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IMMUNE RESPONSE OF THE BOVINE FETUS TO *IN UTERO* VACCINATION WITH ATTENUATED CALF DIARRHEAL CORONAVIRUS: A GNOTOBIOTIC STUDY

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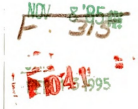
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By

Louis E. Newman

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ABSTRACT

IMMUNE RESPONSE OF THE BOVINE FETUS TO *IN UTERO* VACCINATION WITH ATTENUATED CALF DIARRHEAL CORONAVIRUS: A GNOTOBIOTIC STUDY

By

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Bovine fetuses were vaccinated during the last 7 weeks of gestation by inoculation of either cell culture attenuated calf diarrheal coronavirus (14 fetuses) or sterile physiological saline solution (16 fetuses) into the amniotic fluid. Six additional fetuses were not inoculated. Two virus vaccinated fetuses were aborted on days 2 and 3 postvaccination and 4 virus vaccinated fetuses were delivered prematurely on days 9 and 10 postvaccination. All 22 saline inoculated and non-inoculated (control) cows maintained normal pregnancies. The data are from 8 gnotobiotic calves which survived all phases of this research and were challenged with virulent calf diarrheal coronavirus.

Calves were cesarean section delivered into a gnotobiotic environment. A 150 cm loop of ileum (Thiry-Vella loop) was surgically isolated at 1 day of age and sections for histopathologic examination were collected at this time.

Intestinal secretions were collected on days 3 through 7 by flushing the ileal loop with phosphate buffered saline solution. Immunoglobulins present in the loop fluid were characterized and quantitated and specific neutralizing antibody determinations were performed. Immunoglobulin and antibody determinations were also carried out on

serum samples collected from cows before and after vaccination and calves the day of cesarean section delivery. Calves were challenged at 6 days of age by oral administration of virulent calf diarrheal coronavirus and necropsied at 9 days of age.

All calves vaccinated with attenuated coronavirus intra-amniotically and subsequently challenged with virulent virus failed to develop clinical signs of coronavirus infection. Control calves developed diarrhea 19 to 22 hours following oral administration of virulent virus. Tissue sections of colon, ileum and ileal loop from control calves collected at necropsy and stained by fluorescent antibody technique (FA) were coronavirus positive. Tissue sections from vaccinated calves were FA negative.

Comparison of histopathologic sections of ileum revealed no difference between control and vaccinated calves at 1 day of age. *In utero* vaccinated calves euthanatized 72 hours following oral challenge with virulent coronavirus did not have gross or microscopic lesions. Control calves euthanatized 72 hours following challenge had liquid contents in small and large intestine and histopathological changes caused by coronavirus infection were observed in ileum, ileal loop and colon.

Cow sera from all cows contained high levels of immunoglobulin (Ig) before and after vaccination. There were significant increases in serum Ig levels of vaccinated calves compared to control calves on the day of cesarean section delivery. No detectable Ig's were found in intestinal loop washings of control calves. Oral vaccination of the fetus with attenuated coronavirus resulted in significant increases of IgA, IgM, and IgG in the intestine.

The serum from all cows contained antibodies to calf diarrheal coronavirus before and after vaccination and there was no significant difference between before and after vaccination titers. Coronavirus serum neutralization antibody tests on serum samples from control calves were negative. Newborn vaccinated calves had serum neutralization antibody titers from 4 to 128. Neutralization antibody titers of intestinal loop washings from vaccinated calves were significantly higher than those from control calves. These results are evidence of the ability of the near-term fetus to respond to an orally administered attenuated coronavirus by the production of specific serum and secretory humoral antibody.

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INTRODUCTION

Neonatal enteritis is one of the most common causes of morbidity and mortality of man and higher animals. Diarrheal disease in newborn calves is of major importance to worldwide cattle production because it is widespread and progresses beyond control in many herds. It is estimated that 10% of the annual calf crop in the United States is lost due to diarrheal diseases and an annual loss of approximately $400 million occurs from death alone (USDA, 1976). Calf mortality is a serious cause of economic loss to the Michigan food animal producer (Oxender et al., 1973; Speicher and Hepp, 1973).

A voluminous amount of literature is available pertinent to calf mortality. Much of the information concerns clinical treatment of afflicted calves. This has consisted of fluids and electrolytes to counteract dehydration, antibiotics to control pathogenic microorganisms, protectants to coat the digestive tract, absorbants to neutralize toxins, serum to supply antibodies, and good nursing care. Information concerning preventive measures such as isolation, proper use of colostrum, and managerial practices has been known for many years (Selman et al., 1971a). It has been 56 years since the use and importance of colostrum to the newborn calf was emphasized (Smith and Little, 1922). These measures apparently are either not practiced by cattle producers or are inconvenient to incorporate into modern cattle raising operations, since California workers concluded recently

that "there has been no published evidence to indicate a decrease in this rate [of mortality] since the turn of the century" (Martin et al., 1975). Although research involving food producing animals is both expensive and time consuming, it would seem that in view of the importance of this problem, consumer concern for a healthy supply of food and the threatened curtailment of antibiotic use, research emphasis on prevention is urgent.

Neonatal calf diarrhea is not a single disease but is recognized as a complex of several distinct entities having diarrhea as a common clinical expression. Most cases of neonatal enteritis are caused by infectious agents, and nutritional, environmental and management factors predispose to infection. Calf diarrheal coronavirus is a frequently diagnosed cause of acute infectious enteritis in neonatal calves, and the disease can be reproduced experimentally. A satisfactory method has not yet been developed to protect the newborn calf from infection by this virus.

This research investigated the immune response of calves to *in utero* vaccination with a cell culture attenuated coronavirus. The response to immunization was measured by the production of immunoglobulins and antibodies in secretions from isolated intestinal loops (Thiry-Vella loops) and serum and by the response of the cesarean section collected and gnotobiotically maintained calf to challenge with virulent virus.

LITERATURE REVIEW

Introduction

The greatest mortality of animals born alive occurs during the neonatal period (Barnum et al., 1967). Infectious enteritis, an acute disease characterized by diarrhea and exhaustion, is the most serious cause of economic losses in newborn calves (Fey, 1972; National Academy of Sciences, 1968). Diarrhea in calves for many years was considered as a distinct disease entity rather than as a clinical sign. Colibacillosis infection caused by *Escherichia coli* was the most common diagnosis (Gay, 1965; Barnum et al., 1967; Fey, 1972).

Neonatal calf diarrhea is recognized as an etiologically complex disease and many bacteria and viruses are incriminated as causal agents; however, considerable uncertainty exists about which agents are most important (Acres and Radostits, 1976; USDA, 1976). A limited number of serotypes of *E. coli* can act as primary enteropathogens. They must produce enterotoxin and proliferate to high numbers in the anterior small intestine. Cultures of *E. coli* which caused dilatation of ligated gut loops were isolated from 5.5 to 37% of calves with diarrheic feces in four studies (Acres et al., 1975). Enteropathogenic *E. coli* alone were isolated from 6 of 55 dairy and beef calves with acute neonatal calf diarrhea and from 4 additional calves from which more than 1 pathogen was isolated (Morin et al., 1976).

Other etiological diagnoses made in this study included: calf diarrheal coronavirus (13), rotavirus (16) and *Cryptosporidium* (11).

The viral etiology of neonatal calf diarrhea has assumed increasing importance since a rotavirus was isolated from a field outbreak by Mebus and co-workers at Nebraska (1969a). Experimental reproduction of the disease, virus isolation and purification, successful cell culture, and techniques for fluorescent antibody staining led to recognition of the widespread incidence of this agent (Mebus et al., 1969b; White et al., 1970; Welch, 1971; Mebus et al., 1971; Mebus et al., 1972a). A cell culture attenuated rotavirus as an oral vaccine was effective in preventing diarrhea caused by the rotavirus; however, diarrhea occurred in several herds when the vaccinated calves were 5 to 21 days of age (Mebus et al., 1973a; Mebus et al., 1973b; Mebus et al., 1973c). The Nebraska researchers detected a coronavirus in diarrheal fecal material from neonatal calves in 12 vaccinated and 7 nonvaccinated herds and the disease was reproduced experimentally (Stair et al., 1972). The calf diarrheal coronavirus has since been widely recognized as a major cause of neonatal calf diarrhea, and it is the most frequently diagnosed viral etiologic agent of this disease in the Animal Health Diagnostic Laboratory at Michigan State University (Trapp and Roberts, 1978). The voluminous literature on calf enteric infections includes as etiologic agents infectious bovine rhinotracheitis virus, bovine viral diarrhea virus, bovine adenoviruses, bovine parvoviruses, coronaviruses, rotaviruses, enteroviruses, *Escherichia coli, Salmonella* sp., chlamydial agents and other bacteria, and coccidia of the genus *Cryptosporidium*. This literature review will pertain primarily to the calf diarrheal

coronavirus and immunologic, gnotobiotic, surgical and laboratory techniques essential to this research.

Calf Diarrheal Coronavirus

Coronaviruses were proposed as a new taxonomic group of viruses in 1968 to account for structural differences between avian infectious bronchitis virus and myxovirus. The prefix corona- was proposed to describe the petal-shaped projections from the central core which resembled the solar corona. Coronaviruses are widely distributed in nature and have been demonstrated to infect man, calves, mice, pigs, rats, dogs, chickens, turkeys, and possibly foals (Mebus, 1976; Sharpee et al., 1976). The classification of the neonatal calf diarrheal coronavirus used in this study was based on the 6 major properties of the coronavirus group: 1) characteristic surface structure, 2) size, 3) replication within cytoplasmic vesicles, 4) ribonucleic acid content, 5) presence of an essential lipid envelope, and 6) low particle density (Sharpee et al., 1976; Estola, 1970).

The calf diarrheal coronavirus was first identified as a problem in the spring of 1971 by Mebus and associates at the University of Nebraska (Mebus et al., 1972a; Stair et al., 1972). It was adapted to cell culture by passage on secondary fetal bovine kidney cells originating from a single fetus and remained virulent after 19 passages. Attenuation was accomplished by passage of the virus on consecutively higher passages of fetal bovine kidney cells (Mebus et al., 1973d). In contrast to this work, most researchers have been unable to propagate this virus on several types of primary fetal cells. However, Inaba and co-workers in Japan (1976) reported multiplication

and a cytopathic effect in culture of a continuous cell line, BEK-1, derived from bovine embryonic kidney.

The identification and isolation of viral agents of neonatal calf diarrhea, adaptation to cell culture and cell culture attenuation, and development of experimental vaccines culminated many years of enteric disease research by Mebus and co-workers in Nebraska. This work also led Mebus to speculate concerning research ramifications and studies on the cutting edge of new knowledge which needed to be investigated, and this research was developed in part following discussions and suggestions by Dr. Mebus in 1975 and 1976.

Clinical Signs

Neonatal calf diarrhea is characterized by depression and profuse, watery, yellow feces. Morbidity may reach 100% in severe outbreaks; mortality varies from less than 1% to over 50%. Diarrhea caused by the coronavirus may appear shortly after birth, but calves in coronavirus infected herds usually develop diarrhea between 5 and 21 days of age (Mebus et al., 1973a).

The incubation period following oral inoculation of coronavirus infected fecal material varies from 19 to 24 hours. The calves become depressed and anorectic and feces become watery and yellow, with volume depending on amount of milk fed (Mebus, 1976a; Mebus et al., 1973c). The liquid feces contains curds and mucus following initial diarrhea. Coronavirus infected calves continue to have diarrhea for 5 to 6 days; some calves become moribund or die 48 to 62 hours following the onset of diarrhea. These calves have severe dehydration with blood packed cell volumes of 45 to 50%.

Electrolyte and Fluid Alterations

Research over several years by Phillips and Lewis at Colorado State University has contributed to an understanding of diarrheic induced body changes. Diarrhea causes the following biochemical alterations: dehydration, acidosis, and changes in sodium, bicarbonate, chloride and potassium ion concentrations. The normal calf gains about 22 ml of water per kg per day while the diarrheic calf loses up to 72 ml/kg/day. This negative water balance results from lack of absorption of ingested fluid and digestive fluids and increased fluid loss due to the effects of endotoxins (Phillips et al., 1971; Lewis and Phillips, 1973).

Acidosis is both extracellular and intracellular and results primarily from a loss of bicarbonate ions. Cellular acidosis occurs first, for as HCO_3^- ions are lost in the feces, tissues exchange K^+ for H^+ to maintain normal extracellular pH in the cerebrospinal fluid and central nervous system. The exchange of K^+ for H^+ plus decreased renal excretion of K^+ because of dehydration results in increased plasma K^+ and decreased cellular K^+ concentrations. This change in transmembrane K^+ concentration ratio causes improper nerve conduction and muscular contraction and cardiac dysfunction (Lewis, 1977; Phillips and Lewis, 1973).

In addition to the loss of HCO_3^-, there is also an increase in lactic acid production. As dehydration develops, the body attempts to maintain normal blood pressure through peripheral vasoconstriction. Vasoconstriction in turn causes a deficiency of oxygen to muscle and an increase in lactic acid production from anaerobic muscular metabolism. Lactic acid contributes to acidosis since the liver cannot utilize

the lactic acid in gluconeogenesis because of venous congestion (Phillips and Lewis, 1973).

Earlier work demonstrated that there is also a significantly increased loss of serum proteins via the intestinal tract in diarrheal calves. Calves with severe diarrhea may lose significant amounts of all serum proteins as well as unabsorbed milk proteins. Normal calves have proteinuria with a number of serum proteins and some milk proteins appearing in the urine (Howe, 1924). The proteinuria in newborn calves is related directly to colostrum absorbed (Smith and Little, 1924). Diarrheal calves have less proteinuria than normal calves, indicating that they may not be absorbing enough protein from the intestine to induce proteinuria (Marsh et al., 1969).

The Mucosa of the Intestine

The small intestine is the main organ for absorption of dietary nutrients. The villus and the epithelial cells on the surface of the villus constitute the functional absorptive unit (Kenworthy, 1970). The mucosa of the tubular crypt is continuous with that of the villus. The integrity of the villus is essential to normal function of the intestinal tract. Intestinal epithelial cells, in general, have a very fast turnover rate (1 to 6 days) and the entire epithelial lining is renewed every few days as the result of rapid cell replacement (LeBlond, 1958; Moon, 1971). Villous cell replacement times were in excess of 48 hours in neonatal calves and lambs and decreased somewhat with age (Moon and Joel, 1975).

The crypt epithelium has the specialized function of cell division; as cells are lost from the extrusion zone of the villus they are replaced by continual movement of epithelial cells from the crypt.

Epithelial cells arise by repeated division of relatively undifferentiated cells in the crypt of Lieberkühn and, once division has taken place, differentiation is complete relative to eventual function (Kenworthy, 1970). The crypts are almost solely for epithelial cell production, while the villi provide a large surface area almost wholly for absorption. The cells lost from the tips of the villi disintegrate rapidly so that they are rarely recognizable in the lumen of the small intestine (Moon, 1971).

The lamina propria of the villus is covered with simple columnar epithelium bearing a discernible brush border of microvilli. Goblet cells are interspersed among epithelial cells on the villus. There are increasing numbers of goblet cells in the most distal portions of the small intestine. The epithelium of the villus rests on a basement membrane. The lamina propria of the villus consists of connective tissue and capillaries, lymphatic vessels, a central chyle vessel termed a lacteal, contractile fibers termed Breucke fibers, and variable numbers of reticuloendothelial cells and lymphocytes (Ham, 1965). The villi are longer and the crypts shorter in gnotobiotic pigs than in naturally raised pigs (Christie, 1967; Moon, 1970). Villi in newborn lambs and calves are longer than those from older animals and villi in jejunum are longer than those in duodenum and ileum, and crypt lengths are shorter in newborn animals than in older animals (Moon and Joel, 1975). It is an apparently consistent feature of the small intestine of calves, lambs and pigs to have increasing crypt depth during the first few weeks of life and to have a decrease in intestinal villous length with increasing age (Moon and Joel, 1975).

The secretory and absorptive capabilities of the mucosa vary from crypt to villus. The young, immature or undifferentiated

epithelial cells of the crypt lack digestive enzymes and have minimal absorptive capacity. The differentiated cells which migrate out to the villi and become mature villous epithelial cells carry out the bulk of digestive and absorptive functions of the intestines but have minimal secretory capabilities. Viruses that selectively multiply in and destroy villous absorptive cells result in atrophy of villi and impair absorptive capability. Intestinal villous absorptive cells have digestive as well as absorptive functions and the digestive capacity of the intestine is also impaired in virus diseases which destroy villous absorptive cells (Moon, 1978).

Pathologic Alterations

Lesions of calf diarrheal coronavirus infection, determined in gnotobiotic and colostrum-fed calves, occurred in small and large intestine (Mebus et al., 1973a). Small and large intestines were lined by morphologically normal epithelium which contained a large quantity of viral antigen at onset of diarrhea. All tissues appeared normal upon gross examination of calves necropsied 4 hours after onset of diarrhea. Contents of small intestine and colon were liquid. There was submicroscopic villous atrophy 48 hours after onset of diarrhea.

The coronavirus infected mainly villous epithelium of upper, middle and lower small intestine and superficial and crypt colonic epithelium, and caused lysis of infected cells (Mebus et al., 1973a). Lesions in the small intestine were similar to those reported in animals affected with transmissible gastroenteritis of swine. Calves killed 48 to 96 hours after onset of diarrhea had shortened small intestinal villi. Some adjacent villi were fused and villous

epithelium was composed of low cuboidal to squamous cell types (Mebus et al., 1973a). Colonic ridges in large intestine were atrophied and covered by cuboidal epithelium and there were marked differences in length and spacing of microvilli on individual epithelial cells (Mebus et al., 1975). Scattered colonic crypts were dilated and lined by squamous to cuboidal epithelial cells.

Immunofluorescent, light, scanning, and transmission electron microscopic findings at the University of Nebraska and Michigan State University suggested the following pathogenesis for calf diarrheal coronavirus infection (Mebus et al., 1975a). Following oral inoculation, columnar epithelial cells of villi in the small intestine became infected and the infection rapidly progressed caudally. When the calf became depressed and diarrhea began, villous epithelial cells were morphologically normal, but the cells contained a large quantity of viral antigen and virions (Mebus et al., 1973a). As the infection proceeded, there was an accelerated migration of infected epithelial cells toward the tips of villi and cells were shed off the villi and replaced by cuboidal epithelial cells.

In more recent research Mebus (1975a) postulated that diarrhea initially resulted from decreased intestinal absorption due to a redirection of epithelial cell function from absorption to virus production and accumulation in the intestinal lumen of digestive fluids and partially digested milk. The continuing diarrhea in calves resulted from reduced absorptive capacity of the intestine due to immature villous epithelium, reduced surface area because of small intestine villous and colonic atrophy, and continuing infection with prolonged damage to intestinal epithelium (Mebus et al., 1975a; Mebus, 1976b).

Several years' work by H. W. Moon, primarily with the pig, at the National Animal Disease Center in Ames, Iowa, has contributed greatly to an understanding of the pathogenesis of diarrhea. The selective nature of viral damage has implications for recovery; while villous epithelium is destroyed, crypt epithelium containing the proliferative segment of the population is spared, undergoes hyperplasia, and regenerates the villi. The pathogenetic sequence of this virus infection results in selective destruction of villous absorptive cells which leads to varying degrees of villous atrophy, malabsorption, diarrhea, crypt hyperplasia, and recovery (Moon, 1978; Mebus et al., 1975a; Mebus et al., 1975b).

Immunologic Considerations

Immune responses are adaptive processes which occur only in vertebrates and constitute the principal means of defense against infection. It had been known that man and animals could become immune to certain microorganisms following recovery from infection, but the basis for these immune responses was not revealed until von Behring and Kitasato demonstrated an induced immunity to tetanus due to the appearance in serum of a capacity to neutralize tetanus toxin in 1890.

Antibodies are proteins formed in response to an antigen which react specifically with that antigen and belong to a special group of proteins called immunoglobulins. Early concepts of cellular immunity recognize ingestion and destruction of microbes by polymorphonuclear leukocytes and macrophages and the role of antibodies in coating particles and acting as opsonins to increase particle susceptibility to phagocytosis. The antigen combines specifically with

particular lymphocytes in these cell mediated immune reactions. Humoral immunity is the result of freely diffusible antibody molecules and cell mediated immunity is the result of specifically reactive lymphocytes. The concept of local antibody systems followed work with immunoglobulin A in 1959 by Heremans and Schultze and recognition of this immunoglobulin in human secretions in 1963 by Chodirker and Tomasi.

An appreciation of the complex nature of the immune system and the consequences of a compromised immune system has occurred during the last decade. Several excellent research and review articles by recognized authorities have appeared in recent scientific literature (Osburn, 1973; Osburn et al., 1974; Schultz, 1973a; Logan et al., 1974; Porter, 1973a; Porter et al., 1974; Schultz, 1973b; Logan, 1974; Seto et al., 1977; Mach and Pahud, 1971; Mach et al., 1969; Butler, 1973; Schultz et al., 1973; Butler, 1969; Berman, 1973; Butler et al., 1971).

Immunologic function in mammals consists of two principal components: the thymic or T cell system and the bone marrow or B cell system. Thymic lymphocytes, which constitute approximately 80% of the lymphocytes observed in circulating blood, are involved in cellular or cell mediated immunity. Bone marrow lymphocytes eventually become plasma cells and provide immunoglobulins with specific antibody activity (Osburn et al., 1974; Schultz, 1973b).

Thymic lymphocytes (T cells) apparently function in both cellular immunity and antibody production (Osburn et al., 1974). When the appropriate antigen interacts with receptor sites on the T cell membrane, the cell releases lymphokines. Lymphokines have diverse

biological effects which include mitogenic factor, macrophage-activating factor, migration-inhibition factor, lymphotoxic factor, and chemotactic factor. T cells also act in an accessory role with macrophages to aid the response of bone marrow lymphocytes (B cells) to many immunogens (Davis et al., 1973).

Bovine Immunoglobulins

Three classes of immunoglobulins have been identified in cattle: immunoglobulin G (IgG), immunoglobulin M (IgM) and immunoglobulin A (IgA) (Butler, 1969). The calf, like the adult, is able to produce all 3 classes of antibodies (Schultz, 1973a). It has also been suggested the cow has an immunoglobulin similar to human immunoglobulin E (IgE) (Schultz, 1973b). There are 2 distinct subclasses of IgG: IgG_1 and IgG_2. The distribution and synthesis of immunoglobulins and immunoglobulin-producing cells in cattle are similar to those described in other species (Butler, 1973). Two prominent subclasses of IgG immunoglobulins are demonstrable in serum from normal cattle. Immunoglobulin G_1 comprises about two-thirds of the IgG in serum and nearly all of the IgG in external secretions. Immunoglobulin G_1 is selectively transported by the udder from the circulation to the lacteal secretions and is the principal immunoglobulin for passive immunization of the calf. High concentrations of IgG_2 also appear in bovine serum.

Immunoglobulin M has been detected in serum, colostrum, milk and other secretions (Butler, 1969; Butler, 1973). It is especially concentrated in bovine colostrum, often to levels double the serum value. Immunoglobulin M antibodies are formed in almost every immune response, nearly always early in that response, and usually at low levels. They

are soon overshadowed by larger amounts of IgG antibodies to the same antigen.

An apparent bovine homolog of IgE has recently been detected (Butler, 1973). This IgE-like protein was concentrated in colostrum. The cells that produce IgE in the human are abundant in mucosa of the respiratory and intestinal tracts, and antibodies of the IgE class are found in exocrine secretions. The IgE proteins play an important role in allergic reactions.

Immunoglobulin A is the principal immunoglobulin in exocrine secretions of most animals (milk, respiratory and intestinal mucin, saliva, tears), but was not isolated from the bovine until 1969 (Mach et al., 1969). Secretory IgA (SIgA) is the predominant immunoglobulin in all exocrine secretions of cattle except lacteal secretions (Mach and Pahud, 1971). The fact that SIgA is a minor component of bovine colostrum is understandable when the difference in the route of passive transfer of immunity to offspring of different species is considered. Immunoglobulin G is the universal carrier of passive immunity from maternal to offspring serum. The importance of colostral IgA to the newborn calf should not be lightly considered without additional study in spite of deficient colostral IgA and the relatively low level of IgA synthesis by the bovine mammary gland. The biological function of an immunoglobulin is not related to its quantitative dominance.

Research on serum and secretions indicated that secreted IgA had distinct structural characteristics which differentiated it from serum IgA (Tomasi and Grey, 1972). The additional glycopeptide chain which is complexed into the structure of IgA has been termed secretory component and IgA in external secretions termed secretory IgA

(SIgA). The free secretory component is a protein product of epithelial cells and is attached covalently and extracellularly to an IgA dimer produced as such by local plasma cells (serum IgA occurs mostly as a monomer) (Davis et al., 1973; Butler, 1973). It is interesting to note that SIgA is not derived from serum IgA, but is produced by cells in the lamina propria of small intestine and in the mucosa of exocrine glands.

Porter and co-workers at Unilever Research Laboratory in England have conducted extensive enteric disease research over many years. A two-stage process takes place in construction of the SIgA molecule. Immunoglobulin A antibody is synthesized in plasma cells located in the gut wall and transported across epithelial cells where it is complexed with secretory component (Porter and Allen, 1972). Secretory IgA complex is formed at some point in the epithelium while immunoglobulin A is being transported from the lamina propria to the external surface (Porter, 1973a). The biological functions of secretory component are essential to maintenance of the concentration and stability of the antibody barrier. The secretory component contributes to resistance of SIgA to enzymatic degradation and assists in binding of antibody to the surface mucin layer (Porter, 1976; Porter, 1973b; Logan, 1974; Mach et al., 1969; Butler, 1973).

Most intestinal antibody system studies have occurred in the pig and recent studies suggest earlier descriptions of secretory immune mechanisms were oversimplified. Recent studies have demonstrated that more cells in the gastric mucosa of the young pig are involved in producing IgM than IgA (Allen and Porter, 1973). Porter (1973a) suggested it was possible that IgM in the young pig was mainly responsible

for the primary response in the external secretory system. Porter et al. (1972) also found an abundance of IgM in intestinal immunoglobulins of the neonatal calf. However, SIgA predominates in the secretions and bears the main responsibility for antibody defense of the external surface.

Porter recently discussed the role of lymphoid cells in Peyer's patches (1973a). These cells synthesize predominantly IgM. Porter suggested Peyer's patches act as a source of IgM memory cells which migrate to other lymphoid tissues. He also suggested Peyer's patches supply cells which exhibit preferential homing to the intestine and have the potential to proliferate and differentiate into IgA producing immunocytes in the lamina propria.

Antibodies are formed in response to antigens, but serum may contain immunoglobulins that react specifically with certain antigens to which the animal has had no known exposure. These immunoglobulins are called natural antibodies. Low levels of IgM have been demonstrated in fetal calf serum after mid-gestation and IgG has been found in precolostral serum of newborn calves even though the placenta is incapable of immunoglobulin transfer (Schultz et al., 1973; Schultz, 1973b; Klaus et al., 1969). Precolostral calf serum and bovine fetal serum are usually not agammaglobulinemic; low levels of IgG or IgM are present in most samples collected after 200 days of gestation. IgA has not yet been identified in the bovine fetus (Schultz, 1973b).

Vaccination During Gestation

It has been over 45 years since Theobald Smith described the relationship between colostral immunity and susceptibility to

colibacillosis in calves and suggested it might be possible to protect the calf by vaccination of the dam. There is still conflict concerning the value of vaccination as a means of protecting the newborn calf against *Escherichia coli* and neonatal calf diarrhea (Gay, 1971). Early reports in the literature that dealt with the value of vaccination of the dam as a means of protecting the newborn against colibacillosis described results which were generally unsuccessful; however, promising results have been reported in a number of recent studies at the Ohio Agricultural Research and Development Center in which sows were fed cultures of enteropathogenic strains of *E. coli* during the last month of gestation (Kohler, 1974; Kohler et al., 1975). Pregnant cattle have also been orally inoculated with viable *E. coli* (Ward et al., 1977). Formalin-treated live *E. coli* vaccines administered by intramammary or intramuscular injection have been demonstrated to increase immunoglobulin levels in mammary secretions and reduce neonatal enteric colibacillosis under field conditions (Wilson, 1972a; Wilson, 1972b; Wilson, 1972c; Wilson, 1974; Wilson et al., 1972a; Wilson et al., 1972b; Ward and Bigland, 1976).

It seems unlikely that serotype-specific antibody is the primary protective mechanism in the field even though it can protect against experimental enteric colibacillosis. Enteric colibacillosis and colisepticemia are age limited in calves and occur only under 3 weeks of age. Gay (1971) suggested that this universal age resistance could not be associated with protection by a serotype-specific antibody and postulated that a more likely mechanism was development of local intestinal immunity following colonization of the intestine after birth. He then went on to suggest that *in utero* immunization of the calf might be an effective means of protection.

Surgical procedures utilizing laparotomy were developed for fetal vaccination by intracardiac, intramuscular, and intra-amniotic routes (Gay, 1971; Gay, 1975; Richardson et al., 1968; Richardson et al., 1971; Richardson and Conner, 1972; Cegnar et al., 1975). Conner and Carter (1975) at Michigan State University and Gay (1975) in Australia developed nonsurgical techniques to inject antigens into the amniotic fluid surrounding the bovine fetus which would lead to prenatal immunization by the oral route.

Prenatal immunization of ovine and bovine fetuses vaccinated intra-amniotically with *E. coli* antigen was effective and protected against challenge with the homologous *E. coli* serotype (Conner et al., 1973; Wamukoya and Conner, 1976; Olson and Waxler, 1976; Olson and Waxler, 1977). Conner and Carter (1975) were also able to demonstrate that calves developed serum neutralizing antibodies against calf diarrheal rotavirus following intra-amniotic vaccination with a cell culture attenuated rotavirus. Gay (1975) has also demonstrated that when the fetus was vaccinated *in utero* with a single serotype of *E. coli* the result was heterogeneous protection against neonatal colisepticemia. The finding that *in utero* vaccination of the calf can result in protection against colisepticemia that is non-serotype specific enhances consideration of this method of control for this disease. All calves orally vaccinated by placement of a bacterial antigen in the amniotic fluid surrounding the fetus for periods greater than 9 days prior to birth possessed antibody to the vaccinal strain and resisted challenge at birth (Gay, 1975; Conner et al., 1977).

Gay (1975), Conner et al. (1977), and Hamid et al. (1977) caution that the frequency of premature birth, stillbirth, and abortion following intra-amniotic vaccination precludes recommendation of this

vaccination technique for widespread field trials without further study.

Oral Immunization

It was observed during early research with calf diarrheal rotavirus that recovered calves did not develop diarrhea when reinoculated with virus several days to 4 weeks after initial infection (Mebus et al., 1973b). The possibility that calves could be protected by oral inoculation with an attenuated virus seemed probable.

In research at Nebraska the calf diarrheal coronavirus was attenuated by being passed on a fetal bovine kidney cell line (Mebus et al., 1976). Vaccine safety tests were performed using gnotobiotic calves; all calves remained normal. Potency of attenuated coronavirus vaccine was tested by orally inoculating 23 calves at first feeding. Each calf was given 1 dose of lyophilized reconstituted vaccine diluted to contain approximately $10^{3.3}$ $TCID_{50}$ (median tissue culture infective dose) per test dose. All calves remained clinically normal during the postvaccination observation period. They were observed 96 hours and then challenge-inoculated orally with 5 ml feces from a gnotobiotic calf which had typical signs of coronavirus diarrhea. One of the 23 developed mild diarrhea after challenge-inoculation. Nine nonvaccinated control calves were challenge-inoculated when 5 days old. All 9 developed severe diarrhea and 3 died.

In further research, attenuated coronavirus and attenuated rotavirus were combined into a single vaccine (Mebus et al., 1976). The combined viral vaccine was safety tested in ten gnotobiotic calves; all remained normal. Potency of the combined vaccine was tested by orally inoculating 38 gnotobiotic calves shortly after birth. Each

calf was given one dose of reconstituted vaccine which contained approximately $10^{4.5}$ $TCID_{50}$ attenuated coronavirus and $10^{5.1}$ $TCID_{50}$ attenuated rotavirus per test dose. Fourteen calves were challenge-inoculated with gnotobiotic calf rotavirus infected feces 72 hours after vaccination and 14 calves were challenge-inoculated with coronavirus infected feces 96 hours after vaccination. All calves remained normal throughout postvaccination and postchallenge observation periods. Seven control nonvaccinated gnotobiotic calves rotavirus challenge-inoculated at 4 days of age developed diarrhea. Seven control nonvaccinated gnotobiotic calves coronavirus challenge-inoculated at 5 days of age developed severe diarrhea and 2 died.

More recently, the two viral vaccines were inoculated intramuscularly into 4 to 8 month old fetuses in addition to required safety tests (Mebus, 1976). Six fetuses were inoculated with attenuated rotavirus; 6 fetuses were inoculated with attenuated coronavirus; 6 fetuses were inoculated with combined vaccine; and 2 fetuses were inoculated with a placebo. All calves were normal at birth. Coronavirus serology was performed on precolostral serums from calves which received attenuated coronavirus, combined vaccine and placebo. Calves which received the placebo had antibody titers to coronavirus less than 4 and calves which were injected with attenuated coronavirus had titers greater than 256. Titers are expressed as a reciprocal of the highest neutralizing dilution.

Isolated intestinal loops (Thiry-Vella loops) were prepared in the lower ileum of 2 1-day-old colostrum-deprived calves and in 1 2-day-old colostrum-fed calf. To obtain more insight on the mechanism of resistance following vaccination, these loops were washed daily by introducing phosphate buffered saline into the cranial end of the

loop. The first 25 ml discharged from the caudal end was collected for viral culture and the next 250 ml for protein analyses. When the calves were 4 days old, 4 ml coronavirus vaccine (titer 10^5 $TCID_{50}$/ml) was introduced into the cranial end of the loop (Mebus et al., 1976).

These studies using Thiry-Vella intestinal loops in colostrum-deprived calves demonstrated that at 4 days postinoculation virus was present in the loop fluid and that no detectable neutralizing antibody was present in the loop fluid or serum. No interferon was detected in the loop fluid during the period of viral shedding. There was no detectable virus, but neutralizing antibody was present in the loop fluid 7 days postinoculation. It was therefore proposed that early protection resulted from a viral interference phenomenon. Later resistance to infection was due to antibody present in the intestine. This antibody was identified as IgM and IgA. These intestinal immunoglobulin profiles were similar to those reported in the calf by Porter et al. (1972).

Replication of coronavirus in the intestinal loop of the colostrum-fed calf which had a high serum neutralizing antibody titer confirmed results in a previous report which indicated that calves with circulating coronavirus antibody were not protected against calf diarrheal coronavirus infection (Mebus et al., 1973d). Preinoculation antibody in the loop fluid of this calf was primarily IgM and IgA. This finding supports Porter's observation that a considerable amount of secretory IgA initially absorbed is subsequently resecreted into the intestinal lumen (Porter, 1972). Indirect evidence supporting Porter's observation of decreasing passively acquired IgA excretion with age was obtained in an investigation in which the extent of small

intestinal infection and severity of diarrhea in colostrum-fed calves increased between 5 and 14 days of age (Mebus et al., 1973d).

The failure of circulating antibody to prevent infection of intestinal epithelium by virus does not minimize the importance of colostrum for the newborn calf. Feeding adequate colostrum shortly after birth is important to attain normal levels of circulating antibody; as the quantity of circulating immunoglobulin increases, calf mortality decreases (House, 1968; Selman et al., 1971a; Selman et al., 1971b; Johnston et al., 1977). Failure of passive transfer of colostral immunoglobulins in clinically normal animals, not related to low immunoglobulin content in colostrum or inadequate colostrum ingestion, resulted in increased mortality and emphasized the importance of studies of absorption factors in the intestine and alternative methods of attaining neonatal immunity (McGuire et al., 1976; Sawyer et al., 1977).

Gnotobiotic Equipment

The first use of a flexible film isolator for rearing germfree animals was reported by Trexler and Reynolds (1957) and was followed by improved modifications by Trexler (1959). The flexible plastic film was inexpensive and readily made into microbial tight structures. It could be sterilized with liquid germicides applied as a spray or gaseous germicides. Peracetic acid was used for sterilization of the isolator and supplies were steam sterilized (Trexler and Reynolds, 1957). Formaldehyde gas could also be used for isolator sterilization (Trexler, 1961). Filters were made from layers of fine glass wool material and isolators were checked for leaks by the use of freon and a freon leak detector (Trexler and Reynolds, 1957; Trexler, 1961).

Research related to rearing specific pathogen-free and germfree swine in gnotobiotic flexible film isolators was first reported by Waxler (1961) and Trexler (1961). Techniques for rearing gnotobiotic pigs were discussed by Waxler et al. (1966). Experiments were also conducted with gnotobiotic ruminants in flexible film isolators (Smith, 1961; Trexler, 1971). Oxender et al. (1971), following a procedure to obtain germfree goats by hysterotomy, used an isolator in which the upper portion was made of plastic film and the lower portion consisted of a stainless steel tub. Mebus et al. (1972b) used a surgical bubble fabricated from plastic tubing to decrease the expense of hysterotomy derived calves. Calves were maintained in isolator units fabricated of galvanized sheet metal or stainless steel with plexiglass windows. These workers used formaldehyde gas to sterilize the isolators, steam sterilization for equipment, and peracetic acid in the transfer port.

Microbiological studies, problems and diets of germfree animals were discussed in *Technology in Germfree and Gnotobiotic Life Research* (Heneghan, 1969; Kotake et al., 1969; Nagata, 1969; Wostmann et al., 1969). In recent articles Trexler has discussed the need for reliable control of microbial contamination and ramifications of the uses of isolator systems in industry, research, medicine and surgery (Trexler, 1964; Trexler, 1973; Trexler, 1975).

Instrument Sterilization

Steam, or autoclave, sterilization is the most generally used, most economical and one of the most effective methods for total destruction of all organisms and microorganisms. However, this method

of sterilization is impractical for instruments or parts sensitive to heat or moisture.

Ethylene oxide will destroy all known organisms and microorganisms including bacteria, spores, fungi, and at least the larger viruses. The microorganisms most commonly used in culture tests of the sterilizing effect of ethylene oxide are the spores of *Bacillus subtilis* var *globigii* because they are more resistant to ethylene oxide than the spores of other organisms. Gas concentration, temperature, exposure time, and humidity must be in proper relationship to each other for ethylene oxide to be an effective sterilizing agent (Jorow, 1975). It is for this reason that ethylene oxide sterilization chambers are necessary, they must be used according to manufacturer's directions, and sterilization indicators should be used. A vacuum is used in the sterilization chamber as an aid to gas penetration. Aeration is required following sterilization of most materials to permit elution of residual gas. Ethylene oxide gas and its by-products are highly irritant to human tissue and inadequate aeration of rubber and plastic materials may result in inflammation and necrosis or burns (Glaser, 1977).

Polyethylene, polycoated paper and mylar, and uncoated paper packaging materials are commonly used for gas sterilization. Polyethylene bags which are permeable to ethylene oxide gas result in a transparent package which protects the sterile item from recontamination during storage. A piece of ethylene oxide tape attached to packs as an indicator of sterility is helpful (Aseptic-Thermo Indicator Company).

Anesthetic Agents

Tavernor et al. (1971) described the use of general anesthesia to derive gnotobiotic piglets and calves by hysterotomy. Methohexitone, halothane and oxygen was also the general anesthetic method used by Drummond et al. (1973) for the derivation of gnotobiotic foals. Prolonged anesthesia and depression of the fetus has been troublesome with general anesthesia of the dam. The use of methohexitone sodium as an induction agent followed by halothane and oxygen resulted in viable lively foals for these workers. Research workers at the Institute for Research on Animal Diseases in England also used methohexitone sodium as an induction agent and maintained anesthesia with oxygen-halothane during the derivation of gnotobiotic calves (Hoare et al., 1976). These research workers described an apparatus for rearing gnotobiotic calves following derivation by hysterotomy and salvage of the dam by slaughter (Dennis et al., 1976; Hoare et al., 1976). Workers at Nebraska used 2.5% procaine HCl with 1:10,000 epinephrine to infiltrate the surgical site and no general anesthesia (Mebus et al., 1972b).

Local and general anesthesia have been used during performance of abdominal surgery in the calf. Isolated intestinal loop surgery is possible in day-old calves with local anesthesia; however, considerable restraint is required (Mebus, 1976). Inhalation anesthetics offer a number of advantages over most other general anesthetics used for surgical procedures of the newborn calf; however, there are a number of problems inherent in the use of inhalation anesthetics within a gnotobiotic isolator.

The anesthetic CI-744 has been used in a variety of laboratory, zoo and wildlife animals (Parke-Davis and Company, 1974). One of the

two component drugs in CI-744 is a cataleptoid anesthetic (tiletamine HCl) and the other is a nonphenothiazine tranquilizer (zolazepan). Anesthesia produced in sheep by intravenous injection of CI-744 was found desirable and satisfactory for a variety of major surgical procedures (Conner et al., 1974). This anesthetic was also used experimentally in a few calves (Conner, 1976).

Thiry-Vella Fistulas

A Thiry-Vella fistula is an isolated segment of small intestine with an intact blood and nerve supply. The two ends of the isolated intestine are exteriorized through the skin surface as fistulous openings and the intervening portion of the intestine remains in the abdomen with the mesenteric pedicle (Markowitz et al., 1964). Halliwell et al. (1976) described the mucosal morphologic alterations in Thiry-Vella fistulas surgically placed in dogs. There was a reduction in villous length during the first day after surgery and a significant decrease in villus/crypt ratio. There was a reduction in crypt depth during the second and third days after surgery and no significant change in villus/crypt ratio between days 1 and 2, but there was a significant increase in villus/crypt ratio between days 2 and 3. There was no significant change during the remaining 3 days of the experimental period. Mebus et al. (1976) and Mebus and Torres-Medina (1975) reported the use of Thiry-Vella intestinal loops in 3 calves but did not describe mucosal morphologic alterations.

Summary

Most newborn calves are born with a minimum amount of serum immunoglobulin (Klaus et al., 1969). Passive transfer of antibodies from dam to calf occurs through colostrum. Newborn calves which do

not obtain colostrum or adequately absorb immunoglobulins from colostrum are extremely susceptible to neonatal infections (Fey, 1972; Gay, 1965; Klaus et al., 1969). Circulating and intestinal immunoglobulins are both necessary to protect the neonatal calf from colibacillosis (Logan and Penhale, 1971; Logan et al., 1974).

Immune responsiveness of the neonatal calf is complicated by passive transfer of maternal immunoglobulins in colostrum. Passive transfer of specific antibodies interferes with active immunization by specific antigens (Osburn, 1973). The antigen, instead of being presented to appropriate cells for immunization, interacts with circulating antibodies and is removed from the circulation by the reticuloendothelial system.

Recent studies suggest additional reasons for concern about the postnatal ability of the neonate to develop active immunity. Calves at birth may have plasma corticoid levels 2 to 5 times fetal or adult values, and corticoids have immunosuppressive effects and possibly cause a decline in cellular immunity at this time (Osburn, 1973). Metzger et al. (1978) reported that antigenic stimulation performed before 4 days of age in conventional pigs led to a poor humoral immune response compared with that of pigs immunized later and, although unable to explain the reasons for this inhibition, ruled out a direct relationship with corticoid hormones. There is also experimental evidence that late-term and newborn calves may be immunologically deficient as a result of a compromised cellular immune system, a reduced T cell population and an inability to respond to certain antigens (Osburn et al., 1974).

There appears to be an immunologic paradox in the newborn calf. Colostrum is of paramount importance in protecting the calf, but

passively transferred antibody also prevents initiation of the calf's active immune response to specific antigens. Passively transferred antibody, particularly IgG, is immunosuppressive (Schultz, 1973a).

Recent studies suggest that the intranasal route of vaccination with certain antigens may overcome immune suppression of passively transferred antibody (Schultz, 1973b). Results of *in utero* vaccination with *E. coli* and with calf diarrheal rotavirus are encouraging and would suggest the necessity for additional studies of this technique (Gay, 1971; Gay, 1975; Conner et al., 1973; Conner et al., 1977). This procedure has the advantage of providing protection at birth as well as preventing suppressive effects of colostral antibody on the immune response. Schultz (1973a, 1973b) has also urged continued and expanded studies of intrafetal vaccination as a means of prevention of neonatal calf diseases.

OBJECTIVES

The objectives of this investigation were:

1. To determine if injection of a cell culture attenuated calf diarrheal coronavirus into the amniotic fluid surrounding the fetus would induce sufficient fetal immune response to provide protection to the newborn calf to challenge at 6 days of age with virulent calf diarrheal coronavirus.

2. To determine and characterize secretory immunoglobulins produced in the small intestine of the newborn calf following vaccination *in utero*.

3. To determine if serum antibodies were produced in the fetus and the effect upon serum antibodies in the dam following *in utero* vaccination.

4. To confirm and extend previous research describing the lesions of the intestine resulting from calf diarrheal coronavirus infection.

5. To develop gnotobiotic techniques and surgical procedures for bovine enteric disease research.

MATERIALS AND METHODS

Experimental Animals and Design

Thirty-six pregnant Holstein cows in the third trimester of gestation and ten 1-day-old calves were used in this research. All calves involved in this research were Holstein dairy calves. The research was initiated in late 1976 and completed in the spring of 1978. Techniques were modified and improved as the research progressed and a total of 8 calves were gnotobiotically collected and maintained through all phases of the experiment.

Fourteen of 36 cows in this study were subjected to *in utero* vaccination of the fetus with modified live calf diarrheal coronavirus, 16 were vaccinated *in utero* with sterile 0.85% saline solution as controls, and 6 remained unvaccinated. All experimental procedures were completed on 8 gnotobiotic calves from these 36 cows. Five of these calves had been given modified live virus by intra-amniotic vaccination. Two of these calves were given saline solution and 1 calf was unvaccinated (Table 1).

Cows were maintained in small pastures with an open-sided barn for protection from inclement weather. They were fed a mixture of grass and legume hay free choice and ground corn. Trace mineral salt and dicalcium phosphate were also available free choice. Calving dates and fetal growth were estimated by rectal palpation of cows at frequent intervals. The cows were moved into the Veterinary Clinical

Table 1. Experimental procedure

8 Pregnant Holstein Cows		
	serum samples from dam	
2 *in utero* saline inoculated controls and 1 non-inoculated control	3 weeks preparturition	5 *in utero* attenuated coronavirus vaccinates
cesarean section	day 1	cesarean section
	serum samples from calf and dam	
intestinal loop surgery	day 2	intestinal loop surgery
	intestinal loop washings	
	days 3-7	
virulent challenge	day 7	virulent challenge
necropsy	day 10	necropsy

Center at the time of *in utero* vaccination and maintained there until the time of surgery. Feed was withheld for 12 to 24 hours and water for 8 to 12 hours prior to cesarean section.

Challenge Agent and Method of Exposure

The initial virulent calf diarrheal coronavirus was obtained from Dr. C. A. Mebus at the University of Nebraska. It was propagated for challenge in a germfree calf derived and maintained by methods parallel to those used for other gnotobiotic experimental animals. The calf was cesarean section derived in the morning, encouraged to suckle a nipple in the afternoon, and allowed to nurse 1 liter of 4% homogenized whole milk from a nursing bottle. Virulent virus was drawn into a 10 ml syringe and the calf orally inoculated at 6 hours of age by allowing the calf to suck on the syringe while pressure was applied to the plunger. The calf was given a second liter of milk 1 hour later and an additional 2 liters of milk the following morning.

The calf was carefully observed the day following inoculation so that virus-laden feces could be collected when diarrhea commenced. Diarrhea was first observed at 21 hours postinoculation and virus-laden fecal material was collected from the anus in a beaker by placing a finger in the rectum to stimulate peristalsis. Fecal material was collected over a 5 hour period and pooled in a 2 liter bottle.

An automatic multiple dose syringe[a] was used to transfer 10 ml aliquots of pooled fecal material into screw cap vials. The vials

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[a]Cornwall Pipetter, Continuous, Syringe Type, B-D, 10 ml, Scientific Products, McGaw Park, IL.

were immediately removed from the isolator, labeled, frozen, and stored at -60 C.

Sterility was maintained by carrying out all procedures within the gnotobiotic isolator. The isolator, calf, feces, and thawed challenge virus specimens were cultured and checked for contamination by procedures described in another section (Determination of Sterility). Viral propagation procedures were repeated 4 times before successful collection of challenge virus without a monocontaminant *Bacillus* species.

Vaccine Agent

Characterization, propagation and attenuation of calf diarrheal coronavirus has been described (Mebus et al., 1973; Sharpee et al., 1976). The modified live virus oral calf vaccine for calf diarrheal coronavirus used in this experiment was the only vaccine agent available and had been used extensively in other trials and experiments. This experimental modified live coronavirus vaccine, serial number X-5, propagated on bovine kidney diploid cell line BK4, was obtained from Norden Laboratories (Lincoln, NB). The lyophilized vaccine was refrigerated until it was reconstituted with diluent immediately prior to use. This experimental vaccine contained approximately $10^{6.1}$ $TCID_{50}$ per test dose.

Vaccination Procedure

It was intended fetuses be vaccinated 14 to 28 days prior to normal parturition; vaccination procedures were performed when it was estimated parturition would take place in 21 days. In preparation for vaccination the cow was restrained, the tail tied, and the position of the fetus located by abdominal ballottement of the right flank

(Figure 1). The point of vaccination was normally that point at which the fetus was most easily ballotted. If fetal ballottement was not possible, the point of vaccination was located 5 cm above and 25 to 30 cm anterior to the highest point of the right flank. An area approximately 30 cm square was clipped. This area was scrubbed[b] 6 times and prepped[c] 6 times with an antimicrobial solution. A 25 gauge 1 cm needle attached to a 3 ml syringe was used to infiltrate the skin at the point of vaccination with 2.5% procaine hydrochloride. The area was again surgically scrubbed[b] 6 times and prepped[c] 6 times. Two persons who had surgically scrubbed and donned sterile gloves were required for this vaccination procedure in order to guarantee aseptic technique.

The skin at the point of vaccination was nicked with a scalpel. A 12 gauge 5 cm needle was used as a cannula and inserted until the operator could feel peritoneal puncture. Emission of a small amount of peritoneal fluid was normal. A 16 gauge 30 cm needle was directed transversely, slightly upward or downward through the 12 gauge needle and slowly advanced (Figure 2). It was generally possible to feel passage of the needle through the omentum and uterine wall. The fetus usually kicked when pricked by the needle and in some instances continued vigorous activity throughout the procedure.

A 10 ml syringe was attached to the 16 gauge needle and 2 to 4 ml of amniotic fluid was removed. Amniotic fluid is normally clear, colorless and mucoid. If the tip of the needle was not within the amniotic cavity it was likely that clear, watery, amber-colored

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[b]Betadine Surgical Scrub, Purdue Frederick Company, Norwalk, CT.

[c]Betadine Solution, Purdue Frederick Company, Norwalk, CT.

Figure 1. Abdominal ballottement of the fetus in the right flank.

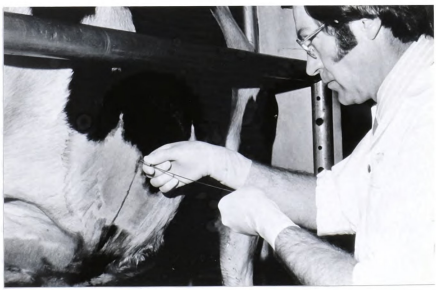

Figure 2. Advancing the needle toward the fetus.

allantoic fluid would be withdrawn into the syringe. It was then necessary to redirect the needle and reassess needle tip location. The syringe containing amniotic fluid was detached from the needle, the vaccine syringe attached to the needle, and the vaccine injected into the amniotic cavity. The needles were removed following vaccination and a topical dressing[d] applied.

A blood sample was collected from the cow prior to vaccination. Serum was separated and stored at -60 C. Amniotic fluid was cultured to determine if bacteria were present at the time of vaccination. Intramuscular injections of vitamins A and D[e] (5 ml) and selenium-vitamin E[f] (5 ml) were administered to all cows at the time of *in utero* inoculation after 3 calves had been lost with lesions of selenium deficiency.

Cesarean Section

Surgery was performed when parturition seemed imminent. This was determined by udder enlargement, presence of colostrum, ligament relaxation, and fetal presentation (rectal palpation). The cow was restrained in stocks and prepped for surgery. The area for epidural anesthesia and the entire left flank were clipped. The clipped area extended to the right of the midline dorsally and to the level of the subcutaneous abdominal vein ventrally. This entire area was surgically scrubbed and the surgical area from the lumbar transverse processes to the

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[d]Topazone, Eaton Veterinary Laboratories, Norwich, NY.

[e]Vitamin A&D Injectable, Pfizer Inc., New York, NY.

[f]BO-SE Injection, Burns-Biotec Laboratories, Oakland, CA.

flank, caudad to the rib cage, was shaved. The surgical site was scrubbed[b] 6 times, defatted 6 times with ether, and prepped[c] 6 times.

Anesthesia of the flank was accomplished with a paravertebral block by placing 20 ml of 2.5% procaine hydrochloride[g] 5 cm from the midline at the points where T_{13}, L_1, L_2 and L_3 cross the transverse processes (Figure 3). A slight lateral bow to the midline and non-reactivity to needle skin pricks were indicative of satisfactory anesthesia. The cow was led to the operating table and 10 ml of 2.5% procaine hydrochloride[g] epidural anesthesia administered immediately prior to tying the cow to the operating table (Figure 4). The cow was tied to the surgical table and the table tipped so the cow was in lateral recumbency with the left side uppermost (Figure 5). A partially inflated inner tube was placed under the right shoulder of the cow and the right leg extended forward to decrease the likelihood of radial paralysis. The grounding plate for the electro-surgical unit was placed under the neck of the cow. The surgical area was again scrubbed[b] 3 times, defatted with ether 3 times and prepped with 70% alcohol 6 times.

The bottom, or surgical aspect, of the surgical isolator was cleaned with alcohol and a sheet of mylar plastic taped to the bottom of the isolator after it dried. An aerosol of 2.0% peracetic acid[h] with 0.1% wetting agent[i] added was sprayed between the isolator and plastic sheet at least 30 minutes prior to the time of surgery. The

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[g]Epidural, Haver Lockhart Company, Kansas City, MO.

[h]FMC Corporation, Industrial Chemical Div., Buffalo, NY.

[i]Nacconol NRSF, National Analine Div., Allied Chemical Corp., New York, NY.

Figure 3. Administration of paravertebral anesthesia.

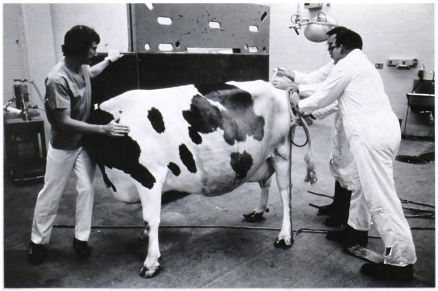

Figure 4. Administration of epidural anesthesia.

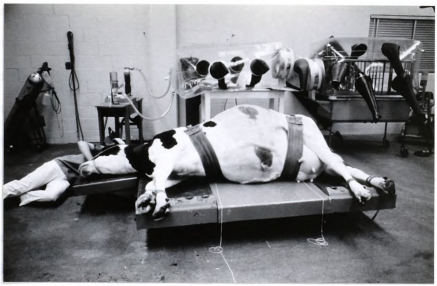

Figure 5. The cow on the operating table.

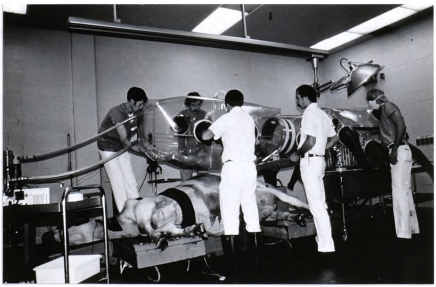

Figure 6. Surgical isolator being positioned on the cow.

sheet of mylar was removed and the isolator bottom dried with a sterile towel at approximately the same time that preparation of the surgical site was completed. When the surgical site and isolator bottom were dry, both were sprayed thoroughly with sterile aerosol adhesive.[j] The surgical isolator was moved into position above the surgical site and lowered onto the cow (Figure 6). The surgeon and assistant surgeon maintained their hands in the isolator gloves so that as the isolator was lowered they could guide it into position, smooth out wrinkles and assure firm attachment to the skin. Care was required to prevent creases which could cause leaks between the incision site and external isolator environment. The isolator was tied to the cow with 4 lines to prevent separation from the cow and possible contamination during surgery.

The incision through the bottom of the surgical isolator and partially through the skin was accomplished with a cautery tip[k] (Figure 7). The incision through the skin and subcutaneous tissues was completed with an electro-surgical pencil.[l] The incision through the muscle layers and peritoneum was accomplished with blunt dissection and scissors. These incisions were made parallel to the muscle fibers of each layer.

The tip of the gravid uterine horn was exteriorized through the incised abdominal wall by manipulation of the feet and hocks of the

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[j]Vi-Drape Adhesive, Parke-Davis and Company, Medical-Surgical Division, Detroit, MI.

[k]National Electric Instrument Company, Inc., Long Island, NY.

[l]Solid-State Electrosurgery Model SSE2 with IsoBloc and Cautery Pencil Lectro Switch Model Number E2502, Valley Lab, Boulder, CO.

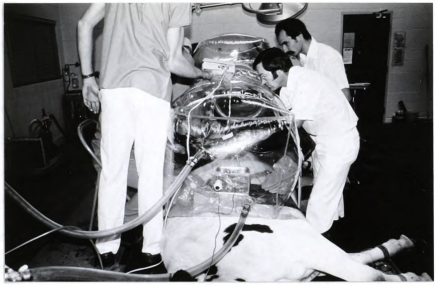

Figure 7. Skin incision with electrocautery.

Figure 8. Exteriorized gravid uterine horn.

fetus (Figure 8). An incision through the uterine wall and fetal membranes was made with scissors and extended until it was long enough to allow delivery of the fetus. Obstetrical chains were placed over each foot and obstetrical handles were attached to the chains close to the feet (Figure 9). The covers on the transfer port were removed and assistants placed their hands in gloves located in the transfer sleeve so they could grasp the obstetrical handles and help deliver the fetus. The fetus was immediately conveyed through the transfer port into the transport isolator and the cover replaced (Figure 10).

The surgical and transport isolators were disconnected and the surgical isolator removed from the cow. Two senior students, who had previously prepared for surgery, draped the surgical site and proceeded with closure of the surgical incisions (Figure 11).

The nose and mouth of the delivered calf were cleaned with linen towels immediately upon arrival in the transport isolator. Thumb forceps were inserted into the nostrils of the calf to induce sneezing and the calf was briskly rubbed and dried. A self-locking clamp[m] was placed on the umbilical stump to control hemorrhage. Blood samples were collected from the jugular vein and intramuscular injections of vitamins A and D[e] (2 ml), selenium-vitamin E[f] (2 ml), iron dextran complex[n] (2 ml) and B-vitamins[o] (2 ml) were administered. The transport isolator with the calf was moved by van to the veterinary research farm.

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[m]"Double-Grip" Disposable Cord Clamp, Hollister, Inc., Chicago, IL.

[n]Nonemic (Iron Dextran Injection), Burns-Biotec Laboratories, Omaha, NB.

[o]Vi-B Complex Injectable, W. A. Butler Company, Columbus, OH.

Figure 9. Attachment of obstetrical chains.

Figure 10. The newborn calf in the transport isolator.

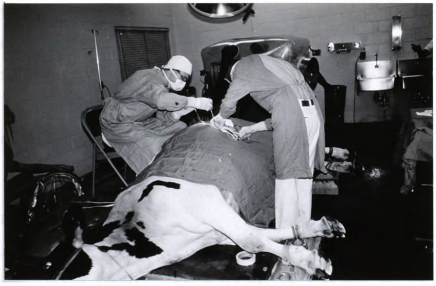

Figure 11. Closure of the surgical incisions.

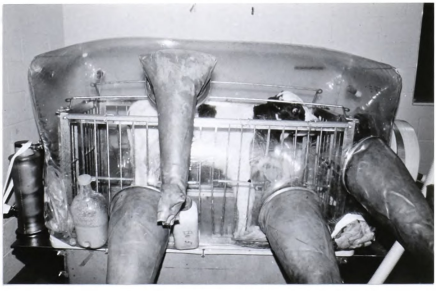

Figure 12. The calf in the primary gnotobiotic isolator.

The transport isolator was connected to the primary gnotobiotic isolator in which the calf was to be maintained by a transfer sleeve sprayed in with 2% peracetic acid. The calf was transferred to the primary gnotobiotic isolator 30 minutes later (Figure 12). Surgery was normally completed in the morning and by late afternoon the calf could be trained to suckle and allowed to nurse 1 liter of 4% homogenized whole milk from a nursing bottle.

Isolated Intestinal Loop Surgery

The Thiry-Vella loop surgery was performed on day 2 (1 day of age) using modifications of procedures described by Markowitz et al. (1964) and Mebus (1976b). The calf was tied to one corner of the isolator with a rope halter. Anesthesia was induced with an intravenous injection of an experimental anesthetic agent[p] (0.5 ml of a 10% solution). Most calves required an additional 0.5 to 1.0 ml of the anesthetic solution as the surgery progressed. The calf was transferred to a surgical isolator fitted with latex surgical gloves and a 110 volt electrical connection (Figure 13).

The calf was maintained in lateral recumbency on the left side and a towel placed under the calf's head to absorb saliva (Figure 14). The right flank was clipped and the hair gathered in a towel and placed in a plastic sack. The surgical site was draped with 4 linen towels and a surgical drape with a 30 cm hole. A mid-flank dorsoventral incision approximately 7 cm long was made through the skin. Scissors were used to incise the muscle layers and peritoneum dorsoventrally. Hemorrhage was controlled with mosquito forceps. A finger

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[p]CI-744, Parke-Davis and Company, Detroit, MI.

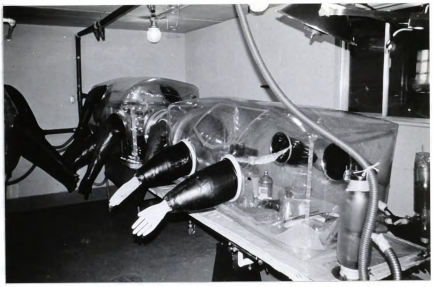

Figure 13. The surgical isolator for intestinal loop surgery.

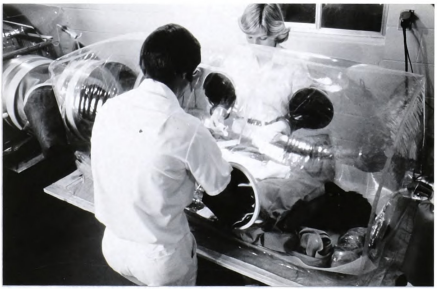

Figure 14. The anesthetized calf being prepared for surgery.

was hooked over the omental fold so it could be displaced in an anteroventral direction exposing the small intestine and cecum. The cecum was identified and exteriorized and the ileocecal junction located (Figure 15). Exteriorized portions of the intestine were kept moist during surgery with physiological saline solution. Color coded Allis tissue forceps were clamped over the ileum 5 cm apart and 30 cm anterior to the ileocecal junction. These color coded forceps were used to avoid confusion after the ileum had been severed at 2 points. The ileum was traced anteriorly an additional 150 cm and 2 additional color coded Allis tissue forceps were placed 5 cm apart (Figure 16).

The ileum was severed 30 and 180 cm anterior to the ileocecal valve. A 2 to 3 cm length of small intestine was removed from the distal ileum. A section of lymph node 1 cm square was carefully dissected from an ileal lymph node. Care was necessary to avoid damage to blood vessels supplying the ileum. The 2 specimens were wrapped in blood-soaked gauze and immediately transferred out of the isolator for use in other research. There was a tendency for severed intestinal ends to roll back upon themselves; excess mucosa was trimmed from the severed ends in preparation for anastomosis. The anterior end was frequently smaller in diameter than the posterior end and, since these ends were to be anastomosed, the anterior end was trimmed at an angle that would increase its effective diameter to equal the diameter of the posterior end. Care was taken to insure that the omental attachments of the loop and intestine to be anastomosed were not compromised or twisted. The Allis tissue forceps were held in close approximation by the assistant surgeon and the esophageal and rectal ends of the ileum were anastomosed using simple

Figure 15. The exteriorized cecum.

Figure 16. The ileum with Allis tissue forceps in place.

interrupted through-and-through sutures. Initial sutures were started through the mucosal surface at the omental attachment and the remaining sutures started through the serosal surface. When the suture line was complete, the Allis tissue forceps were removed, the anastomosis checked for patency, and the intestine replaced in the abdominal cavity.

The 2 ends of the Thiry-Vella loop were positioned in the incision with the distal end of the loop most often in the dorsal terminus of the incision. However, ease of positioning the loop ends and the care taken not to compromise the blood supply or twist the omentum was deemed more important than positioning a particular loop end in a specific incision terminus. Hence, the distal end of the loop was occasionally placed in the ventral terminus of the incision. The antimesenteric side of the loop end was slit to allow the ileum to fold back on itself (Figure 17). The peritoneum was sutured to the ileum at each end of the incision such that 2 to 2.5 cm of ileum (the end of the loop) protruded from the incision. Care was taken not to compromise the blood supply to the ends of the Thiry-Vella loop. Sutures placed in the ileum close to the mesenteric attachment were placed at right angles to the direction of the ileum and sutures close to the antimesenteric side of the ileum were placed in a longitudinal direction. The peritoneum in mid-incision between the 2 ends of the Thiry-Vella loop was brought together with interrupted sutures. The peritoneum was not sutured between the incision tips and the ileal loop ends. Some sutures were placed in muscle layers to bring them into apposition with each other or to fasten them to ileal loop ends in a manner similar to the method by which the peritoneum was sutured.

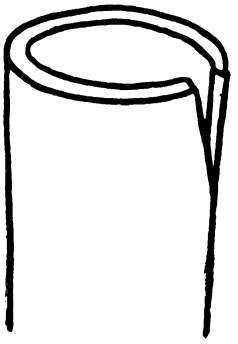

Figure 17. Schematic drawing of the incised antimesenteric ileal loop end.

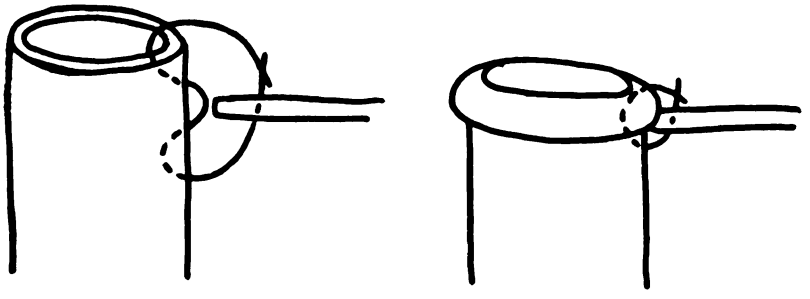

Figure 18. Schematic drawing of the suture pattern and stoma formation at the ileal loop ends.

A stoma was formed in each end of the intestinal loop by placing 4 sutures in a manner such that they held the ileum in apposition with the skin edge and the portion of the ileum folded back on itself rolled out onto the skin surface (Figures 18 and 19). Interrupted sutures were placed in the skin between the 2 loop ends. A liter of normal electrolyte solution[q] was administered intravenously to the calf upon completion of surgery and the calf was encouraged to nurse a liter of milk late the same day. Calves received 2 liters of milk twice each day thereafter until completion of the experiment. Isolator and room temperatures were maintained at 30 C for the first 2 days, then reduced to 22 C.

Intestinal Loop Secretion Collection

Collection of secretions from the ileal Thiry-Vella loop for determination of immunoglobulin and antibody required the use of a flushing solution. Flasks containing 400 to 500 ml of phosphate buffered saline solution (PBS) were fitted with rubber tubing and a drip chamber and autoclaved prior to use. A syringe filter holder[r] which had been individually wrapped and gas sterilized was attached to the air inlet tubing on the bottle of PBS. The tubing from the PBS was attached to a special double capped entry tube in the calf isolator (Figure 20).

A halter was placed on the calf and the calf tied in one corner of the primary isolator. A number 20 French Foley catheter was placed

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[q]Normal Electrolytes with Dextrose 5%, Jensen-Salsbury Laboratories, Kansas City, MO.

[r]Swinnex 25 mm Filter Holder with Millipore Filter, Type HA, 0.45 microns, Millipore Corp., Bedford, MA.

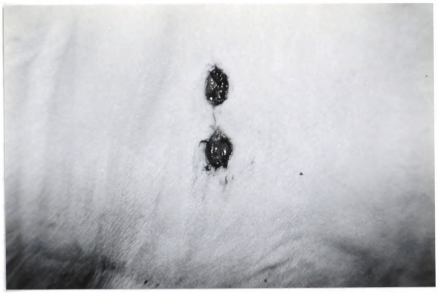

Figure 19. The intestinal loop ends fastened to the skin.

Figure 20. The primary calf isolator with collection apparatus attached.

in the anterior end of the Thiry-Vella loop and a number 24 French Foley catheter in the distal end (Figure 21). Phosphate buffered saline was allowed to run freely for a few moments to flush out any peracetic acid which might remain in the tubing. The tubing was then attached to the proximal Foley catheter and the flow of PBS adjusted so that rapid formation of drops could be seen in the drip chamber. The distal Foley catheter was attached to another length of rubber tubing which in turn entered a side arm flask (Figure 22).

The gravity flow of PBS was stopped before all of the solution drained out of the bottle. The inside tubing was disconnected from the special entry tube and the special entry tube capped. The proximal Foley catheter was removed from the Thiry-Vella loop after several minutes. The distal Foley catheter was left in place until peristaltic movement resulted in the collection of approximately 250 ml of material. The distal Foley catheter was removed at this point and the calf released. The side arm flask was stoppered and placed in the isolator port for removal. The special PBS entry tube was sprayed with 2% peracetic acid and capped immediately upon removal of the exterior tubing.

Sterilization Procedure

Surgical packs, clippers, etc., were sterilized in an ethylene oxide gas autoclave. This allowed a great deal of flexibility and the use of many items within the gnotobiotic isolators that otherwise would not have been possible (electric surgical clippers, electrosurgical pencil, and esophageal probe with plastic tubing).

Three milliliter amounts of BO-SE, Nonemic, Vi-B Complex and Injectable A&D were placed in 25 ml rubber-stoppered metal-capped vials.

Figure 21. Foley catheters in intestinal loop ends.

Figure 22. Secretion collection in a side arm flask.

These vials were wrapped in paper and steam autoclaved for 15 minutes at 15 pounds per squre inch and 121 C. Physiological saline solution, normal electrolyte solution and PBS were steam autoclaved at 121 C for 20 minutes. Homogenized 4% whole milk was steam autoclaved in 2 liter bottles for 35 minutes at 121 C. Vials and bottles were handled with rubber gloves following sterilization and were sprayed into the isolators with 2% peracetic acid. A 10% solution of CI-744 anesthetic was prepared by adding 100 mg of dry powder per ml of distilled water. This solution was sterilized by forcing through a filter[r] into a steam autoclaved sterile ampule. The glass ampule was sealed over a propane torch flame. Challenge virus was thawed and sealed in sterile glass ampules immediately prior to being sprayed into the gnotobiotic isolator.

Determination of Sterility

The presence of contamination was determined from composite specimens of fecal and waste material collected from the bottom of the isolator with sterile swabs. Sterile swabs were also used to collect rectal specimens. Material was streaked on tryptose blood agar[s] plates and inoculated into thioglycollate medium.[t] The media were incubated aerobically and anaerobically at 25 C, 37 C and 56 C. Aerobic plates were checked daily for 5 days and discarded at 7 days if negative; anaerobic plates were checked daily for 5 days, then weekly for 3 weeks if negative. The material was also inoculated

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[s]Tryptose Blood Agar Base, Difco Laboratories, Detroit, MI; Defibrinated Sheep Red Cells, Colorado Serum Company, Denver, CO.

[t]Bactofluid Thioglycollate Medium, Difco Laboratories, Detroit, MI.

into mycoplasma broth[u] and incubated aerobically at 37 C for 3 days. A blind passage of 0.1 ml was made from this broth onto a mycoplasma agar plate and incubated for 7 days at 37 C. Organisms isolated were further examined and identified.

Necropsy Procedures

Calves were euthanatized by electrocution on day 10 (9 days of age) and immediately placed in dorsal recumbency for necropsy (Jones and Gleiser, 1954). Tissue sections collected for light microscopy were placed in 10% formalin-sodium phosphate solution (buffered neutral formalin). Tissues collected for histopathologic examination were: colon, ileum, ileal loop, lymph node, thymus, spleen, salivary gland, thyroid, skeletal muscle, heart, lung, liver, kidney, adrenal, pancreas and urinary bladder.

Sections of spiral colon, ileum, and ileal loop were removed for fluorescent antibody staining and examination for the presence of coronavirus, and for bacteriologic culturing.

Light Microscopy

Fixed tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) according to established procedures (Luna, 1968).

Sections of colon, ileum and ileal loop were obtained at necropsy for detection of coronavirus by fluorescent antibody techniques. Tissues which could not be processed on the day of necropsy were preserved by freezing.

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[u]PPLO Broth, Difco Laboratories, Detroit, MI.

Tissue sections were trimmed to a thickness of approximately 3 mm, embedded in OCT compound[v] and frozen on a microtome chuck. Frozen sections were cut 8 microns thick and mounted directly on glass slides.

Fixation and staining of sections was accomplished as described by Bedell et al. (1968) using coronavirus fluorescent antibody conjugate.[w] Sections were examined for cytoplasmic fluorescence under a Zeiss microscope with 16X objective and Schott OG4 and OG5 barrier filters in combination with an FITC interference filter.

Immunoglobulin Identification

Immunoglobulins present in intestinal loop washings and serum were identified and quantified. Intestinal loop washings were collected each morning for 5 days beginning on day 3 (2 days of age, the day after calf surgery). Material collected each day was processed separately.

Centrifugation of intestinal loop washings for 5 minutes at 1500 rpm (500 x g) in an IEC Model CS centrifuge was employed to remove large particulate and mucoid matter. The resulting volume was measured and any amount in excess of 250 ml was discarded. Subsequent purification procedures were carried out on volumes of 250 ml or less.

Ammonium sulfate was used to precipitate the immunoglobulin out of solution. Granular ammonium sulfate was weighed to obtain a 50% saturated solution with intestinal loop washings as the solvent (31.3 g per 100 ml). Ammonium sulfate was added to intestinal loop washings over a 30 minute period while stirring slowly with a magnetic

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[v]Ames Company, Division Miles Laboratories, Inc., Elkhart, IN.

[w]Norden Laboratories, Inc., Lincoln, NB.

stirrer. The solution was allowed to stir an additional 60 minutes after the ammonium sulfate was dissolved.

The resulting solution was centrifuged at 10,000 rpm (11,000 x g) for 30 minutes in an IEC Model B-20 centrifuge. The supernatant was decanted and the pellet resuspended in 1 ml of 50% saturated ammonium sulfate in phosphate buffered saline (PBS), pH 7.4. The volume was increased to 20 ml with ammonium sulfate-PBS and centrifuged for 30 minutes at 10,000 rpm. The wash procedure was repeated. The resulting pellet was dissolved in PBS to a final volume of 3 ml.

Following precipitation of protein the samples were dialyzed against 2 liters of PBS, pH 7.4, in a 2 liter Erlenmeyer flask on a magnetic stirrer for 3 days at 4 C. The PBS was changed twice daily. All samples from a single calf were dialyzed in the same Erlenmeyer flask, but each sample was in an individual dialysis tubing bag.

Dialyzed protein fractions were concentrated in separate dialysis bags (Spectrapor Membrane Tubing, number 3; molecular weight cutoff 3,500). Each bag was surrounded with polyethylene glycol (20,000 molecular weight) taking care to crush the polyethylene glycol flakes and carefully surround each dialysis bag with the material. The solution in the dialysis bag was concentrated to a volume of approximately 0.5 ml by removal of water into the polyethylene glycol over a period of 1 to 2 hours. Calf sera were also concentrated from 3 ml to 0.5 ml by this method.

Quantitative immunodiffusion kits[x] composed of 3 calibrated immunodiffusion plates, 4 vials of reference standards and capillary pipettes

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[x]Research Products, Miles Laboratories, Inc., Elkhart, IN.

were used to determine the presence of immunoglobulins (IgA, IgM, IgG, IgG_1 and IgG_2) in calf sera and intestinal loop washings. After incubation of the plates for the specified amount of time (18 or 22 hours), precipitin rings of standards and samples were measured (Figures 23, 24 and 25). Immunoglobulin concentrations were determined using graphs provided with the kits.

Immunodiffusion plates were washed in 2% saline at room temperature for 4 days and rinsed in 3 changes of distilled water for 24 hours to remove any unbound proteins. Moistened pieces of filter paper were overlaid on the agar and the plates were left to dry overnight. The filter paper was removed and the dry plates were flooded with 0.1% Coomassie's Brilliant Blue[y] for 30 minutes. Excess stain was removed in a 10% acetic acid wash for 2 days to 1 week. Plates were considered destained and removed from the 10% acetic acid bath when the background was pale blue and precipitin rings were dark blue and easily distinguishable. Previous measurements were checked on stained plates for accuracy.

Titration of Calf Diarrheal Coronavirus

Secondary bovine fetal kidney cells were used in this procedure (Figure 26). The growth medium was basal medium (Eagle) with Hank's balanced salt solution containing 0.5% lactalbumin hydrolysate plus 10% calf serum. The maintenance medium was Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate (no calf serum).

Cell culture monolayers in 24 well plastic plates were washed 3 times with Hank's balanced salt solution before inoculation.

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[y]Coomassie's Brilliant Blue, 1 gm Coomassie's Brilliant Blue, 450 ml 10% Acetic Acid, 450 ml absolute ethanol, distilled H_2O to 1 liter.

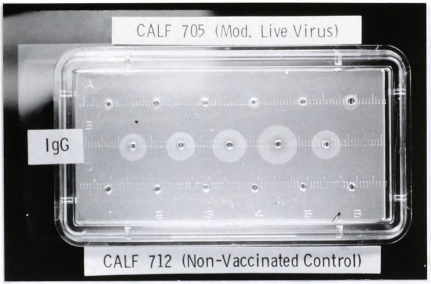

Figure 23. IgG immunodiffusion precipitin rings. The reference standards are in the center wells (row B). Wells 1 through 5 in rows A and C contain concentrated daily intestinal loop washings. The number 6 wells contain concentrated calf serum.

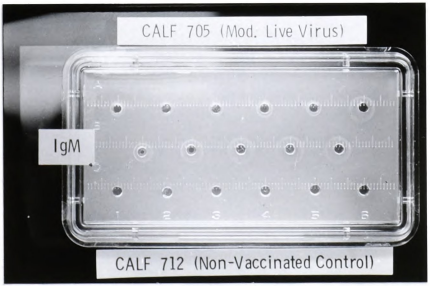

Figure 24. IgM immunodiffusion precipitin rings. The reference standards are in the center wells (row B). Wells 1 through 5 in rows A and C contain concentrated daily intestinal loop washings. The number 6 wells contain concentrated calf serum.

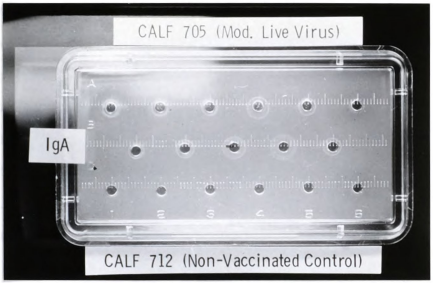

Figure 25. IgA immunodiffusion precipitin rings. The reference standards are in the center wells (row B). Wells 1 through 5 in rows A and C contain concentrated daily intestinal loop washings. The number 6 wells contain concentrated calf serum.

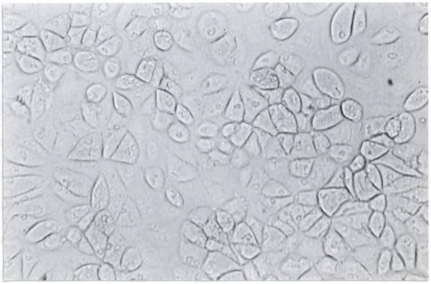

Figure 26. Normal appearance of secondary bovine fetal kidney cell monolayer.

Appropriate tenfold dilutions of virus were prepared in maintenance medium (Figure 27). The last medium wash of the cell cultures was decanted from the plates and 0.2 ml of the viral dilution added to each well. Virus was allowed to adsorb for 6 minutes at 37 C. After adsorption 1.3 ml of maintenance medium was added to each well. Plates were incubated at 37 C under high humidity and 5% carbon dioxide for 5 days.

Test results were read by hemadsorption using hamster erythrocytes. Hamsters were bled, the whole blood added to Alsever's Solution (anticoagulant) at a 1:1 dilution, and the suspension centrifuged at 500 to 800 rpm. The red blood cells harvested were washed 3 times with PBS. A 0.2% solution of red blood cells from the hamster blood was made in PBS. The maintenance medium was discarded from the wells and hamster erythrocytes were added for hemadsorption. One milliliter of 0.2% red blood cell solution was added to each well (Figure 28). Plates were incubated at 4 C for 30 to 60 minutes. Plates were then gently rocked back and forth to dislodge and unsettle red blood cells that were not attached (adsorbed to virus particles). The red blood cell suspension was decanted from the wells and 0.5 ml of PBS added to each well. The plates were gently rocked again and decanted. The individual wells were examined for red blood cell hemadsorption through an inverted microscope and 50% end point titers determined by the method of Karber (Lennette, 1969) (Figure 29). Titers were expressed as the reciprocal of the highest neutralizing dilution.

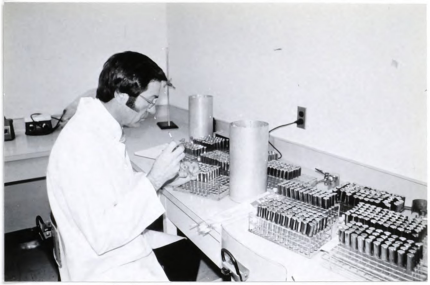

Figure 27. Dilutions of virus being prepared.

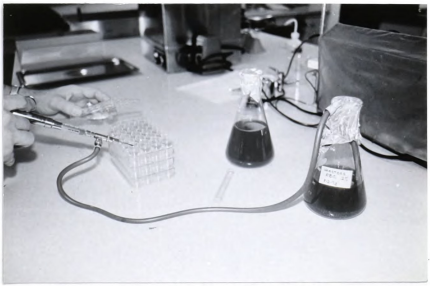

Figure 28. Addition of hamster red blood cell solution to each well.

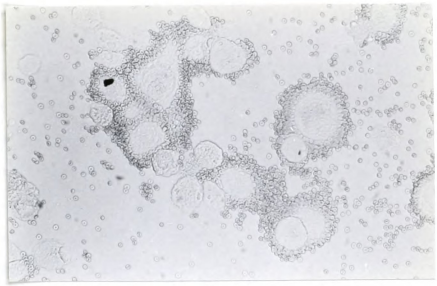

Figure 29. Red blood cell hemadsorption.

Figure 30. Toxic effect on tissue culture cells or virus which prevents hemadsorption determination.

Neutralization of Calf Diarrheal Coronavirus

The initial individual daily samples of intestinal loop washings proved unsatisfactory for neutralization testing. Intestinal loop washings had a toxic effect on the cells, or a toxic effect on the virus, and it was not possible to make accurate virus determinations by hemadsorption with hamster red blood cells (Figure 30). Intestinal loop washings remaining from the 5 day collections for each calf were pooled. A protein determination was made on each of these samples by the method of Lowery et al. (1951). Protein concentrations of the pooled samples from each calf were adjusted by adding PBS until each sample contained 2 to 4 mg of protein per ml of solution (approximately a 1:3 dilution). The intestinal loop washings pooled from each calf were placed in dialysis tubing and dialyzed against PBS in a 2 liter Erlenmeyer flask on a magnetic stirrer for 5 days at 4 C. The PBS was changed twice daily. The samples were filtered[z] into sterile screw cap vials.

Tissue cells and growth and maintenance media were the same as described for the titration process. Twofold dilutions of serum and intestinal loop washings were prepared in maintenance medium. An equal volume of medium containing 100 to 1000 $TCID_{50}$/ml of coronavirus was added to each sample dilution tube. The virus-sample mixture was incubated for 60 minutes at 37 C. Twenty-four well plastic plates containing cell culture monolayers were washed 3 times with Hank's balanced salt solution. The last medium wash was decanted and 0.2 ml of the virus-sample mixture was added to each well (4 wells per dilution). The mixture was allowed to adsorb for 60 minutes at 37 C. After

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[z]Millex Disposable Filter Units; 0.45 µm pore size; 25 mm diameter, Millipore Corp., Bedford, MA.

adsorption, 1.3 ml of maintenance medium was added to each well. Plates were incubated at 37 C under high humidity and 5% carbon dioxide for 4 days. The medium from the wells was discarded, hamster erythrocytes added for hemadsorption, and titers determined as described under the titration section of this report.

RESULTS

In utero Vaccination

Fourteen of 36 cows utilized in this research were vaccinated *in utero* with cell culture attenuated calf diarrheal coronavirus. Two cows aborted on days 2 and 3 postvaccination. Four cows gave birth to live premature calves on days 9 and 10 postvaccination.

Twenty-two cows were utilized as controls. Sixteen were inoculated *in utero* (intra-amniotically) with sterile 0.85% saline solution and 6 were not inoculated. All 22 cows maintained normal pregnancy until delivery by cesarean section.

Calf Survival

Ten 1-day-old calves which had received colostrum were successfully maintained in early phases of this research. Control colostrum deprived calves developed diarrhea and died early in the research when attempts were made to raise them in a conventional environment. This necessitated the use of strict isolation facilities (unavailable) or alteration of the research proposal to a gnotobiotic study.

More cows and calves than anticipated were required to develop the methodology for this research and to replace calves lost in unsuccessful experiments. The use of gnotobiotic calves to propagate virulent challenge virus (4), early isolator failure (4), surgical error (1), anesthetic death (1), selenium deficiency (3), failure to survive cesarean section delivery (2), intestinal loop stoma patency

failure (1) and inadequate time to prepare for gnotobiotic delivery prior to normal parturition (3) resulted in the need to increase the number of research cows from 12 to 36. Eight calves survived all phases of the final modified research proposal and were challenged with virulent virus to produce the data in the following sections.

Resistance to Challenge

All calves vaccinated with attenuated coronavirus intra-amniotically and subsequently challenged with virulent virus failed to develop clinical signs of coronavirus infection. Vaccinations were accomplished 9 to 36 days prior to parturition.

Oral vaccination of the fetus with a cell culture attenuated calf diarrheal coronavirus was effective in protecting neonatal calves from challenge with orally administered virulent calf diarrheal coronavirus at 6 days of age. The feces from vaccinated calves remained unchanged following challenge. Control (saline inoculated and non-inoculated) calves developed diarrhea 19 to 22 hours following oral administration of virulent virus (Table 2).

Immunofluorescent Microscopy

Tissue sections of colon, ileum, and ileal loop from saline control and non-inoculated calves collected at necropsy and stained by fluorescent antibody (FA) technique were coronavirus positive. Tissue sections from vaccinated calves were FA negative (Table 2).

Light Microscopy

No gross pathologic changes were noted in the calves at the time of Thiry-Vella loop surgery. Microscopic differences were not

Table 2. Results of oral challenge with virulent calf diarrheal coronavirus

Calf Number	Diarrhea	FA
Non-inoculated control		
712	+	+
Saline inoculated controls		
704	+	+
710	+	+
Vaccinates		
705	-	-
711	-	-
714	-	-
801	-	-
804	-	-

seen in histopathologic sections of ileum from vaccinated, saline control and non-inoculated calves 1 day of age.

In utero attenuated coronavirus vaccinated calves euthanatized 72 hours following oral challenge with virulent coronavirus did not have visible gross lesions. Histologically, villi in the small intestine appeared normal and the villous epithelium resembled that in previously described gnotobiotic calves (Mebus et al., 1975b). Eosinophils in the lamina propria were prominent and numerous. Normal colonic ridges and furrows were visible histologically.

Saline control and non-inoculated calves euthanatized 72 hours following oral challenge (49 to 73 hours after onset of diarrhea) had liquid contents in small and large intestine. No other gross changes were observed. Histologically, villi of both ileum and intestinal loop appeared shortened and covered by cuboidal epithelial cells. Many villous tips were denuded of epithelial cells. Epithelial cells in the colon were cuboidal, the number of goblet cells was reduced, and scattered crypts were dilated, lined by cuboidal epithelial cells and contained dead cells in the lumen. There was loss of epithelial cells from the lumen surface of colonic ridges and some fusion of ridges. These observations concurred with the findings of an earlier study with coronavirus infected gnotobiotic calves described by Mebus et al. (1975a). Histopathological changes were not seen in other tissues collected at necropsy.

Bacterial Contamination

The 8 calves reported in this research were contaminated with *Bacillus* sp., *Micrococcus* sp., *Acinetobacter* sp., *Citrobacter freundii* or *Streptococcus* sp., or a combination of these organisms (Table 3).

Table 3. Culture results from swabs taken from the primary gnotobiotic isolator of each calf at 1, 4 and 7 days of age

Days of Age	Non-inoculated control	Saline-inoculated controls		Vaccinates				
	712	704	710	705	711	714	801	804
1				*Bacillus*		*Bacillus*	*Bacillus* *Acinetobacter*	*Bacillus*
4	*Micrococcus* *Acinetobacter*		*Bacillus* *Micrococcus*	*Bacillus* *Micrococcus* *Acinetobacter*	*Bacillus* *Micrococcus*	*Bacillus*	*Bacillus* *Acinetobacter*	*Bacillus* *Micrococcus*
7	*Micrococcus* *Bacillus*	*Micrococcus*	*Bacillus* *Micrococcus*		*Bacillus* *Citrobacter*	*Bacillus* *Micrococcus* *Acinetobacter* *Streptococcus*	*Bacillus* *Acinetobacter*	*Bacillus* *Micrococcus*

During the course of this research most isolators were contaminated with *Bacillus* sp. Only 2 isolators used in this research remained germfree throughout their use.

Immunoglobulin Identification

Cow sera from all cows contained high levels of immunoglobulins before and after *in utero* vaccination. There was a significant increase in serum immunoglobulin (Ig) concentrations of vaccinated calves compared to control calves on the day of cesarean section delivery (Table 4). These results are indicative of the ability of the near-term fetus to respond to certain immunogens by the production of serum Ig. The Ig immunodiffusion tests were performed on concentrated serum samples and the data converted to mg per 100 ml of serum for comparison.

The 5-day combined intestinal loop washings contained Ig amounts similar to the individual daily samples (Table A-1). The 5-day pooled sample immunoglobulin content is reported in Tables 5 and 6. The immunodiffusion tests were performed on concentrated samples and the values reported have been calculated on the basis of mg Ig per 100 mg protein (Table 5) and mg Ig per 100 ml intestinal loop washing (Table 6). No Ig's were found in intestinal loop washings of control calves. Oral vaccination of the fetus with attenuated virus resulted in significant increases of IgA, IgM, and IgG in the intestine. The quantity of IgA present in intestinal loop washings was 3 times the amount of either IgM or IgG and indicates the production of high levels of secretory Ig which may be stimulated by some antigens.

Table 4. Calf serum immunoglobulin content on the day of cesarean section delivery (mg/100 ml serum)

Calf Number	IgA	IgM	IgG	IgG_1	IgG_2
Non-inoculated control					
712	0.0	7.2	0.0	0.0	0.0
Saline inoculated controls					
704	0.0	5.0	t*	4.0	0.0
710	0.0	5.0	t	t	t
Vaccinates					
705	9.6	70.8	43.9	6.8	0.0
711	10.7	153.4	t	5.2	t
714	0.0	86.7	51.7	33.3	13.0
801	20.0	18.0	31.0	28.0	11.0
804	18.3	32.1	146.8	56.5	29.4

*t = trace (a ring was present on the immunodiffusion plate, but it was below measurable levels on the graph).

Table 5. Intestinal loop washing immunoglobulin content in the 5-day pooled sample (mg/100 mg of protein)

Calf Number	IgA	IgM	IgG	IgG_1	IgG_2
Non-inoculated control					
712	0.0	0.0	0.0	0.0	0.0
Saline inoculated controls					
704	0.0	0.0	0.0	0.0	0.0
710	0.0	0.0	0.0	0.0	0.0
Vaccinates					
705	13.2	3.9	t*	0.0	0.0
711	18.2	2.7	11.8	0.0	0.0
714	47.6	7.1	15.5	t	0.0
801	14.7	0.0	t	0.0	0.0
804	21.1	1.6	6.8	1.5	0.0

*t = trace (a ring was present on the immunodiffusion plate, but it was below measurable levels on the graph).

Table 6. Intestinal loop washing immunoglobulin content in the 5-day pooled sample (mg/100 ml)

Calf Number	IgA	IgM	IgG	IgG_1	IgG_2
Non-inoculated control					
712	0.0	0.0	0.0	0.0	0.0
Saline inoculated controls					
704	0.0	0.0	0.0	0.0	0.0
710	0.0	0.0	0.0	0.0	0.0
Vaccinates					
705	.2016	.0605	t*	0.0	0.0
711	.4334	.0650	.2817	0.0	0.0
714	1.0681	.1602	.3471	t	0.0
801	.2220	0.0	t	0.0	0.0
804**	16.5289	1.2397	5.3719	1.1570	0.0

*t = trace (a ring was present on the immunodiffusion plate, but it was below measurable levels on the graph).

**Intestinal loop washings from this calf were not obtained by the described protocol.

Specific Coronavirus Neutralization Antibody

The serum from all cows contained antibodies to calf diarrheal coronavirus before and after vaccination. No serum samples had an antibody titer less than 256 (256 to 2048) and there were no significant differences between before and after vaccination titers (Tables A-2 and A-4).

The coronavirus serum neutralization antibody tests on samples from non-inoculated and saline control calves were negative. In contrast, the vaccinated calves had serum neutralization antibody titers of 4 to 128 (Table 7 and Tables A-5 and A-4). Neutralization antibody titers of the concentrated pooled 5-day intestinal loop washings from vaccinated calves were significantly higher than those from non-inoculated and saline control calves (Table 7 and Tables A-3 and A-4). These results are indicative of the ability of the near-term fetus to respond to an orally administered attenuated virus by the production of specific serum and secretory humoral antibody.

Table 7. Coronavirus neutralization antibody titers of calf sera collected the day of cesarean section delivery and of pooled 5-day intestinal loop washings

Calf Number	Interval (days) between Vaccination and Delivery	Antibody Titer of Calf Sera	Antibody Titer of Concentrated Loop Washings
Non-inoculated control			
712	---	0	4
Saline inoculated controls			
704	49	0	4
710	24	0	0
Vaccinates			
705	9	32	128
711	9	64	64
714	10	4	64
801	36	64	32
804	24	128	128

DISCUSSION

Introduction

The logistics involved in large animal research, especially when coupled with gnotobiotic techniques and multiple facilities, can be awesome. In addition, colostrum deprived control calves are not easily reared (Barnum et al., 1967; Fey, 1972) and dairy calves appear to be immunologically less responsive than beef calves (Brown, 1978; Wamukoya, 1975). In spite of the associated problems this type of research offers a number of unique advantages in obtaining relevant data concerning pathogenic agents without interference by other pathogens, but the required resources are high when compared with other research.

In utero Vaccination

Abortion and/or the birth of premature calves following *in utero* vaccination, encountered in this research, has been a problem for a number of research workers (Olson, 1975; Conner et al., 1977; Gay, 1975; Hamid et al., 1977). This problem precludes the use of *in utero* vaccination in extensive field trials at this time. Since control saline inoculated fetuses are not affected, the vaccination technique would not appear to be at fault. The factors present in the vaccine agents used to date which cause abortion and premature birth have not been identified.

It is speculated that abortion is precipitated by a fetal stress response and increased fetal corticoids following vaccination, since increased fetal corticoids have been demonstrated to induce parturition (Liggins, 1968). However, there have been no reports of elevated fetal corticoid levels following vaccination. Premature delivery 9 or 10 days following *in utero* vaccination may be related to the onset of antibody production, other immune responses or altered fetal corticoid levels.

Further research should be directed toward evaluating fetal response to vaccine agents. The effect of further attenuation of modified live agents and vaccine dose reductions should also be investigated as means to avoid vaccine induced abortion and premature birth.

Immunoglobulin and Antibody Levels

In utero (intra-amniotic) vaccination is an effective means of introducing some antigens to the fetus (Conner et al., 1973; Gay, 1975) and there are numerous examples of effective oral vaccination with modified live viral agents. This research confirmed the ability of an orally administered attenuated coronavirus to stimulate an immune response in the fetus. These calves were protected during the neonatal period against challenge by virulent coronavirus and developed significant serum Ig, intestinal Ig and specific neutralizing antibody levels.

The development of immunity to calf diarrheal coronavirus and intestinal secretion of IgA and IgM in these experiments was in agreement with results of earlier work by Porter in England (Porter et al., 1972) and Mebus at Nebraska (Mebus et al., 1976). However, secretion

of IgG into the intestinal lumen was not anticipated. Immunoglobulin G is not considered to have an important role in intestinal secretions. A humoral immune response had been expected; however, the serum concentrations of IgA were higher than expected. The immunoglobulin concentrations and neutralization antibody titers lead one to conclude that the near-term bovine fetus is capable of an excellent immune response to this attenuated virus vaccine.

The variation in the results of Ig levels from 2 intestinal loop collections may reflect variations induced by differences in surgical procedures. It was noted that the isolated intestinal loop collection procedure was more time consuming for calf 714 and that the loop was longer than others at necropsy. The stomas of the intestinal loop of calf 804 were damaged by trauma and licking which caused them to become non-patent. In this case satisfactory intestinal loop washing collection was not possible and laboratory procedures were performed on loop washings collected at necropsy. Physical problems with, and differences between, isolated intestinal loops may have added to variation in results.

Apparent field vaccination failures have been reported in groups of calves vaccinated at birth with commercial oral modified live virus vaccine. Speculation regarding the cause of vaccine failure centers on the role of colostrum and colostral antibody. It is theorized that colostral antibody obtained soon after birth neutralizes vaccine virus. All of the cows in this research had serum neutralization antibody titers against coronavirus prior to vaccination and it is likely that specific antibody was also present in colostrum. This would support the theory advanced to explain vaccine failures.

This research utilized colostrum deprived gnotobiotic calves to prevent possible colostral antibody interference. Further research is needed to define the role of colostral antibodies and their effect on the immune response of the newborn calf to attenuated calf diarrheal coronavirus.

Light Microscopy

Small intestinal lesions of coronavirus infection in calves and those of transmissible gastroenteritis in swine are similar. The major difference between coronaviral infections of the 2 species is that the former agent also infects colonic epithelium. Infection of intestinal loop epithelium with coronavirus and presence of lesions identical to those in ileum and the humoral immune response following oral vaccination of the fetus suggest the possibility of a viremic phase of infection; however, there is no experimental evidence to support this hypothesis.

Bacterial Contamination

The difficulty in preventing contamination of gnotobiotic isolators was disappointing. Early failures were attributed to old equipment and cracks, holes and tears in the plastic isolators. Occasionally a torn or punctured glove was identified as a possible source of contamination. Consideration was also given to the isolator juncture of the plastic and stainless steel tub as a possible source of contamination. Four isolators were used for the derivation and maintenance of each calf. The number of isolator manipulations and transfers necessary enhanced the likelihood that one or more contaminants would gain entrance.

More elaborate skin preparation and thermic skin incision procedures were also adopted in an attempt to prevent contamination during cesarean section. It was speculated that *Bacillus* spores, which are more resistant to ethylene oxide than spores of other bacterial species, might have survived sterilization. However, the source of contamination was not determined.

Fortunately, all contaminants were commensal organisms and none was considered pathogenic. It is not likely within the time frame of this research that any of the contaminant organisms played a role in the immune responses measured.

SUMMARY

Bovine fetuses were inoculated during the last 7 weeks of gestation by the deposition of either cell culture attenuated calf diarrheal coronavirus (14 fetuses) or sterile physiological saline solution (16 fetuses) into the amniotic fluid. Six additional fetuses were not inoculated. Two virus vaccinated fetuses were aborted on days 2 and 3 postvaccination and 4 virus vaccinated fetuses were delivered prematurely on days 9 and 10 postvaccination. All 22 saline inoculated and non-inoculated (control) cows maintained normal pregnancies. The data from 8 gnotobiotic calves which survived all phases of this research and were challenged with virulent calf diarrheal coronavirus are included in this report.

This research was carried out under gnotobiotic conditions. Calves were cesarean section delivered into a gnotobiotic surgical isolator and immediately transferred into a transport isolator. Calves were maintained at the veterinary research farm for 9 days in a primary gnotobiotic isolator. Surgery to isolate a loop of ileum 150 cm long (Thiry-Vella loop) was performed at 1 day of age. Ileal sections for histopathologic examination were collected at this time.

Intestinal secretions were collected on days 3 through 7 by flushing the ileal loop with phosphate buffered saline solution. Immunoglobulins present in the loop fluid were characterized and quantitated and specific neutralizing antibody determinations were performed.

Immunoglobulin and antibody determinations were also carried out on serum samples collected from cows before and after vaccination and calves the day of cesarean section delivery. Calves were challenged at 6 days of age by oral administration of virulent calf diarrheal coronavirus and necropsied at 9 days of age.

All calves vaccinated with attenuated coronavirus intra-amniotically and subsequently challenged with virulent virus failed to develop clinical signs of coronavirus infection. Control calves developed diarrhea 19 to 22 hours following oral administration of virulent virus. Tissue sections of colon, ileum and ileal loop from control calves collected at necropsy and stained by fluorescent antibody technique (FA) were coronavirus positive. Tissue sections from vaccinated calves were FA negative.

Microscopic differences were not seen in histopathologic sections of ileum from control and vaccinated calves at 1 day of age. *In utero* vaccinated calves euthanatized 72 hours following oral challenge with virulent coronavirus did not have gross or microscopic lesions. Control calves euthanatized 72 hours following challenge had liquid contents in small and large intestine and histopathological changes caused by coronavirus infection were observed in ileum, ileal loop and colon.

Cow sera from all cows contained high levels of immunoglobulin (Ig) before and after vaccination. There were significant increases in serum Ig levels of vaccinated calves compared to control calves on the day of cesarean section delivery. No detectable Ig's were found in intestinal loop washings of control calves. Oral vaccination of the fetus with attenuated coronavirus resulted in significant increases of IgA, IgM and IgG in the intestine.

The serum from all cows contained antibodies to calf diarrheal coronavirus before and after vaccination and there was no significant difference between before and after vaccination titers. Coronavirus serum neutralization antibody tests on serum samples from control calves were negative. Newborn vaccinated calves had serum neutralization antibody titers from 4 to 128. Neutralization antibody titers of intestinal loop washings from vaccinated calves were significantly higher than those from control calves. These results are evidence of the ability of the near-term fetus to respond to an orally administered attenuated coronavirus by the production of specific serum and secretory antibody.

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VITA

VITA

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APPENDIX

TEST RESULTS

Table A-1. Individual intestinal loop washings (Ig/100 ml)

	IgA	IgM	IgG
Attenuated virus vaccinates			
705-1	0.104	0.040	t
-2	0.100	0.020	t
-3	0.100	0.042	t
-4	0.400	0.060	t
-5	0.140	0.042	t
711-1	0.658	0.066	t
-2	0.200	0.060	t
-3	0.400	0.120	t
-4	0.128	0.026	0.0
714-1	0.329	0.099	t
-2	0.481	0.099	t
-3	1.887	0.307	0.448
-4	0.565	0.099	0.339
801-1	0.0	0.0	0.0
-2	0.200	0.042	0.0
-3	0.225	0.047	0.0
-4	0.304	0.046	0.0
-5	0.080	0.0	0.0
804 - no indiv. samples			
Saline inoculated controls			
704-1	0.0	0.0	0.0
-2	0.0	0.0	0.0
-3	0.0	0.0	0.0
-4	0.0	0.0	0.0
-5	0.0	0.0	0.0
710-1	0.0	0.0	0.0
-2	0.0	0.0	0.0
-3	0.0	0.0	0.0
-4	0.0	0.068	0.0
-5	0.0	0.088	0.0
Non-inoculated control			
712-1	0.0	0.0	0.0
-2	0.0	0.0	0.0
-3	0.0	0.0	0.0
-4	0.0	0.0	0.0
-5	0.0	0.0	0.0

Table A-2. Coronavirus serum neutralization (cow sera)

Sample	Date	Titer 4	16	64	256	1024	4096	16384	Antibody titer 3Xconc/unconc	
Attenuated virus vaccinates										
705	10/21/77	0/2	0/2	0/2	0/2	0/2	2/2		2048	512
	10/30/77	0/2	0/2	0/2	0/2	0/2	1/2		4096	1024
711	12/8/77	0/2	0/2	0/2	0/2	0/2	0/2	2/2	8192	2048
	12/17/77	0/2	0/2	0/2	0/2	0/2	0/2	2/2	8192	2048
714	12/8/77	0/2	0/2	0/2	0/2	0/2	0/2		8192	2048
	12/18/77	0/2	0/2	0/2	0/2	0/2	2/2		2048	512
804	1/19/78	0/2	0/2	0/2	0/2	0/2	1/2		4096	1024
	2/12/78	0/2	0/2	0/2	0/2	0/2	2/2		2048	512
Saline inoculated controls										
704	10/5/77	0/2	0/2	0/2	0/2	0/2	2/2		2048	512
	11/24/77	0/2	0/2	0/2	0/2	0/2	1/2		4096	1024
710	9/27/77	0/2	0/2	0/2	0/2	0/2	2/2		2048	512
	11/21/77	0/2	0/2	0/2	0/2	0/2	0/2		8192	2048
Non-inoculated control										
712	11/10/77	0/2	0/2	0/2	0/2	0/2	0/2		8192	2048

Sample: bovine serum (cow)

Cells: BK-4 p32

Planted: 2/24/78
Test on: 2/27/78
Test off: 3/3/78

Table A-3. Coronavirus neutralization (pooled intestinal loop washings)

Sample	Titer 4	8	16	64	256	1024	Antibody titer
Attenuated virus vaccinates							
705	0/4	0/4	0/4	0/4	4/4	4/4	128
711	0/4	0/4	0/4	1/4	4/4	4/4	64
714	0/4	0/4	0/4	3/4	4/4	4/4	64
804	0/4	0/4	0/4	0/4	4/4	4/4	128
Saline inoculated controls							
704	1/4	4/4	4/4	4/4	4/4	4/4	4
710	4/4	4/4	4/4	4/4	4/4	4/4	neg
Non-inoculated control							
712	1/4	4/4	4/4	4/4	4/4	4/4	4
Virus control	10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}		
S#X-5 10^{-1}	4/4	4/4	3/4	0/4	0/4		464 $TCID_{50}$

Sample: pooled intestinal loop washings

Cells: BK-4B p32

Planted: 3/20/78
Test on: 3/23/78
Test off: 3/27/78

Table A-4. Coronavirus neutralization (bovine sera, intestinal loop washing); attenuated virus vaccinate number 801

Sample	Date	Titer 4	16	64	256	1024	4096	Antibody titer
Serum								
Cow 801	2/17/78	0/4	0/4	0/4	0/4	4/4	4/4	512
	3/25/78	0/4	0/4	0/4	1/4	4/4	4/4	256
Calf 801	3/25/78	0/4	0/4	0/4	0/4	0/4	4/4(5X)	2048/64
Intestinal loop wash								
801		0/4	0/4	4/4	4/4	4/4	4/4	32
Virus control		10^{-0}	10^{-1}	10^{-2}	10^{-3}	cc		
S#X-5 10^{-1}		5/5	5/5	0/5	0/5	0/4		32 $TCID_{50}$

Sample: bovine serum, intestinal loop washing

Cells: BK-4B p31

Planted: 5/15/78
Test on: 5/19/78
Test off: 5/23/78

Table A-5. Coronavirus serum neutralization (calf sera)

Sample	Date	Titer 4	16	64	256	1024	4096	Antibody titer 6Xconc / unconc	
Attenuated virus vaccinates									
705	10/30/77	0/2	0/2	0/2	0/2	1/2	2/2(4X)	1024	32
711	12/17/77	0/2	0/2	0/2	0/2	0/2	2/2	2048	64
714	12/18/77	0/2	0/2	0/2	2/2	2/2	2/2	128	4
804	2/12/78	0/2	0/2	0/2	0/2	0/2	1/2	4096	128
Saline inoculated controls									
704	11/24/77	2/2	2/2	2/2	2/2	2/2	2/2	neg	neg
710	11/21/77	0/2	0/2	2/2	2/2	2/2	2/2	32	0
Non-inoculated control									
712	11/10/77	2/2	1/2	2/2	2/2	2/2	2/2(4X)	neg	neg

Sample: bovine serum (calf)

Cells: BK-4 p32

Planted: 2/24/78
Test on: 2/27/78
Test off: 3/3/78