum* oocysts in 10 microscopic fields per fecal sample per animal at 15 days of age using light microscopy. Values are means ± SEM, n = 36 for controls, and 30 for each of the treatment groups. Values with * are significantly different from the control group; $p \leq 0.05$.

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