THE ROLE OF A BOVINE VIRAL DIARRHEA VIRUS IN NEONATAL CALF ENTERITIS

by

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INTRODUCTION

Enteritis of neonatal calves is characterized by watery, white or yellowish feces, with a rapid onset and course. Dullness and depression are prominent symptoms and mortality is high. The disease is thought to be seasonal, occurring more frequently in winter and early spring, however this period coincides with the peak calving season.

Losses from enteric disease of neonatal calves (calf scours) range frequently from 10 to 50 percent of the annual calf crop. Most losses occur during the first 48 hours after birth. The remainder of the calves usually contract the disease during the first two weeks of life. It is estimated that losses from deaths due to this disease alone are in excess of 40 million dollars annually in the United States. This loss is increased appreciably by the many survivors of the disease that remain unthrifty and never reach their potential levels of productivity and performance.

Although the disease has been the subject of numerous investigations during the past century and the pertinent literature is voluminous, the etiology remains controversial. Early workers isolated the bacterium Escherichia coli from affected calves and proclaimed that it had a significant role in the etiology. However, difficulty in reproducing the syndrome with various E. coli isolates has created some question as to the true etiologic agents or factors involved. The

relatively few reports on viral diarrhea in neonatal calves have been concerned primarily with isolation and characterization of the virus.

This study was initiated to determine if the virus of bovine viral diarrhea (BVD) was significant in the etiology of enteric disease of neonatal calves and also if transmission of virus occurred from dam to calf. An additional objective was to ascertain what effect neonatal or paranatal exposure of a calf to BVD virus would have upon the re-exposure response of the same animal in later life to the BVD virus. This was particularly intriguing since several workers have postulated that an "immune tolerance" mechanism may be operative in cattle affected with the typical mucosal disease form of bovine viral diarrhea.

REVIEW OF LITERATURE

Enteric Disease of Neonatal Calves

Significance of Escherichia coli

Jensen (36) in 1893 was the first to associate E. coli with "white scours" in calves. He cited evidence that the syndrome had been prevalent for over a century.

Joest (37) in 1903 confirmed the observations of Jensen and concluded that fatal calf diarrhea was the result of sufficient numbers of E. coli reaching the intestine, finding suitable growth conditions and then penetrating into the general circulation through the unprotected intestinal mucosa. He was able to reproduce diarrhea by administering cultures of E. coli to healthy newborn calves. Colostrum when given immediately after birth had a prophylactic action. Joest considered that colostrum stimulated the formation of bactericidal gastric juice. The gastric juice was mildly laxative and removed the meconium, assisting the growth of bacteria in the intestine.

Hagan (32) in 1917 found evidence of in utero contamination of the calf intestine, however the main colonization of the intestine occurred after birth originating from the environment.

Smith and Orcutt (72) in 1925 considered that there existed in the young calf a delicate balance between certain strains of E. coli and the mucous membrane of the digestive

tract. When the balance was upset in favor of E. coli, "scours" was produced. Smith (70) also conducted extensive experiments on the beneficial effects of the dam's colostrum in the prevention of neonatal calf enteritis. Lovell (42) in 1937 examined several strains of E. coli isolated from calves which died from enteritis. He placed 79 of 110 strains isolated from 45 calves, into 1 of 8 capsular or K antigenic types. Two of the types accounted for 48 of the strains. Frequently more than one type was isolated from the same herd.

A major step towards uniformity in serotyping E. coli isolates resulted in 1947 when Kauffmann (38) established the diagnostic coli antigenic schema according to the serologic investigations of Kauffmann-Knipschildt-Vahlne. Strains of E. coli could now be classified according to their O (somatic), K (capsular), and H (flagellar) antigens.

Wramby (79) in 1948 was the first to use the Kauffmann scheme for serological typing of E. coli isolated from calves. His extensive studies involved 4262 strains isolated from 484 scouring calves and 1699 strains isolated from 492 normal calves. He found little difference between the frequency of the occurrence of the various O groups in the intestines of scouring calves and those of normal calves. He also noted that 76.1 percent of the strains from sick or scouring calves possessed no K antigen.

Aschaffenburg et al. (1) in 1949 published the results of extensive studies on the nutritive value of colostrum for

the neonatal calf and its effect upon the incidence of scours. These authors (2, 3) reported additional confirmatory data in 1951.

Ewing (22) in 1956 enlarged and confirmed the coli antigenic schema of Kauffmann, Knipschildt-Vahlne.

Fey (24) in 1957 found that 37.5 percent of 145 strains isolated from calves with colisepticemia were of the O 78:K80 serotype. Despite this frequent isolation of O 78:K80 from calves dying of colisepticemia, in an examination of 8,630 strains of E. coli isolated from the intestines of healthy calves and cows this serotype was found only eight times and none of these isolations was from calves. However, this serotype was found in the environment where calves were dying from infection with this serotype.

Reisinger (63) in 1957 confirmed many of the previous observations of Theobald Smith (70) with respect to colostrum and management. He also showed that healthy calves had few or no E. coli in the anterior 25 to 30 feet of the small intestine. Calves suffering from diarrhea were found to have large numbers of E. coli in the anterior portion of the small intestine, and increased numbers of E. coli in the posterior portion of the ileum. He postulated that a virus may enhance the effect of E. coli but concluded that the virus per se probably could not produce diarrhea without E. coli; whereas E. coli could produce the disease alone. He was able to obtain healthy, colostrum-deprived calves in midwinter when they were stringently

protected from aerosol contact with other cattle, including their dams, and also protected from cold and dampness by efficient drying soon after birth. However, other colostrum-deprived calves, likewise unexposed to aerosol from other cattle, but subjected to greater stresses of cold, dampness, etc., died with typical signs of infectious diarrhea.

Glantz et al. (28) in 1958 studied the serotypes of E. coli isolated from calves afflicted with scours in Pennsylvania. Four of six types caused a typical infection when administered to calves which were colostrum deprived. One strain was able to establish experimental infection in calves which had received colostrum. Two E. coli serotypes isolated from normal calves had no effect when experimentally fed to calves.

H. Williams Smith (69) in 1962 found considerable variations in E. coli serotypes which he isolated in England from calves with diarrhea. He noted that serotypes may change during an outbreak and concluded that the presence of the K antigen was not related to virulence. He demonstrated that little significance should be attached to postmortem findings on the number of E. coli present in various portions of the digestive tract due to the marked proliferation of E. coli after the calf's death. Consequently, Smith examined intestines from normal and moribund calves. Only minor differences in the numbers of E. coli in the upper portion of the small intestine were found between sick and normal calves. Similarly,

the same coliphage types were present in both sick and normal calves. In a natural outbreak of calf diarrhea, the colostrum-deprived calves were more severely affected and died sooner than colostrum-fed calves. In transmission experiments, however, he reported a complete failure in attempting to transmit diarrhea to colostrum-fed calves. In contrast, colostrum-deprived calves when artificially infected with E. coli had bacteremia, collapse and death in 18 to 36 hours, but showed no evidence of scouring. Smith concluded that E. coli played little if any role in the etiology of enteritis in neonatal calves.

Osborne (57) in 1965 reported on experiments to determine the LD_{50} of selected serotypes of E. coli for neonatal calves. He concluded that for his strains the LD_{50} for two-day-old calves was 4.5×10^{10} cells daily for 10 days.

Gay (25) in 1965 reviewed the literature on E. coli infection in neonatal calves. He concluded that at least three syndromes are involved--septicemia, enteric toxemia and enteritis. He indicated that susceptibility of calves to E. coli infection is related to their respective gamma globulin levels. Experimental evidence revealed that calves with agammaglobulinemia or hypogammaglobulinemia shortly after birth generally developed scours and died.

Significance of other bacteria

Many other species of bacteria have been isolated from neonatal calves dying of enteritis. These include members of the genera Salmonella, Klebsiella, Clostridium, Aerobacter, Pseudomonas, Staphylococcus, Streptococcus, Pasteurella, Shigella and Corynebacterium (25, 69). It is generally considered that with the exception of certain members of Salmonella and Clostridium other bacteria have very minor roles, if any, in the syndrome of neonatal calf enteritis.

Significance of viral agents

Baker (4) in 1943 reported on a filterable virus which he isolated from a case of enteritis and pneumonia in calves in New York State.

Moll (50, 51) in 1952 was able to transmit a calf pneumonia-enteritis complex with bacteria-free filtrates of blood, lung, spleen, mesenteric lymph nodes and intestinal mucosa.

Subsequently, McClurkin (44, 45) also in studies on calf pneumonia-enteritis characterized a viral agent as being stable at 56° C for 90 minutes, and stable in the presence of diethyl ether for 24 hours. Filtration results suggested a particle of less than 25 mu. He concluded that the disease was airborne in nature.

Moll (50) in 1957 in further studies reported that when *E. coli* was added to the viral material and administered to calves there was a marked increase in the number of "frank"

cases of calf pneumonia-enteritis complex. Subsequently, Moll (52) has suggested that such factors as stress, nutrition, electrolyte imbalance, endocrine dysfunction in the dam or calf, as well as other management and environmental factors may be of considerable etiologic significance in the syndrome. He has pointed to adrenal-pituitary relationships and their effect on membrane permeability particularly in respect to absorption of colostrum from the digestive tract.

Bogel and Mussgay (8) in 1960 isolated an enterovirus from calves with diarrhea. They were successful in reproducing the disease in an extremely mild form characterized by mild fever, slight leukopenia, and diarrhea between the second and ninth day after inoculation. One calf died of septicemia, while a second remained weak and depressed for two days. In 1963 Bogel et al. (9) inoculated enterovirus into 14 calves but were unable to produce a clinical disease, although they were able to re-isolate the virus from calves which subsequently produced antibodies against the enterovirus.

Burki (12) in 1962 suggested that the age of the calf, seasonal changes and environment were factors which affected the transmission of enteroviruses.

Cliver and Bohl (14) in 1962 reported that enteroviruses were never isolated from calves less than four weeks of age. They attributed this to a high antibody content in the dam's colostrum and suggested that the antibody neutralized

enteroviruses in the intestinal tract before they could enter the intestinal mucosa to initiate infection.

McFerran (46) in 1962 reported that he was able to reproduce a marked diarrhea in calves by inoculation of an enterovirus strain, but was unable to produce any other clinical symptoms.

Neiderman et al. (53) in 1963 isolated two enteric cytopathogenic bovine viruses (ECBO) from bovine feces. They inoculated these into 11 calves but produced no visible symptoms.

Van Der Maaten (77) in 1964 isolated a bovine enteric virus from bovine feces. He inoculated the virus into four one-month-old calves. Two of the calves developed diarrhea and one died.

Bartha and Aldasy (6) in 1964 isolated three identical strains of adenoviruses from calves ill with or dead of virus diarrhea. However, no etiologic role could be attributed to the agents since antibodies to the isolates were missing from survivors. In addition, calves artificially infected with the agents did not develop the disease. They suggested, however, that the virus may have etiologic significance under different conditions.

Mohanty and Lillie (49) in 1965 inoculated calves aged 6 to 12 weeks with an adenovirus strain isolated from bovine feces. Respiratory symptoms were severe and diarrhea was observed in one calf from day 5 to day 7 postexposure.

Bovine Viral Diarrhea-Mucosal Disease Complex

Olafson (55, 56) in 1946 first described the occurrence of a newly recognized, contagious and transmissible disease of cattle in New York State. This disease was characterized by fever, leukopenia, nasal discharge, depression, anorexia, dehydration and abortion in some pregnant cows. Lesions most frequently observed were ulcers and necrosis of the mucous membranes of the lips, tongue, pharynx, esophagus, small intestine and cecum. A high proportion of animals in the herd were affected and the mortality was from 4 to 8 percent. The disease could be transmitted with bacteria-free filtrates and the name "virus diarrhea" was applied to this syndrome.

Childs (13), also in 1946, reported a disease in Canada affecting young cattle. This disease was characterized by fever, anorexia, excessive lachrymation, salivation, early watery then late viscid nasal discharge; early eruptions that later become erosions or ulcerations of the mucosa of the alimentary canal; watery diarrhea with blood in the later stages, violent tenesmus, and eruptions on the skin of the inguinal and perineal regions, inner sides of the legs and inside the ears. Necropsy revealed erosions and ulcerations of the mucosa of the alimentary canal, particularly of the lips, tongue, gums, esophagus, abomasum and small intestine. Swollen lymph nodes were a frequent finding. Only a small

percentage of the cattle in a herd were affected, but most cattle that developed clinical signs died in 7 to 10 days.

Hedstrom and Isaksson (33) in Sweden in 1948 reported on a contagious diarrhea of cattle not unlike that described by Olafson. Animals were sick from 4 to 5 days and the mortality rate was low. They were unable to reproduce the disease experimentally but postulated it was due to a virus.

Ramsey and Chivers (61) in 1953 described a syndrome occurring in young cattle in Iowa and surrounding states. This condition was characterized by fever, anorexia, profuse salivation, depression, dehydration, a foul smelling mucopurulent nasal discharge, and watery diarrhea frequently mixed with blood. The main pathologic changes described were hyperemia, hemorrhages, erosions, ulcerations and necrosis of the mucosa of the alimentary canal from the lips to the anus. The morbidity rate varied from 2 to 50 percent within herds while over 90 percent of those affected died. They called this syndrome "mucosal disease of cattle."

Baker et al. (5) at Cornell University in 1954 isolated two strains of virus from field cases resembling the syndrome described by Olafson. When these viruses were re-inoculated into calves they produced a diphasic temperature curve response, leukopenia and general malaise. Diarrhea occurred in only about half of the calves and oral lesions only in an occasional animal. They were unable to produce either detectable complement-fixing or viral-neutralizing antibodies in the inoculated

calves. They found that the two viruses were antigenically related in cross-protection studies with calves. The agents would not grow in embryonating eggs nor in guinea pigs, but could be passaged in rabbits.

Pritchard (58) in 1955 reported on the widespread occurrence in Indiana of a disease in cattle which closely resembled the syndromes previously described by Olafson in New York and by Hedstrom and Isaksson in Sweden. The morbidity was approximately 100 percent in affected herds and the mortality was about 10 percent. He also described a second syndrome in Indiana cattle indistinguishable from that described by Childs and the mucosal disease of Ramsey and Chivers. Preliminary studies on transmission of this second disease with blood, tissues and filtrates suggested viral etiology, however only a very mild form of the disease was produced. Successful attempts at virus isolation were later reported by Pritchard et al., (59) as well as by Schipper et al. (66) in North Dakota, by Neilsen et al. (54) in Canada and by Dow et al. (20) in Scotland. Huck (34) in England and others have also reported successful virus isolations. Subsequent studies by Kniazeff et al. (39) have revealed considerable immunologic relationship among isolates in cross-protection tests.

A chronic form of BVD lasts two to six months. The animals become emaciated and develop continuous or intermittent diarrhea. Frequently the skin over the neck and shoulders of such cases becomes wrinkled, leathery and scurfy resembling

hyperkeratosis. Cattle weighing 1000 pounds frequently lose 150 to 250 pounds during a month of severe illness. Milk production is markedly reduced and abortions may occur.

One factor which has necessitated learning more about the syndrome has been its close relationship to rinderpest from the standpoint of clinical and pathologic changes. The mortality rate in rinderpest is much higher, although morbidity rates are often similar. Cattle that had recovered from viral diarrhea were still susceptible to rinderpest (78).

The gross and histopathology of the disease have been described in considerable detail by Ramsey (60). Lesions were primarily erosive, ulcerative and cystic and were confined generally to the mucosa of the alimentary canal and the lamina epithelia. Necrosis of the lymphoid tissue of the intestine, lymph nodes and spleen was frequent. No lesions have been observed in the nervous system.

Much research has been directed towards obtaining more knowledge of the etiologic agents of this complex. Underdahl et al. (76) in 1959 isolated a virus from cattle with mucosal disease. The virus was cytopathogenic when cultivated in embryonic bovine kidney cell cultures. Gillespie et al. (26) in 1960 isolated a cytopathogenic strain of BVD virus from a calf in Oregon. They were able to show cross protection with other strains of BVD virus isolated in New York and with viruses of the mucosal disease complex. This cytopathogenic strain designated as C24V provided a quick and efficient

method for determining the incidence of BVD as evidenced by neutralizing antibodies in the blood serum of cattle. Later Kniazeff and Pritchard (40) were able to show a serologic relationship among BVD and MD isolates.

Robson et al. (64) in 1960 showed that the virus neutralization test is a satisfactory indicator of the immune status of an animal for BVD.

Darbyshire (16) at Weybridge in 1962 described an agar gel diffusion test for BVD. He used tissue from clinically affected cattle as an antigen and tested this against known immune and negative sera as well as unknown sera. He concluded that the agar gel diffusion test had a high degree of accuracy and was useful and practical for the diagnosis of the bovine viral diarrhea-mucosal disease complex.

Darbyshire (17) in 1962 extended the agar gel diffusion studies and was able to show a consistent serologic relationship between the virus of hog cholera and the virus of bovine mucosal disease. He suggested that the diffusible antigen in hog cholera was not the whole virus particle and that probably the phenomenon was due to soluble antigens being involved in both diseases. As an alternate hypothesis he suggested that a cellular product was liberated as a result of virus multiplication within infected cells. This product may be identical in the case of each virus and thus may act as an auto-antigen in the stimulation of specific antibodies. He postulated that

this was a common antibody and, as such, would be detected in the gel diffusion reactions.

In absorption experiments Darbyshire (17) found that hog cholera antigen removed the antibodies from anti-hog cholera serum as well as the BVD antibodies from the bovine serum. Likewise, the BVD antigen removed the precipitating antibodies from both types of antiserum. By contrast normal tissues did not remove the precipitating activity of either antiserum and furthermore the antigens were not affected by normal sera.

Beckenhauer et al. (7) in 1961 reported that pigs inoculated with Oregon C24V-BVD virus were resistant to challenge with virulent hog cholera virus 14, 24 and 37 days later. Two other isolates, however, did not protect hogs against subsequent challenge with virulent hog cholera virus.

In 1962, Sheffy et al. (67) inoculated calves with hog cholera virus and subsequently challenged them with BVD virus. They found that sera which contained BVD antibodies did not neutralize hog cholera virus, and in reciprocal tests sera that contained hog cholera antibodies did not neutralize BVD viruses. They concluded that since BVD and hog cholera viruses failed to cross neutralize, the mechanism of protection appeared to be related to an accelerated secondary response induced by previous exposure to a heterotypic virus.

In 1962 Kumagai et al. (41) in Japan found hog cholera and BVD viruses to be antigenically different when convalescent sera were used in cross-neutralization tests. However, they

detected only a "slight" cross neutralization when hyperimmune sera were used in the test.

In 1963 Gutekunst and Malmquist (30) separated a soluble antigen and infective virus particles from tissue culture fluids of two isolates of BVD. They found that the soluble antigen of either virus would form a single "line of identity" in agar double diffusion plates when reacted with anti-hog cholera serum or anti-BVD serum. However, infective virus particles failed to form precipitin lines with either type of antiserum. No neutralizing antibodies were found in the anti-hog cholera sera when tested against the BVD viruses except in a commercially prepared serum. Later these same workers (31) demonstrated that the soluble antigens of BVD viruses could be used to determine complement-fixing antibody levels in anti-hog cholera serum. Normal swine serum failed to fix complement when tested with the BVD soluble antigen.

Mengeling et al. (48) in 1963 demonstrated the antigenic relationship between BVD and hog cholera viruses by immunofluorescence.

Dinter (18) in 1963 compared BVD virus and hog cholera virus. Both were RNA-containing lipoviruses, moderately sensitive to trypsin and not stabilized by $M_g\ Cl_2$. On the basis of ultrafiltration, he proposed that both viruses were < 50 mu in size. He was able to show that only hyperimmune sera with very high titers against hog cholera neutralized BVD virus. Similarly only cattle sera with high titers against BVD virus were

able to neutralize hog cholera virus. However, in both cases, some sera with high titers against the homologous virus showed no neutralizing activity towards the heterologous virus. He was unable to show any antigenic relationship between BVD virus and para-influenza 3 virus, infectious bovine rhinotracheitis virus, or bovine enteroviruses.

Huck and Cartwright (35) in 1964 reported the presence of clinical mucosal disease did not increase the incidence of the excretion of enteroviruses and consequently concluded that cytopathogenic enteroviruses were not the causal agents of the disease.

Gutekunst and Malmquist (30) in 1964 reported on comparative studies with the complement-fixation and the serum-neutralization tests. Sera were from cattle infected with cytopathogenic and noncytopathogenic strains of BVD virus. The responses as measured by the complement-fixation test were similar to both strains. However, the serum-neutralizing antibodies indicated strain specificity by reciprocal cross-neutralization tests.

Gillespie et al. (27) in 1962 described an interference technique for detecting noncytopathogenic strains of BVD virus. Essentially the technique consisted of inoculating cell cultures with the suspected material; if no change was observed in the cells after 72 hours, then a strain of known cytopathogenic virus was introduced into the cell cultures. If a noncytopathogenic strain was already present in the suspected material

then the cellular sites were no longer available to the cytopathogenic strain to exert its detectable effect. Malmquist et al. (47) in 1965 have described an interference technique which is somewhat modified from the above procedure.

Fernelius (23) in 1964 has described an immunofluorescence technique for detecting noncytopathogenic BVD viruses in cell culture systems. His results agree closely with serum-neutralization tests on the same strain of the virus in cell cultures.

In 1962 Malmquist* observed that cattle which succumb to mucosal disease fail to develop either neutralizing or complement-fixing antibodies. He demonstrated a viremia in clinical cases for periods up to four months prior to an animal's death. In each case antibodies against the virus were not detectable, whereas other "apparently healthy" cattle in the same herds developed significant levels of antibodies. He suggested that the inability of the clinical cases of mucosal disease to develop antibodies resembled the immune tolerance phenomenon.

Dinter et al. (19) in Sweden and Borgen (10) in Denmark in 1962 have reported similarly on cases where they isolated BVD viruses from cattle dying of mucosal disease, although antibodies against the BVD virus could not be detected.

In 1963 Thomson and Savan (74) in Canada reported on several outbreaks where sera collected from fatal cases

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*Malmquist, W. A., National Animal Disease Laboratory, Ames, Iowa. Observations on immune tolerance associated with the bovine viral diarrhea-mucosal disease complex. Private communication. 1962.

immediately before death revealed only those cattle with a negative antibody titer in the affected group succumbed to the disease. This finding was true in chronic and acute cases. Seven such cattle were described, one of which was studied for over two months before its death. During this two-month period it remained serologically negative although BVD virus was consistently recovered.

Reproduction and transmission of BVD by artificial methods has been characterized by limited success. Various methods and techniques have been employed. These include drenching susceptible cattle with fecal suspensions from infected animals; administering suspensions of spleen intranasally and intravenously; intravenous inoculation of defibrinated blood from an acutely ill animal; inoculation of defibrinated infected blood into the prescapular lymph node, and intravenous administration of infected cell culture fluids. In general all methods have produced only mild forms of the disease and reproduction of typical "mucosal disease" remains an existing problem.

Although Romvary (65) has recently reported on an epidemic of viral diarrhea in neonatal calves in Hungary, the infectivity of most American isolates of BVD virus for neonatal calves remains virtually unknown. Since this virus has played an extensive role in the etiology of diarrhea in older cattle, it seemed worthy to ascertain its role in neonatal diseases of calves. An additional incentive was the inference that an "immunologic tolerance" phenomenon was operative in the

development of fatal cases of viral diarrhea. Since immunologic tolerance depends often on a previous contact with the antigen at or before the time of birth it appeared to be a fruitful area of research. Further stimuli to determine the role of the virus of viral diarrhea in neonatal enteric disease of calves has been indirectly provided by Smith (69) in England. He concluded after an extensive experiment that "neither E. coli nor any other bacterium was primarily concerned in the causation of the disease."

MATERIALS AND METHODS

Virus

The bovine viral diarrhea virus used in this study was the NADL-MD strain which has been previously described by Gutekunst (29). This virus was isolated from several tissues of a naturally occurring fatal case in a group of yearling Holstein-Friesian heifers at the National Animal Disease Laboratory in 1962. Since the original isolation the virus had been passaged three times in primary embryonic bovine kidney cells (EBK). A sufficient quantity of this third passaged virus was stored in the frozen state at -100 F and used throughout the entire experiment as needed.

Tissue Culture System

Primary embryonic bovine kidney (EBK) cell cultures were used for the entire study. Cell cultures were prepared by the general method of Youngner (80) as modified by Gutekunst (29). Kidneys were collected aseptically from selected six to nine months bovine fetuses and placed in a sterile container of Hank's balanced salt solution (HBSS). The capsule was removed from the kidney and the cortical region separated from the medulla. The cortical tissue was minced until pieces were approximately one cubic millimeter in size. The minced cortical tissue was washed three times with cold GKN* solution, with the washing fluids being discarded. The tissues were placed

*See Appendix for formula.

in a sterile trypsinization flask* containing a sterile teflon covered magnetic stirring bar. Sterile trypsin solution (0.25 percent) chilled to 4 C was added to the trypsinization flask and incubated at 4 C for 4 to 6 hours. After incubation the supernatant fluid which contained toxic debris was discarded and fresh cold trypsin was added. Trypsinization was continued for an additional 18 hours at 4 C. Following trypsinization, fibrous tissue was separated from the trypsinized cells by filtration through sterile cheesecloth. The cells were sedimented by centrifugation at 1,000 g in a refrigerated centrifuge and the supernatant fluid was discarded. The cells were then washed three times with cold GKN and diluted in HBSS containing 10 percent specific-pathogen free (SPF) calf serum to give a final concentration of approximately 1×10^6 cells/ml. The EBK cells were propagated in sterile tissue culture tubes, 4-oz. prescription bottles or Blake tissue culture bottles, depending upon the requirements of the experiments. The cells were planted in medium containing 89.5 percent HBSS, 0.5 percent lactalbumin hydrolysate, 10.0 percent SPF calf serum, and penicillin and streptomycin at a concentration of 100 units and 100 ug/ml respectively. After three days' incubation at 37 C, the medium was changed to 89.5 percent Earle's balanced salt solution (EBSS), 0.5 percent lactalbumin hydrolysate, 10 percent SPF calf serum, and also antibiotics were added.

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*Bellco Glass Company, Vineland, New Jersey.

After complete monolayers had formed (approximately five days), the medium was removed and EBSS media with 5 percent SPF calf serum was added for maintenance.

Animal Experimentation

Experiment 1

Three Holstein-Friesian cows were selected from the herd of the Animal Services Section at the National Animal Disease Laboratory. Cows were required to be in the last month of gestation, and serologically negative to two consecutive BVD viral neutralization tests at 60-day intervals. The three cows were removed to the large animal laboratory building, where each cow was maintained in an isolated room for the duration of the experiment. Separate feeding and cleaning utensils were kept in each room. As an additional precaution against transmission of infectious agents, the animal caretakers disinfected their boots and gloves routinely between each room.

Pre-inoculation blood, nasal and rectal specimens from the three cows were examined for BVD virus. Artificial exposure of these pregnant cows was accomplished by the intravenous inoculation of 10 ml of a tissue culture fluid containing approximately 10^7 tissue culture infective doses (TCID) per ml of the NADL-MD strain of the virus.

Three calves were born to these dams at 6, 16 and 25 days, respectively, after exposure. The calves were permitted to nurse their dams to obtain the benefits of colostrum. The

postexposure responses of the dams and their calves were studied from the standpoint of the clinical, serologic, microbiologic, immunologic and pathologic aspects. At approximately three months of age the calves were weaned and their dams were euthanatized and subjected to examination at necropsy.

Experiment 2

A group of three Holstein-Friesian calves was collected from their serologically BVD negative dams by a modified SPF technique. The placenta was incised and manipulated to minimize or eliminate contact between the calf and the birth canal of the dam. Calves were "caught" and dropped from the dam into sterile galvanized steel trash cans, then delivered immediately to the research laboratory. At from one to four hours of age the calves were exposed orally to a dose of BVD virus ranging from 10 ml of 10^4 TCID per ml to 10 ml of 10^7 TCID per ml.

All three calves were permitted their dam's colostrum for the first four feedings then were fed a commercial milk replacer without antibiotics. The amount of colostrum fed to each calf was equivalent to the volume of milk replacer recommended by the manufacturer of the milk replacer for neonatal calves. Colostrum was taken from the dam, refrigerated till just before feeding, then warmed to body temperature and pail-fed to the calf. The various postexposure responses of the calves were measured by methods which will be described later in the section.

Experiment 3

A second group of seven Holstein-Friesian calves was collected from their dams in the same manner as described in Experiment 2. Similarly these calves originated from dams that were serologically negative for BVD antibodies. These calves, however, were deprived of their dams' colostrum and instead were given adequate amounts of a commercial milk replacer without antibiotics. Each of the seven calves was exposed orally to a 10 ml dose of BVD virus containing from 10^6 to 10^7 TCID per ml. Various postexposure responses were measured and recorded. Calves bearing numbers 8 and 9 were twins, the former being a bull and the latter a heifer. When the calves were two to 10 hours old they were exposed to BVD virus.

Experiment 4

Another group of three Holstein-Friesian calves was collected by the modified SPF technique. These calves differed from those in Experiment 2 in that they originated from dams having a high titer of serum neutralizing antibodies for BVD during the last month of pregnancy. All three dams were naturally infected. The three calves were colostrum deprived. Within six hours after birth they were exposed orally to 10 ml of tissue culture fluids containing 10^7 TCID of BVD virus per — ml. The methods for measuring the postexposure responses of this group are described later also.

Experiment 5

Another group of three Holstein-Friesian calves originating from dams that were serologically negative for BVD antibodies were also collected by the modified SPF technique. These calves were colostrum deprived. They were exposed when two to four hours of age to a dose of BVD virus ranging from four to eight ml of a tissue culture fluid containing approximately 10^7 TCID per ml. In contrast to previous groups these three calves were exposed by the nasal route. An equal quantity of exposure material was deposited in each nostril and the head was held up to prevent loss of exposure fluids. As in previous groups the methods for measuring the various postexposure responses will be described later.

Experiment 6

Two Holstein-Friesian cows, naturally infected with BVD, were placed in this group. Their calves were born naturally and were permitted colostrum then weaned at 72 hours of age. Four hours after weaning, both calves were exposed orally to 10 ml of tissue culture fluids containing 10^7 TCID of BVD virus per ml. The postexposure responses of these calves were studied also.

The various methods of handling the experimental animals are summarized in an outline form (Table 1).

Table 1. Outline of experimental methods for studying effect of BVD virus on neonatal calves

Experiment number	Number of calves	Serologic status of dam	Type of birth	Colostrum status of calf	Route of exposure
1	3	Negative	Natural	Permitted	Dam, intravenously, prepartum
2	3	Negative	SPF	Permitted	Oral
3	7	Negative	SPF	Deprived	Oral
4	3	Positive	SPF	Deprived	Oral
5	3	Negative	SPF	Deprived	Nasal
6	2	Positive	Natural	Permitted	Oral

Measurement of Postexposure Responses

Clinical

Pre-exposure temperatures were recorded each morning and evening for several days before the cows were exposed to the BVD virus. However, each calf received only one pre-exposure body temperature check just before inoculation. Postexposure body temperatures were recorded morning and evening on both cows and calves for at least 10 days. Temperatures were recorded more frequently if an animal was seen to be acutely affected.

All experimental cattle were observed at least twice daily for evidence of clinical abnormalities such as anorexia, nasal discharge, excessive lachrymation and salivation, listlessness, diarrhea, constipation, polyuria, lameness and for evidence of lesions on the tongue, gums, dental pad, muzzle, lips, and hard palate.

Laboratory

Hematology Blood samples were collected from each calf for hematologic studies. Pre-exposure samples were obtained and the postexposure bleeding schedule was as follows: daily samples for 10 days, then on alternate days till 20 days, followed by weekly samples till 60 days, then monthly samples until re-exposure or necropsy. When animals were re-exposed a similar bleeding schedule was followed. Samples of blood were collected in 10 percent ammonium and potassium oxalate in

sterile 10 ml tubes. The anticoagulant and the blood were mixed thoroughly and taken to the laboratory, where determinations were made immediately of erythrocyte, leukocyte, and differential white cell counts. The latter were accomplished by using the standard Wright's stain method.

Serology Pre-exposure and postexposure blood samples for serologic studies were collected at identical times to the blood collection schedule described previously.

The serum neutralization test was used to study the antibody responses of the cattle. The beta method of constant virus and varying serum dilutions was used. Test sera were heat-inactivated in a 56 C water bath for 30 minutes. Serial two-fold and four-fold dilutions of each serum to be tested were prepared in 1.0 ml of HBSS. An equal amount of HBSS containing approximately 100 TCID per ml of NADL-MD virus was added to the serum dilutions. The mixtures were incubated at 37 C for one hour and then 0.2 ml of each serum-virus mixture was inoculated into each of four primary EBK cell tube cultures. The cultures were incubated at 37 C and observed daily for evidence of cytopathic effect until the seventh day.

Virology Pre-exposure and postexposure samples of blood for virus isolation were collected also at the previously described times. Generally, samples were collected in a 50 ml centrifuge tube containing 5 ml of 10 percent sodium citrate solution. Samples were immediately placed in a PR-2

refrigerated centrifuge* and spun at 2000 rpm for 50 minutes. After the plasma had been pipetted off, the leukocytes were carefully removed with a Pasteur pipette. When necessary, additional separation of erythrocytes and leukocytes was accomplished by centrifugation in a narrow bore tube and then removing the leukocytes with a pipette. The white cells were placed in one ml of GKN solution and stored at -20 F until examined (usually less than seven days). Approximately 0.2 ml of the leukocyte-GKN solution was inoculated into each of four primary EBK cell tube cultures. The inoculated cultures were incubated at 37 C and were observed daily for evidence of cytopathic effect (CPE). In selected instances where CPE was not apparent, the fluids were subcultured into four additional tubes of primary EBK cell cultures and observed daily for six days for evidence of CPE.

At the previously described times the nares were examined by pre-exposure and postexposure swabbings for the presence of BVD virus in the nasal secretions. Swabs were placed immediately into EBSS and stored at -20 F until examined for BVD virus. Approximately 0.2 ml of this solution was inoculated into each of four primary EBK cell tube cultures. These were likewise incubated at 37 C and observed daily for evidence of CPE.

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*International Equipment Co., Boston, Massachusetts.

Rectal swabs were taken from each experimental animal at the identical intervals as for collecting nasal swabs. Samples were placed in EBSS containing antibiotics. Samples were likewise stored at -20 F until examined in primary EBK cell tube cultures. Maximum storage time before examination was six days. Inoculation and incubation of primary EBK cell cultures was similar to the procedures described for nasal swabs. In selected instances tissue culture fluids were subcultured into four additional tubes of primary EBK cell cultures and these in turn were examined daily for evidence of CPE.

Samples of colostral milk were obtained from each of the dams in the experiment. The colostral milk was centrifuged in a PR-2 refrigerated centrifuge and samples of the cream, sediment and whey were examined in an EBK cell culture system for presence of BVD virus. Portions of tissues from infected animals such as lymph nodes, various organs, bone marrow and sections of the gastrointestinal tract were examined in an EBK cell culture system for BVD virus. Cultures not showing evidence of CPE at the end of seven days' incubation at 37 C were discarded. Cultures showing positive CPE were retained and their fluids removed and subcultured in a similar EBK cell system. After the second subculture in which CPE was demonstrable the culture fluids were removed and titrated for BVD virus using a positive BVD antiserum of known antibody content. Titrations for viral content were made in an EBK cell system in which serum neutralization capacity could be measured.

Bacteriology Samples of rectal contents were taken at the previously described collection periods. Swabs were placed immediately in Earle's medium not supplemented with antibiotics. In Experiment 1 the swabs were stored at -20 C until examined for the presence of Escherichia coli or other bacterial agents. In other experiments the swabs were tested immediately for the presence of E. coli. Propagation and identification of E. coli were conducted in accordance with the techniques recommended by Edwards and Ewing (21). Serotyping of various E. coli isolates was performed by Dr. Paul J. Glantz of the Pennsylvania State University, University Park, Pennsylvania. At his suggestion dextrose was eliminated from the medium to facilitate serotyping of the various isolates.

Additional examinations for E. coli were made from the blood, selected tissues and areas of the gastrointestinal tract.

In selected instances isolates of E. coli were inoculated intraperitoneally into female mice in doses from 5×10^2 to 5×10^8. The mice were examined twice daily for a two-week period after inoculation for any evidence of pathogenicity of E. coli.

Selected isolates of E. coli were tested also for sensitivity to the following antibiotics: chloromycetin, neomycin, erythromycin, kanamycin, novobiocin, penicillin, streptomycin, and tetracycline. This was accomplished by suspending an

eight-hour growth of E. coli in tryptose broth and uniformly spreading 0.2 ml of the suspension on the surface of each tryptose agar plate. Sensitivity discs* of the various antibiotics were placed appropriately on the surface of the inoculated agar. After overnight incubation at 37 C the plates were examined for evidence of inhibition of the growth of E. coli.

Pathology Neonatal calves that succumbed to the virus were subjected to an intensive gross and histopathologic examination. Primary emphases in the histopathologic studies were the regional lymph nodes, spleen, heart, liver, kidney, lung and the digestive tract. Additional studies were made on all animals at necropsy.

Immunologic Responses

Calves which survived the neonatal or prenatal exposure to BVD virus were re-exposed orally to the virus at intervals of three to 10 months after the initial exposure. The post re-exposure responses were measured in a manner similar to the postexposure responses. Particular attention was directed to determine if the re-exposure response resembled in any way an immunologic tolerance phenomenon.

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*Difco Laboratories, Detroit 1, Michigan.

RESULTS

Experiment 1

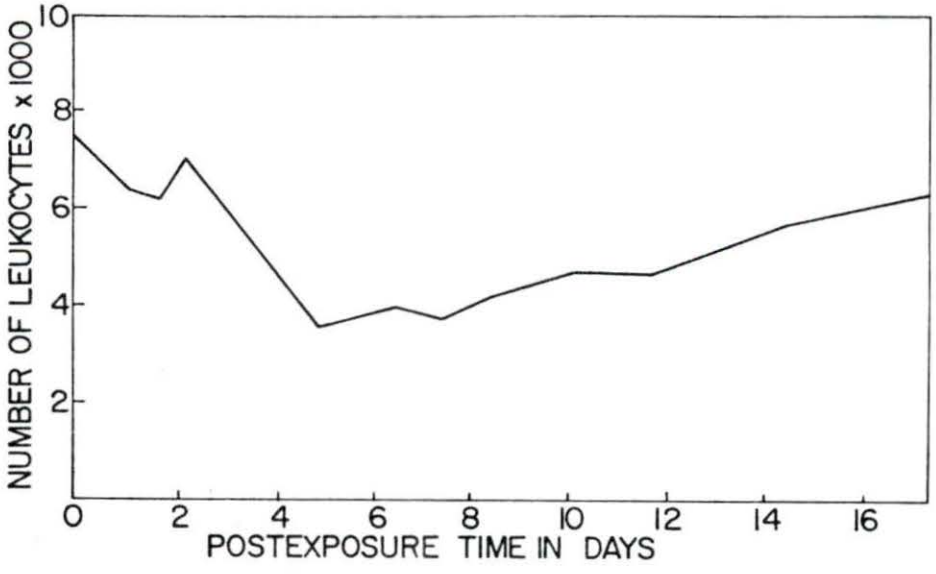
Postexposure responses of cows

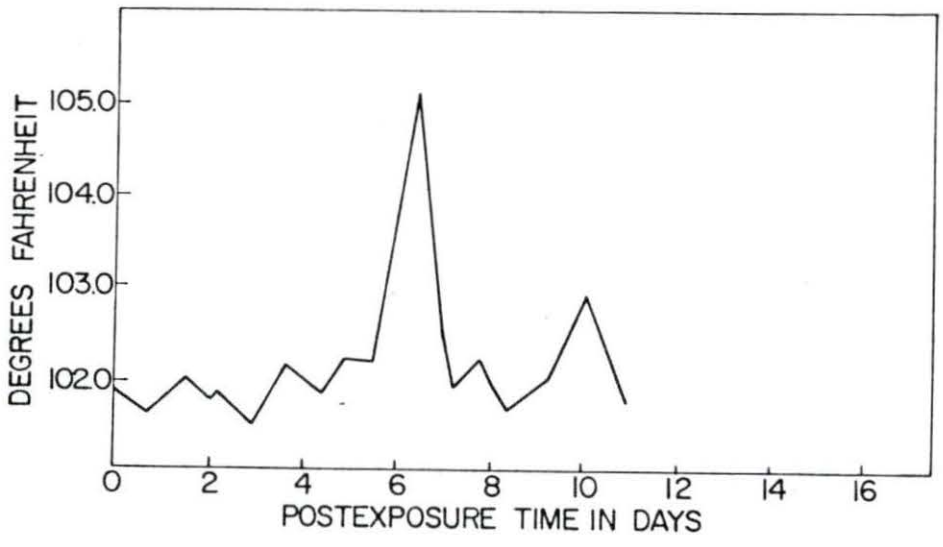
The three cows infected artificially by the oral route had normal parturitions at 6, 16, and 25 days, respectively, postexposure. Each cow experienced a postexposure leukopenia, usually from the fourth to the fourteenth day. Graphic portrayal of the leukocyte count of cow 1 is shown (Figure 1). Leukopenia in each cow was characterized by a marked decrease in the relative number of neutrophiles and a corresponding relative increase in lymphocytes. On day seven postexposure the neutrophile count of cow 3 dropped to 10 percent of the total leukocytes. A moderate increment in the number of eosinophiles was detected in the blood of all three cows particularly from the fourth to the fourteenth day postexposure. Significant differences between the pre-exposure and postexposure levels of monocytes and basophiles were not detected. Erythrocyte levels of all cows remained relatively constant during the study (range of 4.2 to 6.8 million per ml of blood).

Postexposure body temperatures of cow 1 are shown (Figure 2). The peak temperature of 105.2 F occurred on day 6. Parturition of this cow was also on day 6 and may have contributed to the elevated temperature. A second, but lower, peak in body temperature was recorded on day 8. Patterns of

Figure 1. Daily leukocyte count of cow 1 after intravenous exposure to NADL-MD strain of bovine viral diarrhea virus

Figure 2. Record of morning and evening body temperatures of cow 1 after intravenous exposure to NADL-MD strain of bovine viral diarrhea virus


NUMBER OF LEUKOCYTES x 1000
10
8
6
4
2
0
2
4
6
8
10
12
14
16
POSTEXPOSURE TIME IN DAYS


DEGREES FAHRENHEIT
105.0
104.0
103.0
102.0
0
2
4
6
8
10
12
14
16
POSTEXPOSURE TIME IN DAYS

temperature responses for cows 2 and 3 were similar but less marked in respect to peaks.

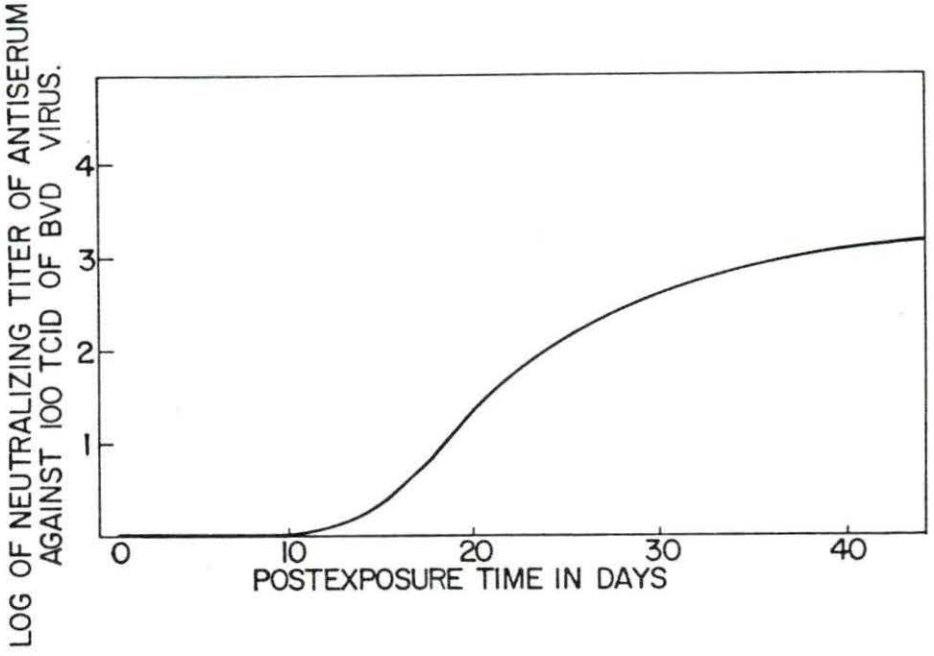
Cow 3 experienced a moderate laminitis of the front feet. The laminitis was most severe on day 5 but persisted until day 11. This was the only animal in the study in which the laminitis syndrome was observed.

The mean serologic response of the three cows is shown (Figure 3). Levels of viral neutralizing antibodies reached detectable levels between 14 and 21 days and were at maximal levels between 42 and 56 days postexposure.

The persistence of viral infections in the three cows is shown (Table 2). Virus was not recovered from the feces of the three cows beyond the tenth day after exposure. Isolation of virus from the nasal passages could not be accomplished after day 13 and was not possible at any time from cow 3. Isolation of the virus from the cells of the buffy coat could not be shown after day 9 in two of the cows, however virus was continuously recovered from the blood of cow 2 up till 43 days' postexposure at which time the cow was subjected to necropsy. Isolations of the virus from the milk from cow 3 were accomplished up to 27 days' postexposure at which time no further samples were collected. Isolations of virus from the milk of cows 1 and 2 were not possible, however the attempts were limited to the first few days postpartum. Each of the three cows experienced a mild diarrhea which persisted from two to four days postexposure.

Figure 3. Mean serologic response of three cows as evidenced by levels of BVD virus neutralizing antibodies in their sera after intravenous exposure to BVD virus

Figure 4. Mean leukocyte count and body temperature of three calves from cows exposed intravenously in last month of gestation to BVD virus


LOG OF NEUTRALIZING TITER OF ANTISERUM AGAINST 100 TCID OF BVD VIRUS.
1
2
3
4
0
10
20
30
40
POSTEXPOSURE TIME IN DAYS

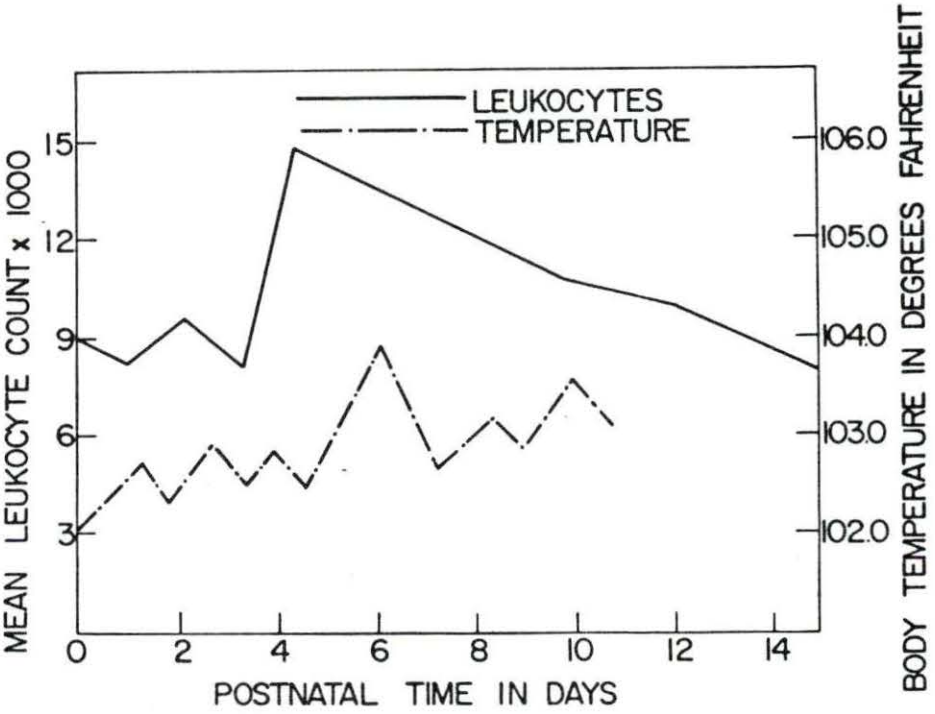

LEUKOCYTES
TEMPERATURE
MEAN LEUKOCYTE COUNT x 1000
3
6
9
12
15
0
2
4
6
8
10
12
14
POSTNATAL TIME IN DAYS
102.0
103.0
104.0
105.0
106.0
BODY TEMPERATURE IN DEGREES FAHRENHEIT

Table 2. Recovery of BVD virus from three cows after intravenous exposure in the last month of gestation to 10^7 TCID of BVD virus

Cow no.	Prepartum time of exposure - in days	Persistence of viral recoveries in days from: Blood	Nares	Rectum	Milk
1	6	9	8	10	Negative
2	16	43	13	10	Negative
3	25	9	0	10	27

Responses of calves

Considerable variation was observed in the leukocyte counts of the three calves born to these three artificially infected cows. The mean daily leukocyte counts of these calves are shown (Figure 4). Generally leukocyte counts rose sharply between the third and fourth day, then gradually returned to lower levels approximating those values observed at birth.

There was much variation in the patterns recorded for postexposure body temperature values. Generally however, there was a moderate increase in body temperature at day 4 or 5 followed by normal levels for three days and then a second elevation about day 9.

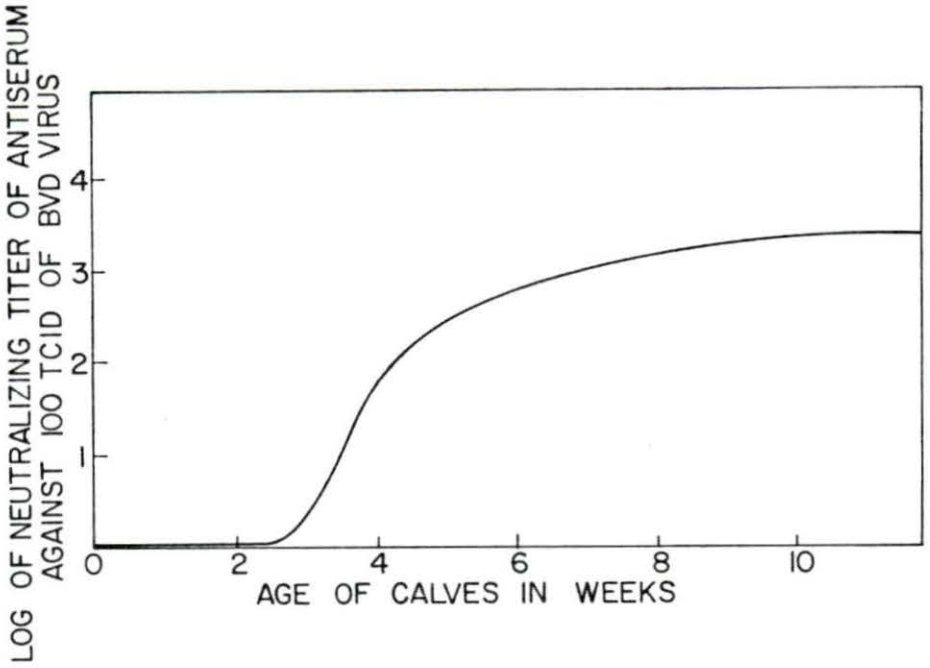
The mean serologic response of these three calves which were allowed colostrum from their dams is shown (Figure 5). Viral neutralizing antibodies were detected in the calves between the second and third weeks of age. Titers increased markedly till about the tenth week and in general persisted at high levels for several months.

The occurrence of the clinical symptoms and their relationship to the time of BVD virus isolations from the calves is shown (Table 3). Moderate symptoms of diarrhea were observed in all calves, being most obvious from the third to the eighth day of age.

Isolations of BVD virus from the buffy coat layer of the blood were much more frequent than isolations of virus from

Figure 5. Mean serologic response of three calves permitted colostrum from dams exposed intravenously in last month of gestation to BVD virus

Figure 6. Mean daily leukocyte count and mean daily body temperature of two SPF calves exposed orally to BVD virus and then permitted colostrum from dams serologically negative for BVD


LOG OF NEUTRALIZING TITER OF ANTISERUM AGAINST 100 TCID OF BVD VIRUS
1
2
3
4
0
2
4
6
8
10
AGE OF CALVES IN WEEKS

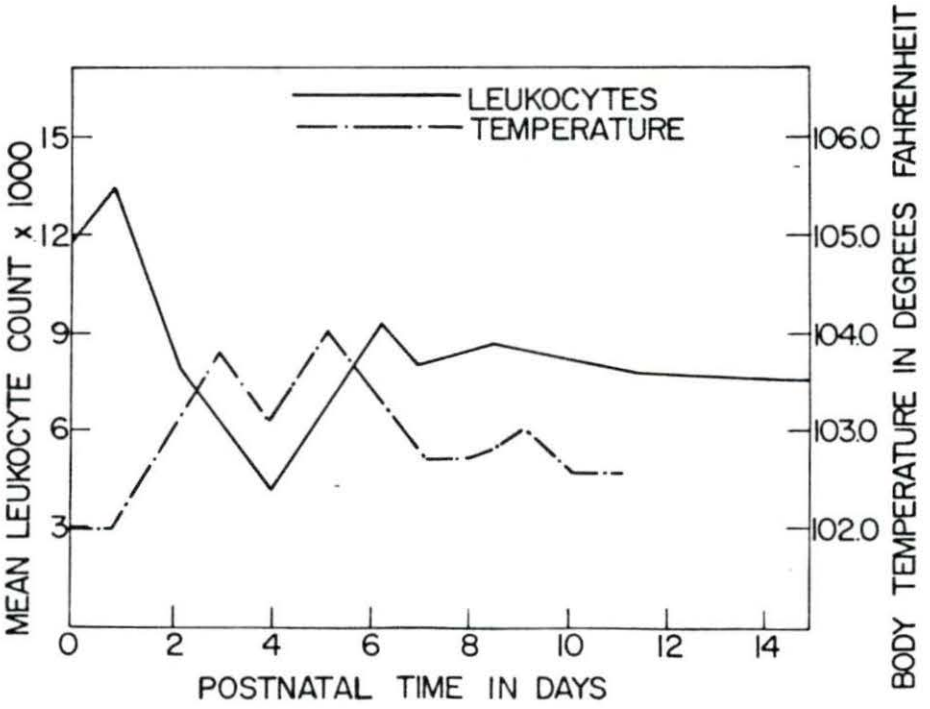

LEUKOCYTES
TEMPERATURE
MEAN LEUKOCYTE COUNT x 1000
3
6
9
12
15
102.0
103.0
104.0
105.0
106.0
BODY TEMPERATURE IN DEGREES FAHRENHEIT
0
2
4
6
8
10
12
14
POSTNATAL TIME IN DAYS

Table 3. Correlation of time of BVD virus isolations and symptoms of calves permitted colostrum from dams exposed intravenously in last month of gestation to BVD virus

Calf number	Severity of diarrhea	Initial day of diarrhea	Persistence of diarrhea (days)	Source of virus isolation	Initial day of virus recovery	Last day of virus recovery
1	Moderate	3	4	Blood	2	93
				Nares	8	14
				Rectum	8	58
2	Moderate	4	3	Blood	2	81
				Nares	2	49
				Rectum	9	35
3	Moderate	4	4	Blood	3	64
				Nares	Not recovered	
				Rectum	8	22

the nares or rectum. The virus persisted in the blood at detectable levels up to 93 days in one calf.

Experiment 2

Responses of calves

The mean daily leukocyte count and mean body temperature of two SPF calves which were exposed orally on the day of birth to BVD virus are shown (Figure 6). A marked leukopenia was observed between the second and sixth days postexposure. Body temperature elevations in this group of calves occurred on day 2 and again on day 5, but they were not substantially above normal levels at any time.

Variations in serologic responses of these three calves are shown (Table 4). Although calf 4 died 28 hours after exposure, calves 5 and 6 survived. The first viral neutralizing antibodies were detected on days 73 and 28, respectively. There was no obvious reason for the delayed immunologic response of calf 5. Subsequent levels of BVD virus neutralizing antibody in this calf were comparable with levels produced by calf 6.

Symptoms of diarrhea in these three calves are correlated with the time of isolations of BVD virus from their blood, nares and rectum, and at necropsy of calf 4 (Table 5). Calf 4 had a profuse watery diarrhea at 18 hours postexposure. Fluid feces were expelled so frequently that within 10 hours (just prior to death) there was evidence of dehydration in the body

Table 4. Serologic responses of three calves exposed orally on day of birth to BVD virus and then permitted colostrum from BVD negative dams

Postexposure time in days	Neutralizing titer of antiserum against 100 TCID of BVD virus		
	Calf 4	Calf 5	Calf 6
0	Negative	Negative	Negative
2	" [a]	"	"
14		"	"
21		"	"
28		"	256
42		"	512
56		"	1024
73		256	1024
100		1024	4096

[a]Died on day 2.

Table 5. Correlation of time of BVD virus isolations and symptoms of calves exposed orally on day of birth to BVD virus and then permitted colostrum from their BVD negative dams

Calf number	Severity of diarrhea	Initial day of diarrhea	Persistence of diarrhea (days)	Source of virus isolation	Initial day of virus recovery	Last day of virus recovery
4	Extreme (death)	1	1	Rectum	1	2
				Small intestine	2 (postmortem)	
				Mesenteric lymph node	2 "	
				Prescapular lymph node	2 "	
				Heart's blood	2 "	
				Spleen	2 "	
5	Mild	4	4	Blood	2	73
				Nares	73	103
				Rectum	4	46
6	Mild	4	4	Blood	2	8
				Nares	2	4
				Rectum	4	56

tissues. At necropsy the BVD virus was isolated from the heart's blood, spleen and several lymph nodes. Moderate lymphoid depletion of the spleen is shown (Figure 7).

Diarrhea was less severe in calves 5 and 6. Isolations of virus ranged from two days postexposure from the blood and nares to 103 days after exposure from the nares of calf 5.

Bacteriologic examination of the carcass of calf 4 indicated the presence of E. coli in the thymus, abomasum, small intestine and bladder. All four isolates showed similar properties with respect to antibiotic sensitivity and mouse pathogenicity. The strains were indistinguishable also from the standpoint of serology (Table 6).

Experiment 3

In this experiment seven SPF calves were colostrum deprived and exposed orally on day of birth to BVD virus.

Responses of calves

There were some variations in the daily leukocyte counts of the seven calves. Calf 11, however, was most representative of the group and the leukocyte and temperature responses of this heifer are shown (Figure 8). Leukopenia was most pronounced on day 6 and the number of neutrophils dropped to less than 20 percent of the total leukocytes. Elevations in body temperatures were usually detected on day 3 or 4 and again on day 6 or 7 postexposure.

Table 6. Comparison of selected characteristics of isolates of E. coli obtained from calf No. 4 at necropsy

Source of isolate	Serologic reactions with:		
	OB-A antisera[a]	OB-B antisera[a]	Saline
Thymus	-	-	-
Abomasum	-	-	-
Bladder	-	-	-
Small intestine[b]	-	-	-

[a]Difco Laboratories, Detroit, Michigan.

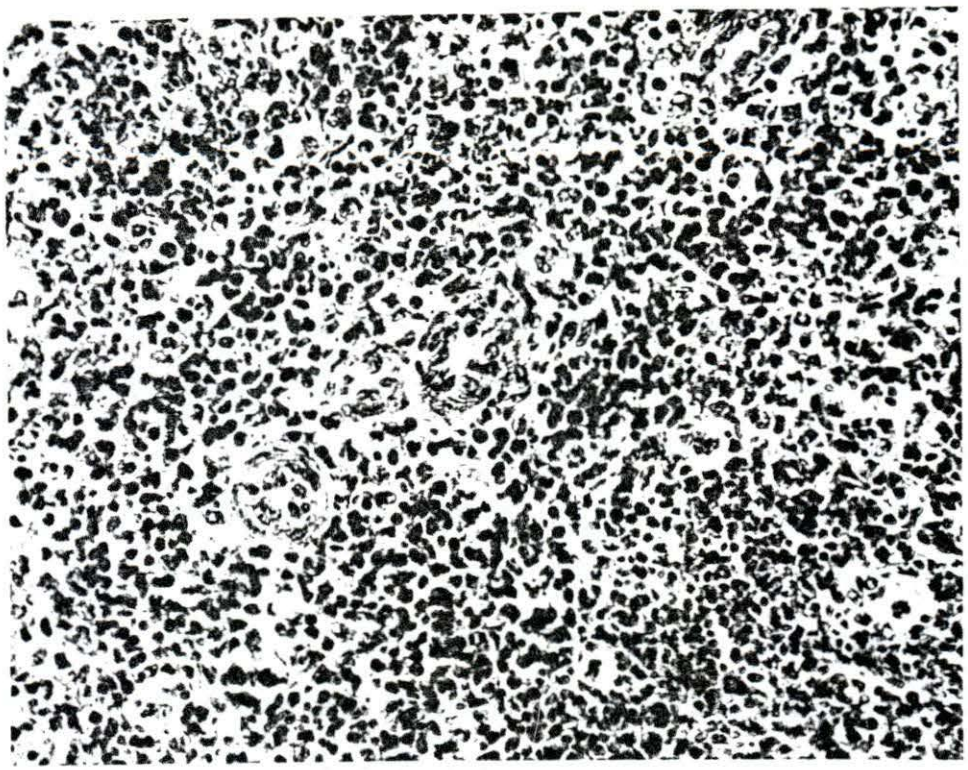
[b]Subsequently typed by Dr. P. J. Glantz, Pennsylvania State University, University Park, Pennsylvania, as O: K30 H 21.

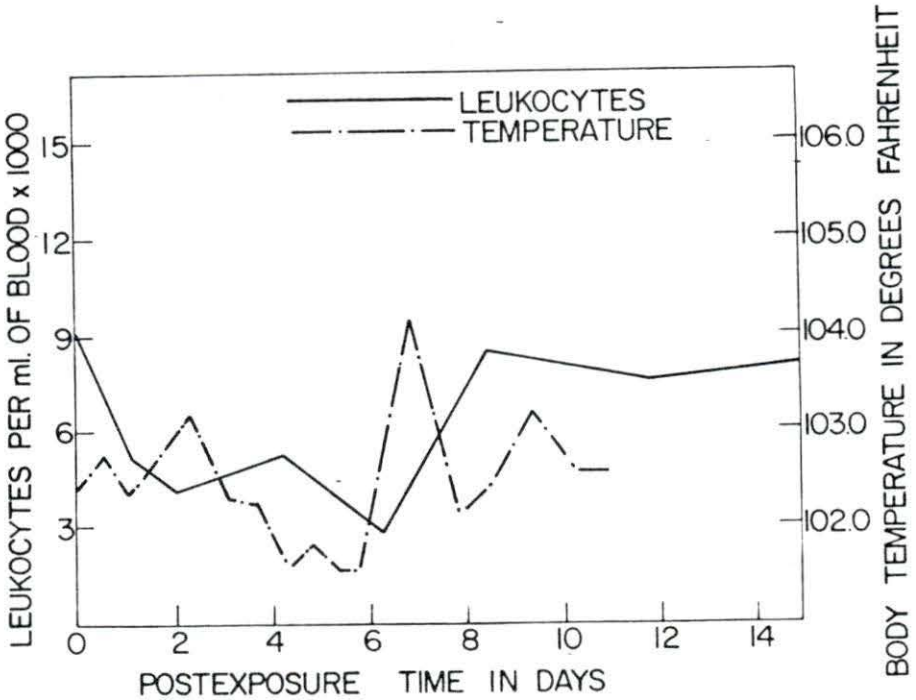
Table 6. (Continued)

Source of isolate	Antibiotic sensitivity[a]		LD_{50} for mice
	Sensitive	Not sensitive	
Thymus	Chloromycetin 10 mcg. Neomycin 10 mcg. Kanamycin 10 mcg.	Erythromycin 5 mcg. Novobiocin 10 mcg. Penicillin 5 units Streptomycin 10 mcg. Tetracycline 10 mcg.	$> 6 \times 10^6$
Abomasum	Same as thymus isolate	Same as thymus isolate	"
Bladder	"	"	"
Small intestine	"	"	"

Figure 7. Section of spleen of calf 4 obtained at necropsy. This SPF calf was exposed orally on day of birth to BVD virus and permitted colostrum from its BVD negative dam. The calf died at 28 hours post-exposure - (X250) H. and E. stain

Figure 8. Daily leukocyte count and body temperature of calf 11 representative of seven SPF calves deprived of colostrum and exposed orally to BVD virus on day of birth




LEUKOCYTES
TEMPERATURE
LEUKOCYTES PER ml. OF BLOOD x 1000
15
12
9
6
3
0
2
4
6
8
10
12
14
POSTEXPOSURE TIME IN DAYS
106.0
105.0
104.0
103.0
102.0
BODY TEMPERATURE IN DEGREES FAHRENHEIT

The serologic responses of the 7 calves are shown (Table 7). Calf 8 did not produce BVD viral neutralizing antibodies at detectable levels until the sixth week after exposure whereas other calves produced detectable levels of antibody at the fourth or fifth week postexposure. In general, titers increased gradually except in the cases of calves 11 and 12 where there was a marked increase in viral neutralizing titer from 1024 to >65,000 during the eighth week.

The symptoms of the seven calves are correlated with the time of isolation of BVD virus (Table 8). The response of calf 7 was extremely acute following oral exposure on day of birth to the virus. Within 18 hours postexposure the calf had an extremely severe and persistent diarrhea. Dehydration was evident as the calf became moribund. Death occurred at 38 hours postexposure. Prior to death virus was isolated from the blood (buffy-coat layer) just slightly more than 24 hours postexposure. At necropsy there was a marked hemorrhagic enteritis (Figure 9). Lymphoid depletion was also observed in the mesenteric lymph nodes (Figure 10). The BVD virus was recovered from the mesenteric lymph nodes, areas of the small intestine and the cecum.

Several isolates of E. coli were obtained from various organs of this calf at necropsy. Studies on these isolates are summarized (Table 9).

Calf 13 had a mild diarrhea at three days postexposure which persisted till death. There was frequent passing of

Table 7. Serologic responses of seven SPF calves exposed orally on day of birth to BVD virus and deprived of colostrum from their BVD negative dams

Postexposure time in days	Neutralizing titer of antiserum against 100 TCID of BVD virus						
	Calf 7	Calf 8	Calf 9	Calf 10	Calf 11	Calf 12	Calf 13
0	Negative	Negative	Negative	Negative	Negative	Negative	Negative
7	Dead[a]	"	"	"	"	"	Dead[b]
14		"	"	"	"	"	
21		"	"	"	"	"	
28		"	"	16	4	4	
35		"	64	64	256	256	
42		64	256	64	1,024	1,024	
56		256	256	256	65,536	65,536	

[a]Died on day 2.

[b]Died on day 5.

Table 8. Correlation of time of BVD virus isolations and symptoms of seven SPF calves exposed orally on day of birth to BVD virus and deprived of colostrum from BVD negative dams

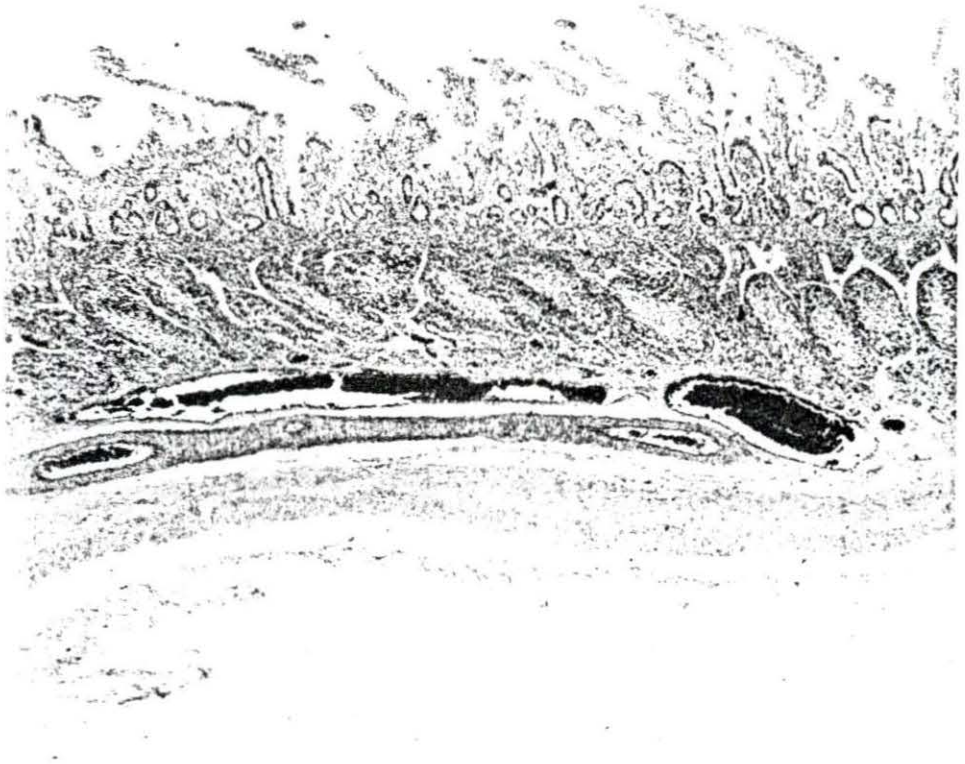
Calf number	Severity of diarrhea	Initial day of diarrhea	Persistence of diarrhea (days)	Source of virus isolations	Initial day of virus recovery	Last day of virus recovery
7	Extreme (death)	1	1	Blood	1	
				Rectum	2 (postmortem)	
				Mesenteric lymph node	2 "	
				Small intestine	2 "	
				Cecum	2	
8	Moderate	3	4	Blood	7	68
				Nares	9	40
				Rectum	5	33
9	Moderate	4	3	Blood	2	68
				Nares	7	40
				Rectum	3	33
10	Marked	4	6	Blood	4	69
				Nares	8	8
				Rectum	4	95
11	Moderate	7	12	Blood	4	8
				Nares	6	9
				Rectum	5	20

Table 8. (Continued)

Calf number	Severity of diarrhea	Initial day of diarrhea	Persistence of diarrhea (days)	Source of virus isolations	Initial day of virus recovery	Last day of virus recovery
12	Severe	6	15	Blood	9	20
				Nares	6	11
				Rectum	6	17
13	Mild, bloody (death)	3	2	Blood	3	5
				Nares	4	5
				Rectum	2	5
				Submaxillary lymph node	5 (postmortem)	
				Atlantal lymph node	5 (postmortem)	

Figure 9. Section of ileum from calf 7 showing severe edema, hyperemia and lymphoid depletion in area of Peyer's patches - (X25) stained with H. and E. This was one of seven calves exposed orally on day of birth to BVD virus and deprived of colostrum from their BVD negative dams.

Figure 10. Section of mesenteric lymph node from calf 7 with areas of lymphoid necrosis - (X250) stained with H. and E.



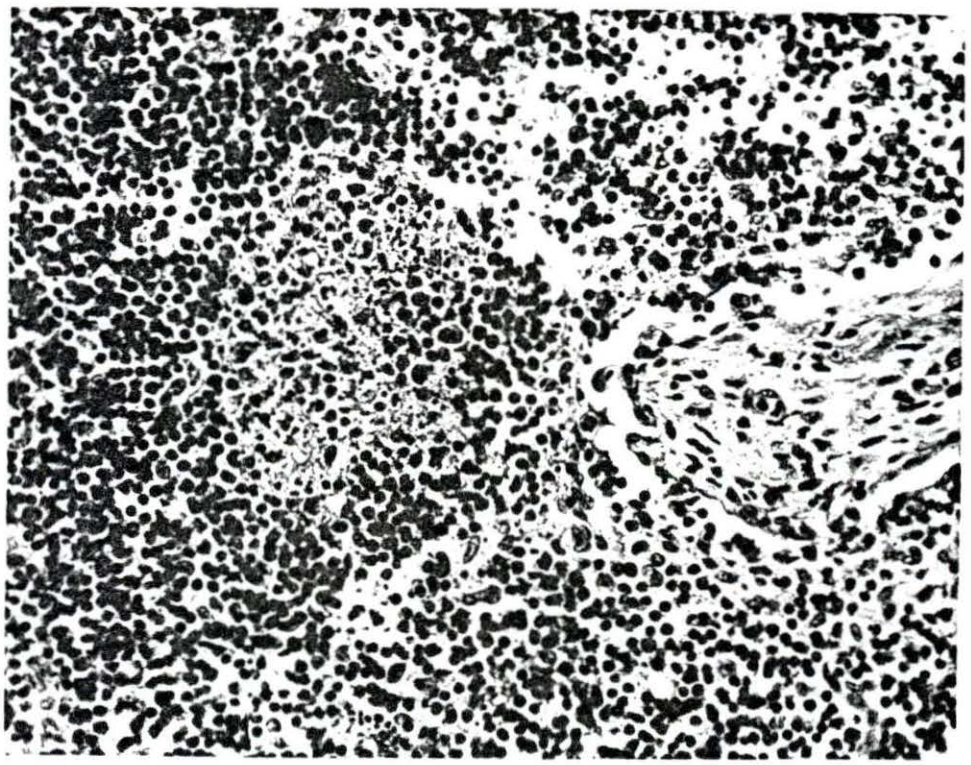


Table 9. Comparison of selected characteristics of isolates of E. coli obtained from calf No. 7 at necropsy

Source of isolate	Serologic reactions with: OB-A antisera[a]	OB-B antisera[a]	Saline
Cecum	-	-	-
Ileum[b]	-	-	-
Rectum	-	-	-
Pancreas	-	+	+
Mesenteric lymph node	-	-	-
Spleen	-	-	-
Heart	-	-	-
Colon	-	-	-

[a]Difco Laboratories, Inc., Detroit, Michigan.

[b]Subsequently typed by Dr. P. J. Glantz, Pennsylvania State University, University Park, Pennsylvania, as O: K15: H 21.

Table 9. (Continued)

Source of isolate	Antibiotic sensitivity[a]		LD_{50} for mice
	Sensitive	Nonsensitive	
Cecum	Chloromycetin 10 mcg. Neomycin 10 mcg. Kanamycin 10 mcg. Tetracycline 10 mcg.	Erythromycin 5 mcg. Novobiocin 10 mcg. Penicillin 5 units Streptomycin 5 mcg.	$> 6 \times 10^6$
Ileum	Same as cecum		"
Rectum	"		"
Pancreas	"		"
Mesenteric lymph node	"		"
Spleen	"		"
Heart	"		"
Colon	"		"

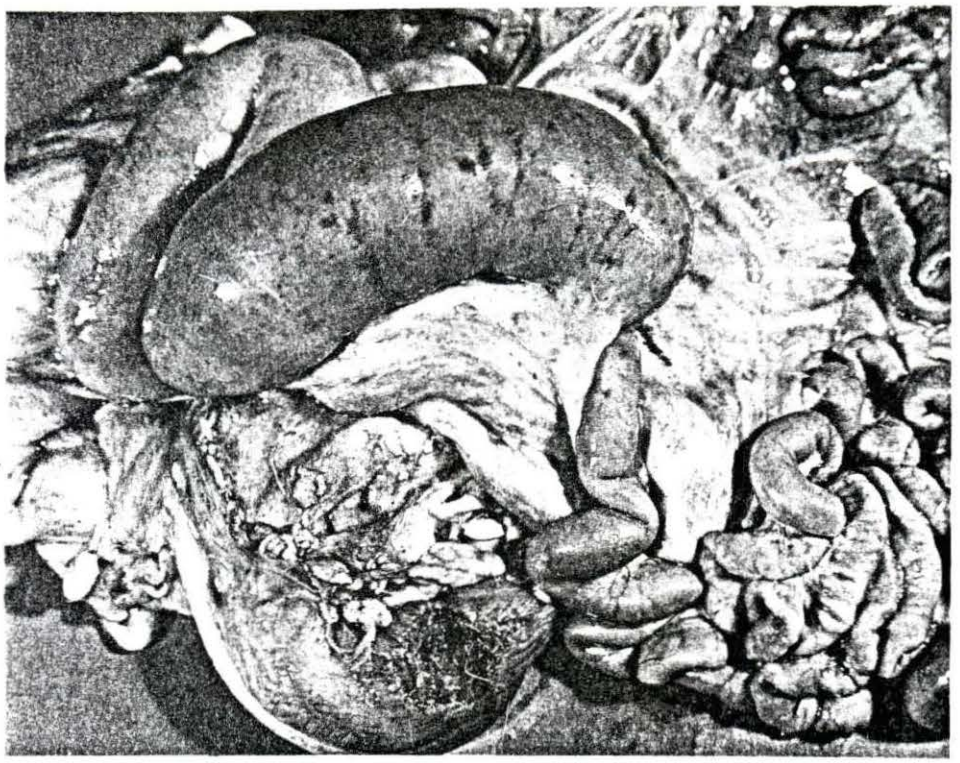
blood in the feces. The calf became depressed on day 3 and the depression increased in severity until death. At necropsy there were several indications of gross pathological changes. The necropsy was performed within five minutes after this calf had expired its last breath. Examination of the viscera revealed a marked generalized enteritis. Hemorrhagic areas were frequent throughout the small intestine and the cecum (Figure 11). Large hemorrhagic areas were also observed in several areas of the internal surface of the abomasal wall. One such area is shown (Figure 12). Smaller and more numerous hemorrhagic foci were prevalent on the walls of the rumen and reticulum and in the regions of the rumen pillars. The spleen was altered grossly with many areas of hemorrhagic foci on its surface. There was little evidence of gross pathology in the other internal organs with the exception of the heart which when opened revealed several hemorrhagic areas in the region of the heart valves (Figure 13).

The regional lymph nodes of this calf, particularly the prescapulars and prefemorals, had areas of hemorrhage and edema.

Several isolations of E. coli were made at necropsy of this calf. The characteristics of these isolates are shown (Table 10). There were only minor differences between the isolates in their serologic, pathogenic and antibiotics sensitivity patterns. The isolate of E. coli from the mesenteric lymph node proved to be less pathogenic for mice than the isolates from the liver and bone marrow. All of the isolates

Figure 11. Hemorrhagic areas in the wall of the cecum and ileum of calf 13. One of seven SPF calves exposed orally on day of birth to BVD virus and deprived of colostrum from their BVD negative dams. Death occurred on day 5 postexposure

Figure 12. Area of extensive hemorrhage on internal surface of abomasal wall of calf 13



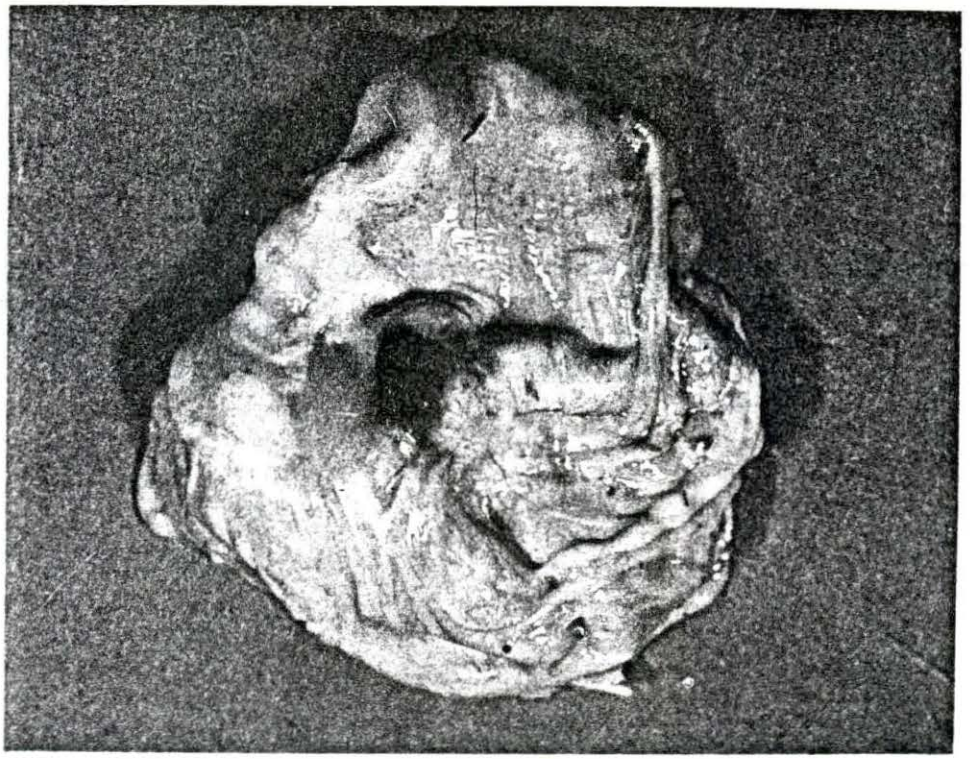
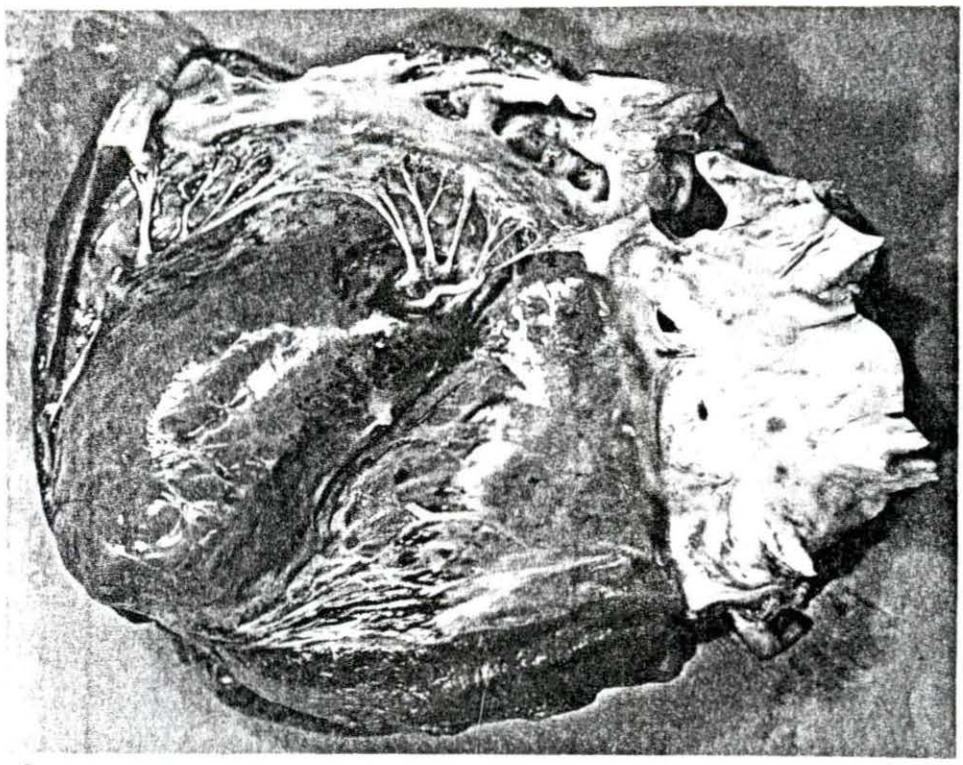


Figure 13. Marked endocardial hemorrhages observed after incising the heart of calf 13

Figure 14. Daily leukocyte count and body temperature of calf 15 representative of three SPF calves exposed orally on day of birth to BVD virus and deprived of colostrum from their serologically positive dams



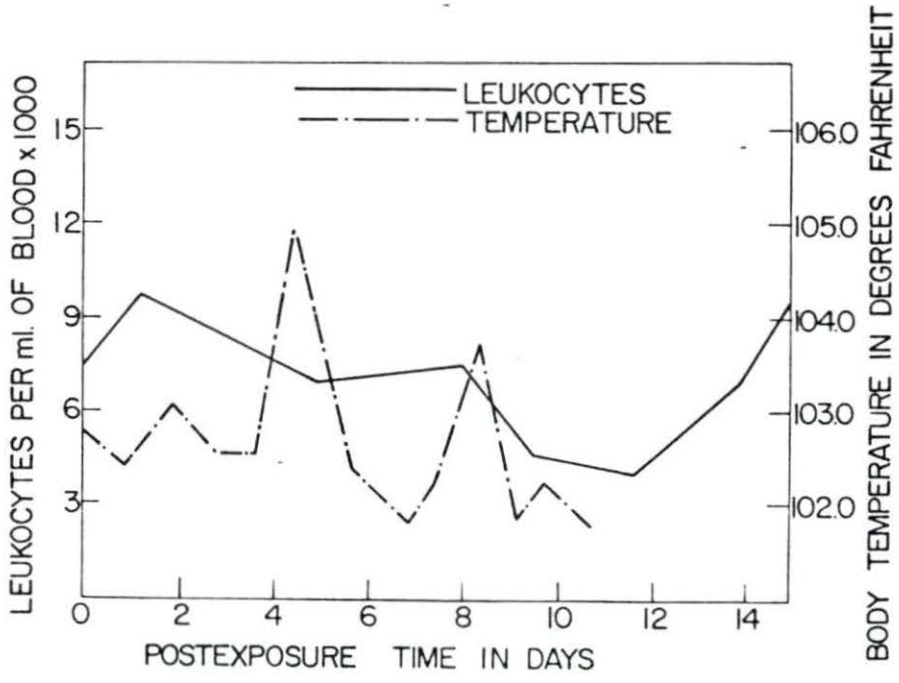

LEUKOCYTES
TEMPERATURE
LEUKOCYTES PER ml. OF BLOOD x 1000
15
12
9
6
3
106.0
105.0
104.0
103.0
102.0
BODY TEMPERATURE IN DEGREES FAHRENHEIT
0
2
4
6
8
10
12
14
POSTEXPOSURE TIME IN DAYS

Table 10. Comparison of selected characteristics of isolates of E. coli obtained from Calf No. 13 at necropsy

Source of isolate	Serologic[a] reactions with: OB-A antisera[b]	OB-B antisera[b]	Saline
Submaxillary lymph node	-	-	-
L. Prefemoral L. N.*	-	-	-
Liver	-	-	-
Mesenteric L. N.**	-	+	-
Atlantal L. N.***	-	-	-
Bone marrow****	-	-	-

[a]Four of the above strains were typed serologically by Dr. P. J. Glantz of Pennsylvania State University, University Park, Pennsylvania, with the following results: * 8:K. :2; ** 104:K.:2; *** 117:K.:19; **** 117:K.:2 (K. = K antigen present but not standard).

[b]Difco Laboratories, Inc., Detroit, Michigan.

Table 10. (Continued)

Source of isolate	Antibiotic sensitivity[b]		LD_{50} for mice
	Sensitive	Nonsensitive	
Submaxillary lymph node	Chloromycetin Neomycin Tetracycline Kanamycin 10 mcg.	Erythromycin Novobiocin Penicillin Streptomycin	10^7
L. prefemoral L. N.	Same as above	Same as above	10^7
Liver	"	"	10^7
Mesenteric L. N.	"	"	$> 10^8$
Atlantal L. N.	"	"	10^6
Bone marrow	"	"	10^7

of E. coli were sensitive to chloromycetin, neomycin, tetracycline, and kanamycin.

The surviving five calves in this experiment had moderate to severe diarrhea for periods ranging from one to three weeks after exposure. There was considerable difference in the length of time that virus could be isolated from the various calves. Maximum persistence of virus occurred in calf 10 where the virus was isolated up to 95 days postexposure from the rectum and up to 65 days from the blood. However in this particular calf, nasal swabs were consistently negative for virus except on day 8.

Several isolations of E. coli were made from rectal swabs of these surviving calves. Rectal swabs were consistently negative for E. coli on days 0, 1 and usually on day 2. From day 3 to the 15th day rectal swabs frequently contained one or more serotypes of E. coli, none of which were pathogenic for mice even in doses greater than 10^8.

Experiment 4

The three SPF calves in this experiment were exposed orally on day of birth and deprived of colostrum from their serologically BVD positive dams.

Responses of calves

The daily leukocyte count and temperature of calf 15 representative of the responses of the three calves is shown

(Figure 14). The leukopenia was observed in this calf at a later date than was generally observed in other calves in this group.

The serologic responses as indicated by presence of viral neutralizing antibodies are presented (Table 11). There was an early and rapid rise in titer in both surviving calves. However, calf 16 which died on day 11 was not producing antibodies at detectable levels on the day of death.

The data in Table 12 show that the period of viral recovery was very short in the two survivors, and its termination coincided with the sudden and marked increases in their levels of viral neutralizing antibodies. Both survivors had a persistent and severe diarrhea.

Calf 16 which died on day 11 had a moderate diarrhea which began on day 3. From day 3 until death this calf was extremely depressed. Respiratory distress and nasal discharge were characteristic symptoms of calf 16 from day 5 to day 10. Although nasal discharge was observed the BVD virus was never recovered from the nasal swabs. However virus was consistently recovered from rectal swabs of this calf. At necropsy of calf 16, virus was recovered from the spleen and the rectum. Various tissues were studied in more detail (Figures 15 through 20).

A marked enteritis was observed at necropsy (Figure 15). This extended throughout most of the small intestine but was

Table 11. Serologic responses of three SPF calves exposed orally on day of birth to BVD virus and deprived of colostrum from their serologically BVD positive dams

Postexposure time in days	Neutralizing titer of antiserum against 100 TCID of BVD virus Calf 14	Calf 15	Calf 16
0	Negative	Negative	Negative
7	"	"	"
14	"	"	Dead[a]
21	4	4	
28	256	256	
35	1,024	1,024	
42	65,536	65,536	
56	65,536	65,536	

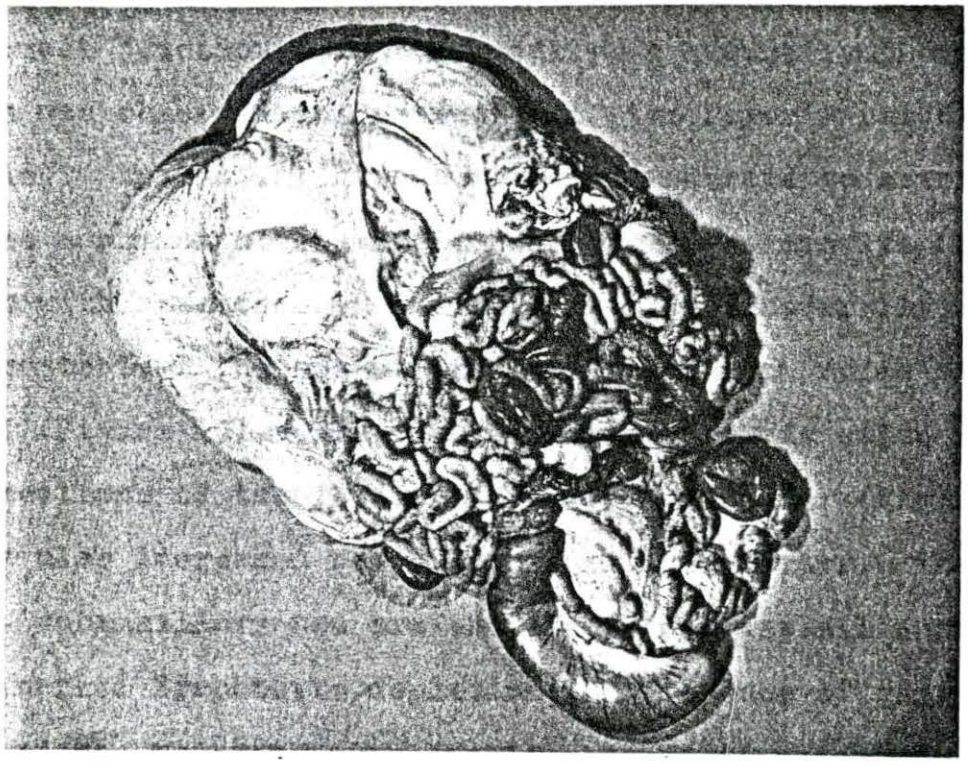
[a]Died on day 11.

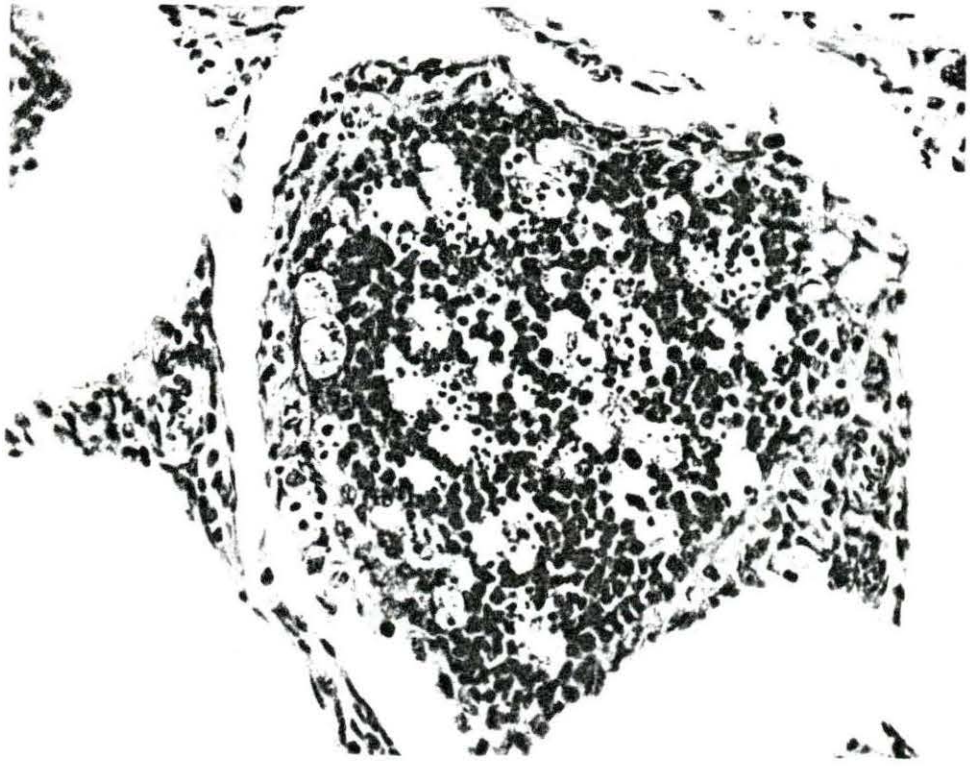
Table 12. Correlation of time of BVD virus isolations and symptoms of three SPF calves exposed orally on day of birth to BVD virus and deprived of colostrum from serologically BVD positive dams

Calf number	Severity of diarrhea	Initial day of diarrhea	Persistence of diarrhea (days)	Source of virus isolations	Initial day of virus recovery	Last day of virus recovery
14	Marked	7	13	Blood	6	32
				Nares	5	11
				Rectum	4	27
15	Extreme	10	19	Blood	4	20
				Nares	5	11
				Rectum	2	32
16	Moderate (death)	3	3	Blood	5	8
				Nares	No isolations	
				Rectum	1	11
				Spleen	11 (postmortem)	
				Rectum	11 (postmortem)	

Figure 15. Viscera from calf 16 showing areas of gross hemorrhages generalized throughout viscera and characterized by a severe hemorrhagic enteritis. This calf died on day 11 postexposure and was one of three SPF calves exposed orally on day of birth to BVD virus and deprived of colostrum from their serologically BVD positive dams.

Figure 16. Section of ileum of calf 16 showing lymphocytic necrosis and phagocytosis in germinal center of Peyer's patch. Stained with H. and E. (X250).





particularly severe in the lower one-third of its length and at the ileocecal junction. Histologically, a severe lymphoid depletion was observed in the region of the Peyer's patches (Figure 16). Epithelial hyperplasia and moderate to severe edema were a common observance. Moderate to severe lymphoid depletion with absence of germinal centers and severe hyperemia characterized the mesenteric and other lymph nodes of this calf (Figure 17). The lymphoid depletion was quite extensive in sections of the thymus gland from this calf (Figure 18). There was also increased phagocytosis of lymphocytes. The prescapular, submaxillary and prefemoral lymph nodes all showed areas of marked lymphocyte depletion with absence of germinal centers, edema, moderate purulent lymphadenitis and reticuloendothelial hyperplasia. No gross visible or microscopic lesions were observed on the trachea, esophagus and kidney. The heart and liver were characterized by marked hyperemia. The tongue contained one small superficial ulcer. Numerous hemorrhagic areas were observed on the surface of the spleen (Figure 19). There was moderate edema of the muscle layers of the rectum with hemorrhages scattered in the submucosal and subserosal layers (Figure 20).

There were several isolations of E. coli from this calf made before and at the time of necropsy. Attempts to isolate E. coli from rectal swabs were unsuccessful until day 3 of age. All of several isolates of E. coli made from the rectal swabs of this calf between day 3 and day 11 were not pathogenic for

Figure 17. Section of mesenteric lymph node from calf 16 showing areas of phagocytosis of necrotic lymphocytes; stained with H. and E. (X250).

Figure 18. Section of thymus gland from calf 16 showing areas of marked lymphoid depletion; stained with H. and E. (X100).

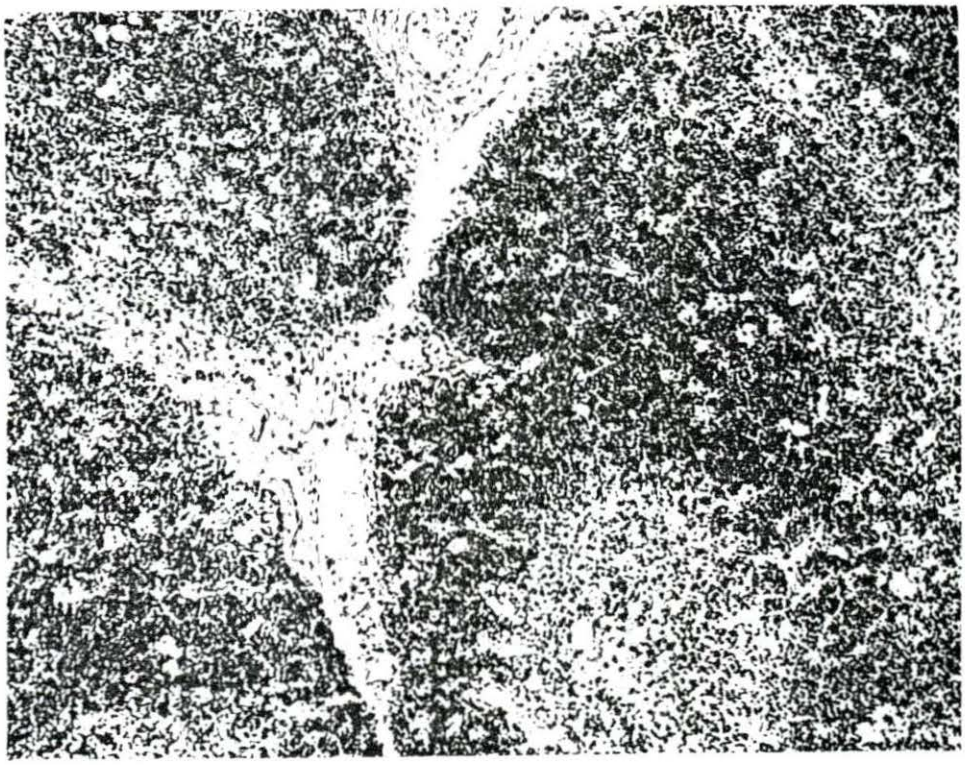
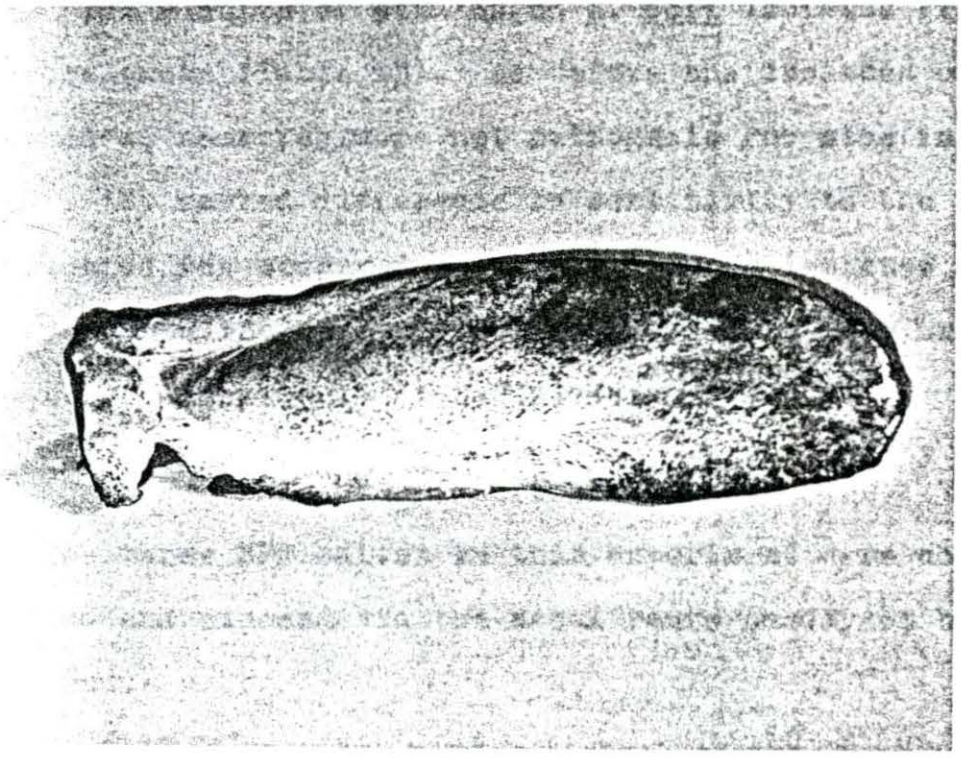
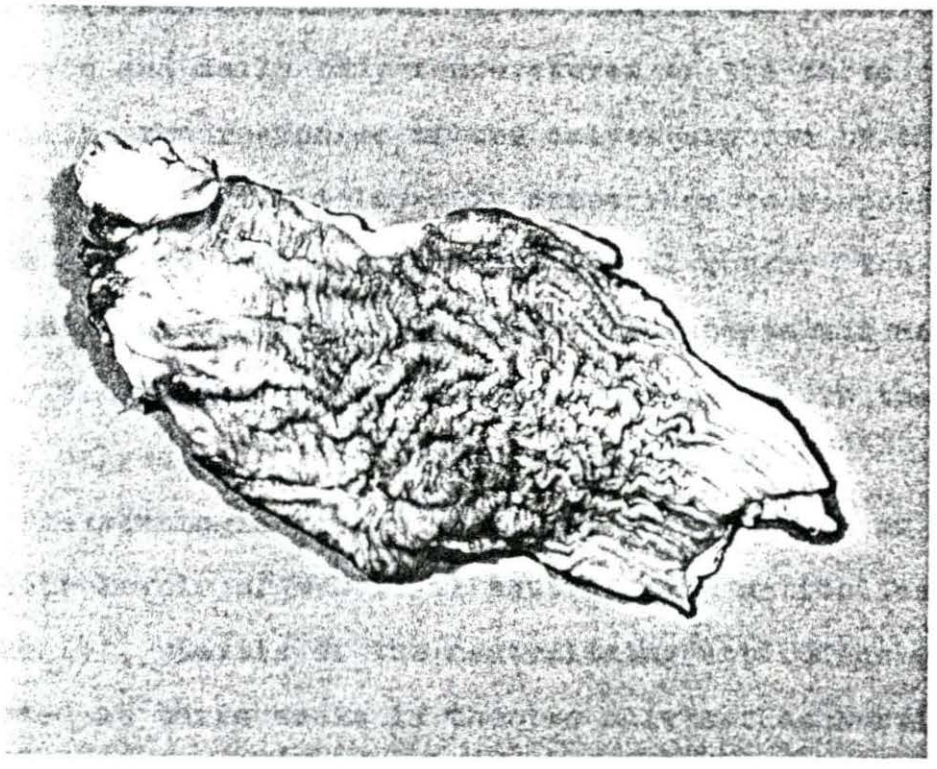


Figure 19. Spleen of calf 16 showing numerous areas of gross hemorrhages on the surface

Figure 20. Rectal wall of calf 16 showing characteristic areas of edema and hemorrhages.





mice. The results of studies on E. coli isolates from this calf are shown (Table 13). As before the isolates of E. coli obtained at necropsy were not pathogenic for mice in doses up to 10^7. No marked difference in sensitivity to the various antibiotics was observed among the various isolates of E. coli. Serologic studies were performed as previously by Dr. Paul J. Glantz of Pennsylvania State University.

Experiment 5

The three SPF calves in this experiment were colostrum deprived and exposed via the nasal route on day of birth to BVD virus.

Responses of calves

There was considerable variation in the postexposure leukocyte and daily body temperatures of the three calves. Generally, the responses of the calves exposed by the nasal route were slightly delayed in comparison to responses of calves exposed to the virus by the oral route. The response of calf 18 is shown (Figure 21). The leukopenia was most apparent on day 7. Temperature peaks were less marked in these experimental calves than in calves exposed orally.

The serologic responses of the three calves as indicated by their levels of BVD viral neutralizing antibodies is shown (Table 14). Levels of the neutralizing antibodies were detected at three weeks in the two calves that survived the exposure. The titers remained stabilized for several months

Table 13. Comparison of selected characteristics of isolates of E. coli obtained from calf No. 16 at necropsy

Source of isolate	Serologic[a] reactions with: OB-A antisera[b]	OB-B antisera[b]	Saline
Heart*	-	+	-
Rectum**	-	-	-
Small intestine	+	+	+
Cecum***	-	+	-
Lungs****	+	-	-

[a]Four of the above cultures were submitted to Dr. P. J. Glantz of Pennsylvania State University, University Park, Pennsylvania, for serotyping. The results were as follows: * 20: K-: 2; ** 117: K.: 2; *** 11: K32: 15; **** 5: K-: 38 (K. = K antigen present but not standard; K- = K antigen not present).

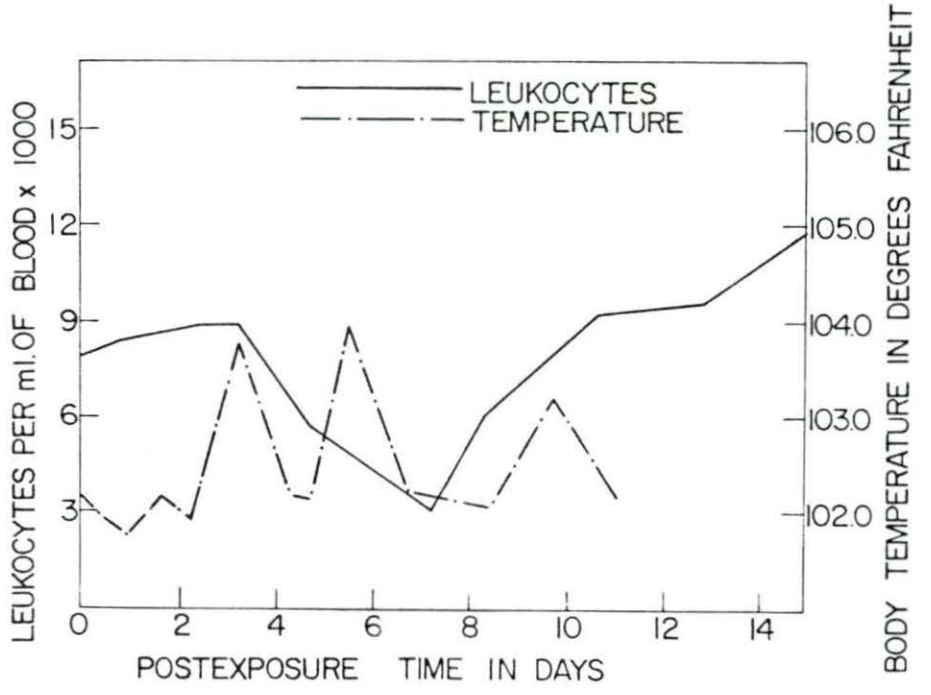
[b]Difco Laboratories, Inc., Detroit, Michigan.

Table 13. (Continued)

Source of isolate	Antibiotic sensitivity[b]		LD_{50} for mice
	Sensitive	Nonsensitive	
Heart	Chloromycetin Kanamycin Tetracycline	Neomycin Erythromycin Novobiocin Penicillin Streptomycin	$> 10^7$
Rectum	Same as heart	Same as heart	$> 10^7$
Small intestine	"	"	10^8
Cecum	"	"	10^8
Lungs	"	"	$> 10^7$

Figure 21. Daily leukocyte count and body temperature of calf 18, one of three SPF calves exposed intranasally on day of birth to BVD virus and deprived of colostrum from their serologically negative dams

Figure 22. Dorsal surface of tongue of calf 17 showing areas of ulceration and erosion. This SPF calf was exposed intranasally on day of birth to BVD virus and deprived of colostrum from its serologically BVD negative dam. The calf died on day 13 post-exposure


LEUKOCYTES
TEMPERATURE
LEUKOCYTES PER ml. OF BLOOD x 1000
15
12
9
6
3
106.0
105.0
104.0
103.0
102.0
0
2
4
6
8
10
12
14
POSTEXPOSURE TIME IN DAYS
BODY TEMPERATURE IN DEGREES FAHRENHEIT

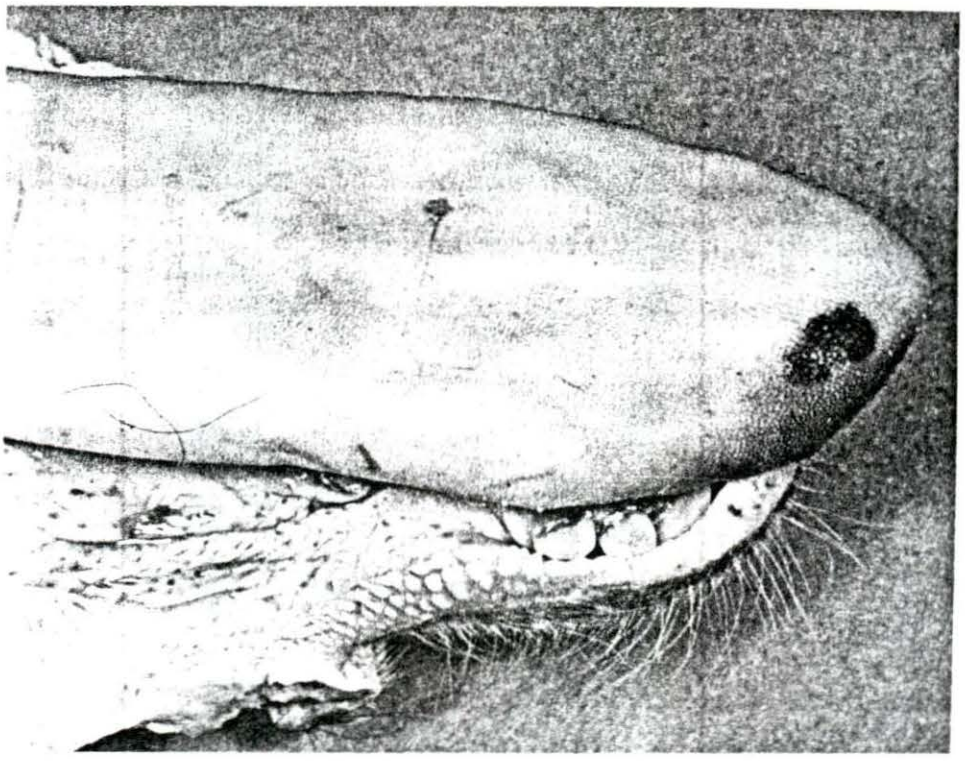


Table 14. Serologic responses of three SPF calves exposed intranasally on day of birth to BVD virus and deprived of colostrum from their BVD negative dams

Postexposure time in days	Neutralizing titer of antiserum against 100 TCID of BVD virus		
	Calf 17	Calf 18	Calf 19
0	Negative	Negative	Negative
7	"	"	"
14	Dead[a]	"	"
21		16	16
28		64	64
35		256	64
42		1024	256
56		1024	256

[a]Died on day 13.

at the levels shown in the table at 56 days. The time of viral isolations and the occurrence of diarrhea in these three calves are summarized (Table 15). Calf 17 died on day 13, whereas the other two calves in this group developed a moderate diarrhea of shorter duration and survived the challenge. However, the surviving calves shed virus in their feces up to 59 days and up to 60 days in nasal secretions. Buffy-coat layer cultures from calves 18 and 19 were positive for BVD virus up to 60 and 59 days, respectively.

Calf 17 had a moderate diarrhea during the first week of life, but seemed to improve slightly during the second week. Death was sudden on day 13. At necropsy BVD virus was isolated from the cecum, small intestine and mesenteric lymph nodes. Three isolates of E. coli were cultured from various portions of the digestive tract of calf 17 at necropsy. The results of bacteriologic studies from this calf are shown (Table 16). Only minor differences existed when the isolates were compared antigenically. Ulcerations and erosions of the dorsal surface of the tongue of calf 17 are shown (Figure 22). Similar but less extensive lesions were observed also on the buccal mucosa and lips of this calf. The esophagus was slightly edematous and had numerous focal areas of hemorrhage. The hemorrhages were larger and more plentiful throughout the rumen, reticulum, omasum, abomasum and small intestine (Figure 23). Enteritis was extremely severe particularly at the ileocecal junction. Hemorrhages and edema were very

Table 15. Correlation of time of BVD virus isolations and symptoms of three SPF calves exposed intranasally on day of birth to BVD virus and deprived of colostrum from BVD negative dams

Calf number	Severity of diarrhea	Initial day of diarrhea	Persistence of diarrhea (days)	Source of virus isolations	Initial day of virus recovery	Last day of virus recovery
17	Moderate-mild (death)	2	11	Blood	4	8
				Nares	4	10
				Rectum	4	10
				Cecum	13 (postmortem)	
				Small intestine	13 (postmortem)	
				Mesenteric lymph node	13 (postmortem)	
18	Moderate	2	5	Blood	2	60
				Nares	7	60
				Rectum	3	32
19	Moderate	2	2	Blood	2	59
				Nares	2	7
				Rectum	3	59

Table 16. Comparison of selected characteristics of isolates of E. coli obtained from calf No. 17 at necropsy

Source of isolate	Serologic reactions with: OB-A antisera[a]	OB-B antisera[a]	Saline
Ileum	-	-	-
Cecum[b]	-	+	-
Rectum	-	+	-

[a] Difco Laboratories, Inc., Detroit, Michigan.

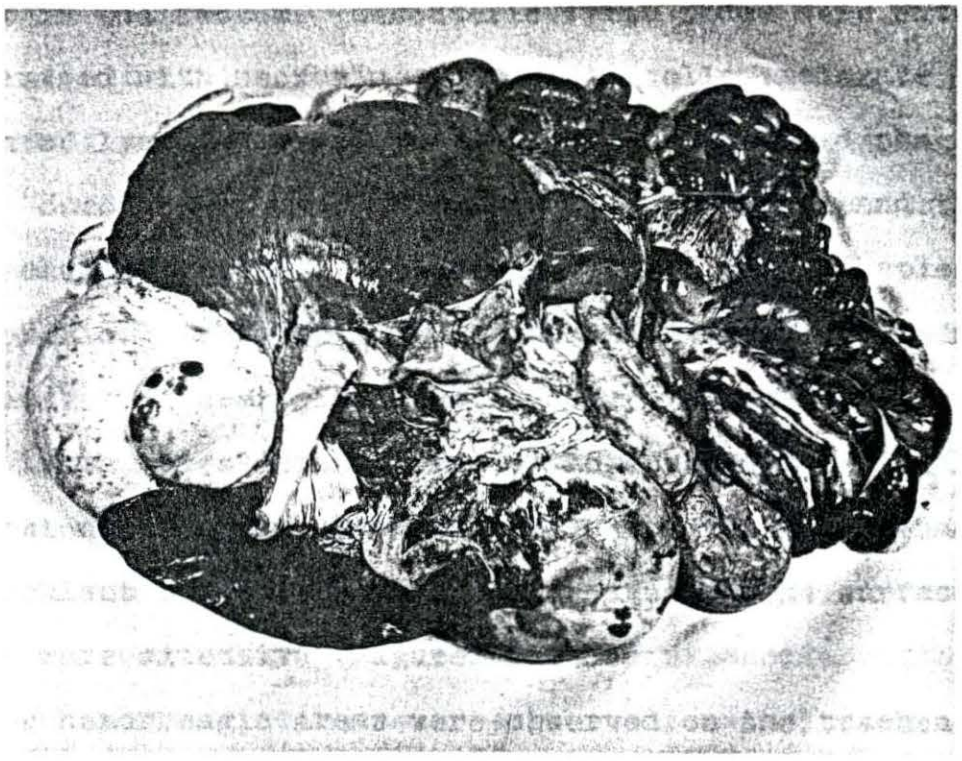
[b] Submitted to Dr. P. J. Glantz of Pennsylvania State University, University Park, Pennsylvania, and serotyped as O: K.: H. (no reactions).

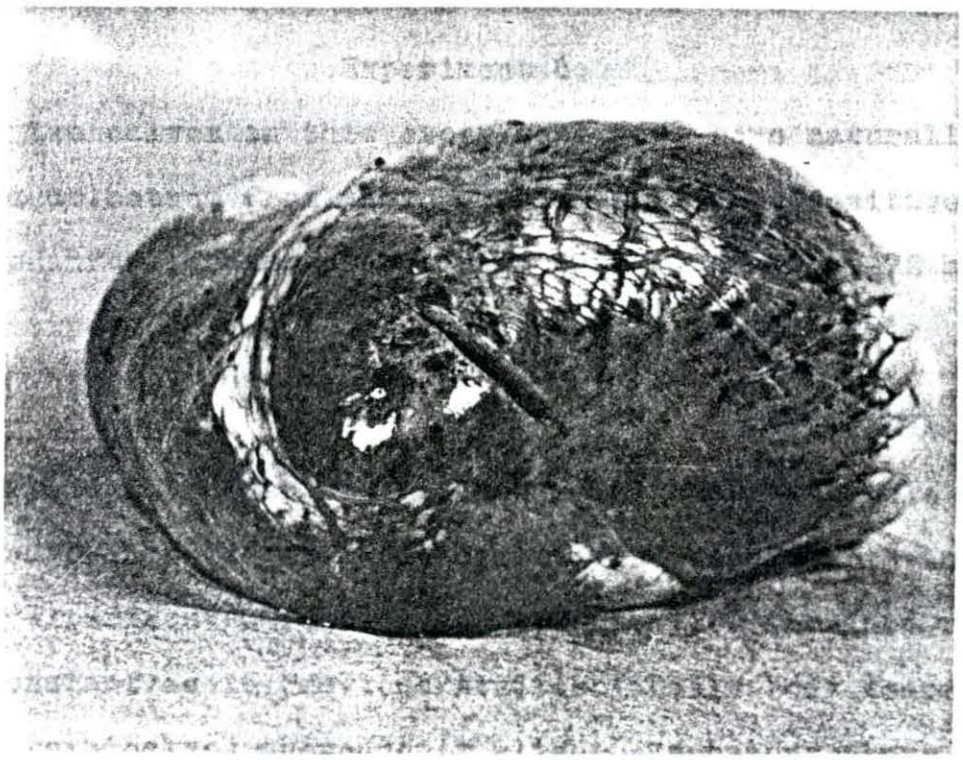
Table 16. (Continued)

Source of isolate	Antibiotic sensitivity[a]		LD_{50} for mice
	Sensitive	Nonsensitive	
Ileum	Chloromycetin 10 mcg. Neomycin 10 mcg. Kanamycin 10 mcg.	Erythromycin 5 mcg. Novobiocin 10 mcg. Penicillin 5 units Streptomycin 5 mcg. Tetracycline 10 mcg.	$> 6 \times 10^6$
Cecum	Same as isolate from ileum		$> 6 \times 10^6$
Rectum	" " " " "		$> 10^7$

Figure 23. Viscera from calf 17 showing areas of extensive hemorrhage and severe enteritis

Figure 24. Multiple hemorrhages on surface of heart of calf 17





prevalent in the rectum. Mesenteric lymph nodes were extensively damaged with necrosis of lymphoid cells, moderate depletion of lymphocytes, severe edema and increased phagocytosis. Hemorrhages were common in the cecal wall and there was much blood in the cecal contents. The liver and spleen were hyperemic. No visible lesions were noted in the kidneys or pancreas. The prefemoral, prescapular, submaxillary and atlantal lymph nodes showed moderate to severe edema and lymphoid depletion. In addition, the bronchial lymph nodes had slight purulent lymphadenitis. Hemorrhages on the surface of the heart were extensive (Figure 24).

Minor hemorrhagic areas were observed on the trachea, bronchi and lungs. Other organs appeared normal grossly and histologically.

Experiment 6

The two calves in this experiment were born naturally and permitted colostrum from their serologically BVD positive naturally infected dams. Both calves were weaned at 72 hours and then exposed orally to BVD virus.

Responses of calves

Daily leukocyte counts of both calves varied little from the leukocyte counts of calves exposed on day of birth in the previous experiments. Similarly, erythrocytes remained relatively constant as in previous studies. Daily body temperatures of both calves showed only slight elevations above

normal ranges during the postexposure period. Both calves experienced a mild but persistent diarrhea. Virus was isolated from the buffy-coat layer of blood and from nasal and rectal swabs of both calves. Attempts to isolate virus from calf 20 were successful up to 56 days postexposure. Similar attempts to isolate virus from calf 21 were successful up to 55 days. Virus could be isolated from the buffy-coat layer of the blood of both calves up to three weeks after isolations from the rectal and nasal swabs were no longer possible.

Although both dams had low colostral titers for BVD the calves were serologically negative at the time of artificial exposure. Within three weeks after exposure viral neutralization titers of both calves were greater than 256.

Immunologic Response

Calves which survived the neonatal exposure to BVD virus and elicited a serologic response were re-exposed to BVD virus at intervals from three to ten months after the neonatal exposure. The postexposure responses of these cattle are summarized (Table 17). Postexposure clinical responses were limited to fever and moderate leukopenia in most cattle. In others clinical responses were not detected. All cattle that were subjected to the re-exposure proved to be immune. There was no evidence of a syndrome resembling "immune tolerance" in any of the animals after re-exposure. Viremia was not detected in any of the cattle after 14 days from the time of re-exposure.

Table 17. Immunogenic response of calves after re-exposure to BVD virus

Exposure - re-exposure interval in months	Number of calves	Mean viral neutralizing titer when re-exposed	Persistence of virus after re-exposure (range in days)	Symptoms after re-exposure	Immune status
3	4	32,768	2 - 8	Fever, leukopenia	Immune
4	4	512	2 - 13	None	Immune
6	4	128	0 - 14	Fever, leukopenia	Immune
9	2	65,536	0 - 0	None	Immune
10	2	32,768	0 - 10	None	Immune

DISCUSSION

Early researchers (32, 36, 37, 71) on enteritis of neonatal calves concluded that E. coli was the sole etiologic agent of the syndrome. More recently, others (25, 28, 62, 69) have realized that this disease is more complex than was originally thought. The complex nature has been demonstrated when repeated attempts to reproduce the syndrome have failed. This has caused some researchers (52, 69) to speculate that perhaps other agents and environmental conditions contributed more to the severity of the disease than does E. coli. In contrast, other equally astute researchers have continued to list E. coli as the prime etiologic agent of calf scours (63, 73). After an exhaustive review of the literature and considerable original study, Gay (25) concluded that:

> There is still very little knowledge of the mechanisms by which E. coli produces these syndromes in calves or of the mechanisms by which the calf is protected from them.

Reisinger (63) concluded that the role of E. coli in calf scours was attributable to quantitative increments of the more pathogenic strains of E. coli in the upper portion of the small intestine. Multiplication of E. coli was accomplished at the expense of the relatively benign species of Lactobacillus. He postulated that E. coli could increase to such large numbers that even colostrum may not contain sufficient antibody to control the infection, or perhaps the colostrum did not contain antibody to the specific strain of E. coli now in predominance.

After an exhaustive study, Williams Smith (69) in England concluded that:

> Apart from one field case in which a virus was possibly involved, the evidence was against diarrhea in colostrum-fed calves being infectious in origin.

One problem encountered in this present experiment has been ascribing a role to the various E. coli isolates. It should be emphasized that E. coli was never isolated from rectal swabs of calves on the day of birth, but isolations were common after the second day of life. Isolations of E. coli were quite frequent also from the calves that survived. To what extent E. coli contributed to the symptoms of diarrhea and the fatal terminations in some calves can only be speculated. In several instances isolates with varying serologic properties and antibiotic sensitivity were obtained from different portions of the intestinal tract and tissues of the same animal at necropsy. Quantitatively fewer numbers of E. coli were isolated from the upper portion than the lower levels of the small intestines of all five calves which died of neonatal enteritis. Since no single serotype of E. coli was predominant, it may be concluded that none of the isolates contributed to the overall syndrome.

McClurkin (44, 45) and Moll (50) clearly showed the significance of viral agents in pneumonia-enteritis of newborn calves. However, Reisinger (63) concluded that:

> Although E. coli and a viral agent may both be incriminated in calf scours, E. coli alone can

cause the disease whereas the "virus" cannot cause scours in the absence of E. coli.

Such a hypothesis is extremely difficult to prove or disprove since a calf raised in the absence of E. coli is indeed "rare". Conditions under which such a calf must be maintained would seriously affect the validity of conclusions drawn from such work. Furthermore, the direct application of such work to the calf scours problem would be questionable.

Although there was considerable variation in the clinical responses of the calves after exposure to the BVD virus, the moderate temperature elevations, leukopenia and varying degrees of enteritis in all exposed calves confirmed the susceptibility of neonatal calves to the virus. Rapid deaths in less than 48 hours in some calves, and persistence of diarrhea up to 29 days postexposure in one calf emphasized the pathogenicity of the BVD virus for neonatal calves. Invasiveness of this virus for neonatal calves was demonstrated by its isolation from several organs and tissues of calves at necropsy. The potential of this virus to persist in tissues was revealed by isolations up to 103 days postexposure in surviving calves. The results suggest that strains of BVD virus may have a predominant role in the etiology of some types of enteritis of neonatal calves and that the role of E. coli may be of secondary importance.

The value of colostrum in preventing or ameliorating this syndrome is indicated by the fact that one of eight calves permitted colostrum died, whereas four of 13 colostrum-deprived

calves died of neonatal enteritis. It should be observed also that the one calf which died in the colostrum-fed group received colostrum from a serologically BVD negative dam.

It was hoped that this experimental design would clarify some of the relationships between bovine viral diarrhea and the phenomenon of immunologic tolerance associated with terminal cases of mucosal disease. A "partial" immunologic tolerance was observed in two calves. One was incapable of producing detectable levels of viral neutralizing antibodies until 73 days postexposure. Detectable levels of viral neutralizing antibodies were not observed in a second calf until 42 days postexposure. In all other animals antibodies were first detected between three and four weeks postexposure. However, one may speculate on what the results would have been from an immunologic standpoint if the methods of exposure were altered in respect to size of dose, route and frequency of administration and timing.

In conclusion, it may be stated that 1) the BVD virus had a significant role in the etiology of experimental enteritis of neonatal calves, 2) the extent to which this role was modified by the presence of *E. coli* remains for future studies, 3) a partial immunologic tolerant state was induced in two calves, but the specific mechanisms by which it was produced remained obscure.

SUMMARY

Research was initiated to determine the role of a bovine viral diarrhea (BVD) virus strain NADL-MD in the etiology and pathogenesis of enteritis of neonatal calves (calf scours). Twenty-one calves were utilized in the experiment.

Thirteen specific pathogen free (SPF), colostrum-deprived calves were exposed orally or intranasally on day of birth to the BVD virus. Four of the 13 calves had severe diarrhea and died of neonatal enteritis from 38 hours to 13 days postexposure. Isolations of BVD virus were made from several of the organs of the four calves at necropsy. All of the nine surviving calves experienced a moderate to severe diarrhea frequently persisting for seven to 10 days. Recovery of BVD virus was possible from one of the survivors up to 103 days postexposure.

Three other SPF calves were permitted dams' colostrum for the first four feedings then given milk replacer. These calves were exposed orally also on the day of birth to BVD virus. One calf experienced a severe diarrhea and died of neonatal enteritis 28 hours postexposure. The BVD virus was isolated from several tissues of the dead calf. The remaining two calves experienced a mild diarrhea persisting until the eighth day of age.

Three calves were from dams exposed intravenously to BVD virus at 6, 16, and 25 days prepartum, respectively. Although

permitted dams' colostrum, each of the three calves experienced a moderate diarrhea persisting until the eighth day of life. The BVD virus was isolated from all three calves and persisted up to 91 days in one calf.

An additional two calves were permitted colostrum from naturally infected dams for 72 hours, then weaned and exposed orally to BVD virus. Both calves experienced a mild persistent diarrhea and viral isolations were possible up to 56 days postexposure.

Several isolates of Escherichia coli were obtained from the calves after the second day of life. These isolates were neither pathogenic for mice nor related serologically to strains of E. coli usually associated with outbreaks of calf scours, nor were they related to each other.

It is concluded that the BVD virus should not be overlooked as a primary cause of the neonatal calf enteritis complex.

LITERATURE CITED

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APPENDIX

Hanks balanced salt solution (HBSS):

NaCl	8.00 grams per liter
KCl	0.40 " " "
$MgSO_4.7H_2O$	0.20 " " "
$Na_2HPO_4.H_2O$	0.06 " " "
Glucose	1.00 " " "
KH_2PO_4	0.06 " " "
$CaCl_2$	0.14 " " "
$NaHCO_3$	0.35 " " "

Earle balanced salt solution (EBSS):

NaCl	6.80 grams per liter
KCl	.40 " " "
$MgSO_4$	.10 " " "
NaH_2PO_4	.125 " " "
$NaHCO_3$	2.20 " " "
Glucose	1.00 " " "
$CaCl_2$	10.0 " " "
Lactalbumin hydrolysate	5.0 " " "

Eagle basal medium (EBM):

NaCl	6.8 grams per liter
KCl	.4 " " "
$NaH_2PO_4.H_2O$	.14 " " "
$NaHCO_3$	2.2 " " "

Eagle basal medium (EBM): (Continued)

$CaCl_2$	0.2	grams per liter
$MgCl_2.6H_2O$	0.17	" " "
Glucose	1.0	" " "
Arginine	.021	" " "
Cystine	.012	" " "
Histidine	.008	" " "
Isoleucine	.026	" " "
Leucine	.026	" " "
Lysine	.026	" " "
Methionine	.008	" " "
Phenylalanine	.016	" " "
Threonine	.024	" " "
Tryptophan	.004	" " "
Tyrosine	.018	" " "
Valine	.024	" " "
Glutamine	.300	" " "
Biotin	1.0	milligrams per liter
Choline	1.0	" " "
Folic acid	1.0	" " "
Nicotinamide	1.0	" " "
Pantothenic acid	1.0	" " "
Pyridoxal	1.0	" " "
Thiamin	1.0	" " "
Riboflavin	0.1	" " "
Phenol red	.04	" " "

GKN solution:

NaCl	8.0	grams per liter
KCl	0.4	" " "
Glucose	1.0	" " "

Trypsin solution:

Trypsin	2.0	grams per liter
NaCl	8.0	" " "
KCl	0.4	" " "
Glucose	1.0	" " "
$NaHCO_3$	0.84	" " "

Dulbecco phosphate-buffered saline (PBS):

NaCl	8.0	grams per liter
KCl	0.2	" " "
Na_2HPO_4	1.15	" " "
KH_2PO_4	0.2	" " "
$CaCl_2$	0.1	" " "
$MgCl_2.6H_2O$	0.1	" " "

Ammonium and potassium oxalate:

Ammonium oxalate	1.2 grams
Potassium oxalate	0.8 grams
Distilled water q.s.	100.0 ml.

Veronal-buffered saline:

NaCl	8.5	grams per liter
Na-5,5-diethyl barbiturate	.0375	" " "

Veronal-buffered saline: (Continued)

5,5-diethyl barbituric acid .0575 grams per liter

Add 0.5 ml of a stock solution containing 1.00 M $MgCl_2$ and 0.30 M $CaCl_2$.

Hematological Data on Experimental Animals

Data of cow 1

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
0	5,460,000	7,550	51	40	4	4	1
1	5,260,000	6,450	55	35	3	6	1
2	5,180,000	6,050	45	42	4	7	3
3	6,820,000	6,450	41	41	4	9	5
4	4,810,000	5,300	45	41	4	7	3
5	6,050,000	3,850	60	35	1	4	0
6	5,550,000	4,050	66	32	1	1	0
7	6,540,000	4,500	44	46	4	4	2
8	5,800,000	4,400	39	58	2	1	0
9	6,450,000	5,200	54	36	2	7	1
10	6,180,000	4,550	27	63	2	5	3
15	6,200,000	4,900	60	30	2	7	1
21	6,400,000	6,200	49	35	4	9	3
28	6,700,000	6,350	42	43	3	12	0

Data of cow 2

0	4,980,000	7,550	59	35	3	2	1
1	5,100,000	7,250	57	33	4	5	1
2	5,530,000	6,200	55	35	3	6	1
3	5,440,000	8,450	40	42	5	12	1
4	5,200,000	4,900	49	39	3	8	1
5	4,930,000	6,000	42	38	4	15	1
6	5,500,000	4,200	30	59	2	8	1
7	4,230,000	6,500	28	62	1	7	2
8	6,330,000	7,300	32	60	2	4	2
9	5,800,000	6,850	42	50	1	4	3
10	5,600,000	5,200	43	46	3	6	2

Data of cow 2 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
14	5,770,000	5,050	46	47	2	9	2
21	5,530,000	9,350	40	47	0	8	1

Data of cow 3

0	5,350,000	4,700	32	64	2	0	2
1	5,400,000	5,000	35	58	3	1	3
2	5,300,000	3,850	39	55	4	0	2
3	5,450,000	3,900	38	59	3	0	0
4	5,370,000	3,750	30	60	6	2	2
5	5,500,000	3,400	40	53	5	2	0
6	5,800,000	6,500	35	53	8	1	3
7	5,450,000	5,700	10	72	6	8	4
8	5,380,000	5,500	29	62	1	7	1
9	3,840,000	6,050	29	58	3	8	2
10	3,900,000	6,600	40	49	3	6	2
14	3,550,000	8,750	32	59	2	6	1
21	4,800,000	10,600	42	53	3	2	0

Data of calf 1

0	7,480,000	8,600	52	39	4	4	1
1	7,660,000	8,900	57	37	1	4	1
2	7,800,000	8,300	37	58	3	1	1
3	7,650,000	6,050	32	62	2	4	0
4	7,500,000	5,300	29	67	2	1	1
5	7,200,000	8,750	10	89	0	0	1
6	8,150,000	15,650	27	70	3	0	1

Data of calf 1 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
7	8,200,000	15,300	30	67	2	0	1
8	8,100,000	13,550	31	62	6	0	1
9	8,200,000	10,000	34	62	2	1	1
10	6,400,000	11,500	39	60	1	0	0
14	6,400,000	9,400	47	48	5	0	0
21	8,100,000	11,700	24	70	5	1	0
28	10,920,000	14,750	30	63	6	1	0
35	14,500,000	9,050	24	67	3	6	0
42	11,800,000	10,950	30	61	4	5	0
49	7,800,000	8,000	23	73	2	2	0
56	8,100,000	7,900	22	73	3	2	0

Data of calf 2

0	7,200,000	7,300	60	33	4	2	1
1	7,500,000	8,500	40	59	0	1	0
2	7,150,000	11,900	39	57	4	0	0
3	8,000,000	13,100	43	55	2	0	0
4	8,750,000	20,000	45	54	1	0	0
5	7,950,000	18,500	46	54	0	0	0
6	8,600,000	17,000	48	51	1	0	0
7	8,200,000	17,850	29	69	2	0	0
8	8,520,000	16,450	31	68	1	0	1
9	8,600,000	14,250	32	68	0	0	0
10	7,300,000	11,950	35	61	4	0	0
14	7,200,000	10,500	37	63	0	0	0
21	7,850,000	10,500	14	84	2	0	0
28	8,500,000	11,800	38	60	0	2	0
35	9,100,000	9,700	30	63	6	0	1
42	10,810,000	8,500	32	64	4	0	0

Data of calf 2 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
49	9,750,000	7,500	21	77	1	1	0
56	8,900,000	9,600	33	66	1	0	0

Data of calf 3

0	7,500,000	12,600	49	46	5	0	0
1	7,480,000	9,000	50	49	1	0	0
2	7,520,000	8,600	42	54	3	0	1
3	7,590,000	8,500	38	56	4	2	0
4	7,980,000	11,100	30	65	2	2	1
5	8,120,000	11,200	32	66	2	0	0
6	8,430,000	11,200	38	59	3	0	0
7	8,570,000	11,000	28	62	9	1	0
8	8,300,000	10,500	26	68	4	3	0
9	8,500,000	7,800	27	67	1	5	0
10	8,900,000	7,850	34	58	1	7	0
14	9,200,000	6,500	26	62	6	6	0
21	11,610,000	6,900	29	59	4	8	0
28	8,500,000	7,500	41	54	0	5	0
35	8,300,000	8,900	35	64	0	1	0
42	8,570,000	7,800	32	63	3	2	0
49	8,300,000	7,500	35	65	0	0	0
56	8,140,000	8,050	54	42	0	4	0

Data of calf 4

0	3,520,000	15,000	65	30	2	1	2
1	4,150,000	13,500	60	32	5	3	0

Data of calf 5

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
0	7,300,000	10,550	55	40	3	0	2
1	7,250,000	9,600	60	36	1	1	2
2	7,600,000	8,200	20	77	1	2	0
3	7,650,000	8,950	40	56	3	1	0
4	8,200,000	4,200	35	58	4	3	0
5	8,380,000	10,000	30	67	3	0	0
6	8,510,000	10,100	19	81	0	0	0
7	8,320,000	4,250	26	67	2	5	0
8	7,930,000	8,400	23	77	0	0	0
9	8,400,000	9,500	17	81	1	1	0
10	8,250,000	6,450	30	69	0	1	0
14	9,400,000	6,000	36	61	1	2	0
21	8,600,000	11,650	33	65	1	1	0
28	11,200,000	14,900	20	71	6	3	0
35	8,850,000	14,450	14	85	1	0	0
42	10,200,000	11,850	26	69	3	2	0
49	10,000,000	10,100	25	73	2	0	0
56	7,250,000	8,500	22	76	0	2	0

Data of calf 6

0	7,270,000	11,300	54	45	1	0	0
1	6,980,000	6,500	39	57	4	0	0
2	7,300,000	3,500	28	72	0	0	0
3	7,200,000	3,800	21	79	0	0	0
4	7,950,000	4,100	28	71	1	0	0
5	7,300,000	4,200	33	64	2	1	0
6	6,950,000	5,800	23	71	5	1	0
7	7,410,000	8,700	38	55	7	0	0
8	7,350,000	9,300	39	56	5	0	0

Data of calf 6 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
9	7,870,000	6,000	26	67	5	2	0
10	7,530,000	4,800	19	72	3	6	0
14	7,400,000	9,650	27	60	9	4	0
21	8,700,000	5,150	15	77	5	3	0
28	7,350,000	4,250	4	84	10	2	0
35	7,800,000	7,000	16	80	4	0	0
42	7,450,000	8,500	35	60	3	2	0
49	7,300,000	9,800	42	53	3	0	0
56	7,100,000	12,150	39	55	5	1	0
Data of calf 7							
0	5,700,000	8,400	64	33	2	1	0
1	5,390,000	6,000	50	48	2	0	0
Data of calf 8							
0	5,800,000	6,000	65	35	0	0	0
1	5,900,000	5,800	56	39	1	2	2
2	6,400,000	8,200	45	50	3	2	0
3	6,200,000	8,700	38	55	4	3	0
4	6,750,000	9,050	47	48	3	2	0
5	6,800,000	9,950	50	44	3	3	0
6	7,250,000	9,500	40	57	3	0	0
7	7,300,000	8,500	42	52	4	2	0
8	7,980,000	8,000	38	60	2	0	0
9	7,450,000	9,000	45	50	3	2	0
10	6,500,000	8,700	40	58	0	2	0
14	7,210,000	6,550	37	61	2	0	0

Data of calf 8 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
21	9,360,000	8,600	29	66	1	4	0
28	9,750,000	9,700	35	59	6	0	0
35	7,450,000	6,200	32	67	0	1	0
42	7,950,000	6,700	30	67	3	0	0
49	8,300,000	6,850	40	56	4	0	0
56	8,000,000	9,900	31	64	5	0	0

Data of calf 9

0	5,500,000	8,500	42	58	0	0	0
1	5,800,000	8,800	39	57	4	0	0
2	6,000,000	12,000	47	49	3	1	0
3	6,800,000	18,850	45	51	4	0	0
4	6,350,000	13,950	38	54	6	2	0
5	7,200,000	9,900	50	47	2	1	0
6	6,900,000	10,300	49	49	2	0	0
7	6,930,000	4,450	43	52	3	2	0
8	6,200,000	6,000	27	71	1	1	0
9	7,320,000	9,500	38	60	2	0	0
10	7,500,000	9,800	31	65	4	0	0
14	7,250,000	10,600	33	67	0	0	0
21	7,580,000	12,200	24	70	5	1	0
28	7,980,000	5,850	27	67	3	3	0
35	8,400,000	7,200	24	69	6	1	0
42	7,600,000	9,000	38	60	2	0	0
49	7,900,000	11,500	22	73	5	0	0
56	8,350,000	10,500	23	75	2	0	0

Data of calf 10

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
0	8,500,000	7,600	40	57	3	0	0
1	8,250,000	8,300	30	67	2	1	0
2	8,300,000	7,850	25	75	0	0	0
3	8,250,000	7,000	24	75	1	0	0
4	8,310,000	7,350	17	83	0	0	0
5	8,275,000	6,500	30	67	3	0	0
6	7,100,000	12,850	36	64	0	0	0
7	6,900,000	10,500	36	62	1	1	0
8	7,500,000	9,500	32	68	0	0	0
9	8,560,000	11,350	40	57	2	1	0
10	8,000,000	17,200	46	46	3	5	0
14	8,400,000	9,100	36	55	6	3	0
21	8,430,000	11,950	42	57	0	1	0
28	8,500,000	8,850	32	68	0	0	0
35	7,100,000	9,300	--	--	-	-	-
42	7,380,000	8,100	24	74	2	0	0
49	7,200,000	6,800	40	59	0	1	0
56	7,400,000	7,700	34	65	1	0	0

Data of calf 11

0	9,900,000	8,850	40	57	2	1	0
1	8,550,000	5,200	54	44	1	1	0
2	9,800,000	4,500	52	43	5	0	0
3	11,300,000	5,900	26	74	0	0	0
4	10,300,000	4,500	27	72	1	0	0
5	9,600,000	3,400	18	82	0	0	0
6	7,650,000	6,400	24	76	0	0	0
7	8,300,000	6,750	44	52	4	0	0
8	8,650,000	6,200	32	64	4	0	0

Data of calf 11 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
9	8,050,000	5,850	31	68	1	0	0
10	9,650,000	6,550	15	81	3	1	0
14	9,700,000	6,700	25	73	1	1	0
21	10,050,000	6,000	28	67	4	1	0
28	8,000,000	5,750	41	56	3	0	0
35	8,880,000	7,950	34	63	2	1	0
42	9,200,000	6,800	34	64	2	0	0
49	6,450,000	7,900	34	60	4	2	0
56	8,900,000	7,300	27	72	1	0	0

Data of calf 12

0	10,550,000	9,300	40	60	0	0	0
1	10,650,000	10,150	31	67	2	0	0
2	11,950,000	11,150	73	23	4	0	0
3	11,550,000	11,500	51	48	1	0	0
4	7,900,000	12,800	44	54	0	2	0
5	11,000,000	10,250	38	60	2	0	0
6	9,600,000	12,600	49	49	2	0	0
7	8,950,000	9,300	64	34	2	0	0
8	9,200,000	5,500	35	64	1	0	0
9	10,500,000	7,350	45	55	0	0	0
10	8,750,000	8,400	42	49	9	0	0
14	10,250,000	8,000	38	61	1	0	0
21	7,700,000	5,900	33	67	0	0	0
28	9,000,000	7,500	28	70	2	0	0
35	9,900,000	10,650	38	60	2	0	0
42	11,650,000	6,400	26	74	0	0	0
49	11,150,000	7,150	27	70	1	2	0
56	11,500,000	7,950	22	72	2	4	0

Data of calf 13

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
0	6,650,000	6,950	38	60	2	0	0
1	8,200,000	4,050	40	60	0	0	0
2	6,550,000	5,550	48	51	1	0	0
3	8,700,000	7,250	36	61	3	0	0
4	5,100,000	10,600	47	51	2	0	0
5	7,700,000	8,650	43	57	0	0	0

Data of calf 14

0	8,700,000	19,500	67	33	0	0	0
1	8,800,000	13,150	50	50	0	0	0
2	6,500,000	5,050	39	59	2	0	0
3	7,600,000	10,700	38	59	2	1	0
4	8,650,000	11,950	28	69	3	0	0
5	7,000,000	7,350	20	76	4	0	0
6	7,250,000	7,550	48	50	2	0	0
7	5,550,000	8,500	9	91	0	0	0
8	8,350,000	10,400	23	75	2	0	0
9	6,500,000	8,200	37	61	2	0	0
10	5,900,000	7,750	48	52	0	0	0
14	6,650,000	6,150	34	63	3	0	0
21	7,600,000	7,350	30	68	1	1	0
28	7,350,000	5,550	23	77	0	0	0
35	6,600,000	5,550	28	71	1	0	0
42	10,400,000	6,700	33	66	1	0	0
49	9,750,000	6,800	35	63	2	0	0
56	9,050,000	6,600	23	74	3	0	0

Data of calf 15

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
0	7,750,000	7,300	65	33	0	2	0
1	8,050,000	9,500	55	42	3	0	0
2	7,600,000	8,250	32	66	0	2	0
3	7,600,000	6,850	29	71	0	0	0
4	9,650,000	6,450	19	79	2	0	0
5	7,400,000	6,400	8	92	0	0	0
6	8,300,000	6,600	18	80	2	0	0
7	7,050,000	6,300	25	75	0	0	0
8	6,250,000	4,500	21	79	0	0	0
9	7,450,000	3,950	41	58	1	0	0
10	6,050,000	6,450	9	90	1	0	0
14	6,500,000	6,500	10	90	0	0	0
21	5,800,000	5,900	3	97	0	0	0
28	5,200,000	8,100	8	91	1	0	0
35	6,350,000	5,450	23	77	0	0	0
42	6,200,000	7,550	12	88	0	0	0
49	9,050,000	8,400	18	82	0	0	0
56	8,250,000	6,500	16	84	0	0	0

Data of calf 16

0	9,450,000	9,950	47	53	0	0	0
1	7,900,000	7,400	45	53	2	0	0
2	9,300,000	8,550	74	25	1	0	0
3	7,900,000	10,700	39	60	1	0	0
4	10,100,000	9,200	37	59	3	1	0
5	8,500,000	8,950	35	64	1	0	0
6	8,800,000	10,200	40	58	2	0	0
7	7,580,000	6,250	49	51	0	0	0
8	6,050,000	4,950	54	45	1	0	0

Data of calf 16 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
9	7,250,000	5,600	52	48	0	0	0
10	10,450,000	9,200	23	74	3	0	0

Data of calf 17

0	7,200,000	15,700	68	30	2	0	0
1	7,300,000	12,000	71	25	3	1	0
2	7,350,000	9,850	59	40	1	0	0
3	6,500,000	9,200	19	77	2	2	0
4	6,910,000	8,800	28	69	3	0	0
5	7,300,000	10,400	26	72	2	0	0
6	7,100,000	13,600	26	74	0	0	0
7	7,500,000	9,000	42	55	1	0	2
8	7,100,000	8,500	21	78	1	0	0
9	7,350,000	6,000	24	75	1	0	0
10	6,850,000	5,700	23	77	0	0	0

Data of calf 18

0	6,320,000	8,050	--	--	-	-	-
1	6,750,000	8,300	18	79	3	0	0
2	7,310,000	8,400	41	58	1	0	0
3	7,200,000	6,650	29	70	1	0	0
4	6,980,000	6,300	39	60	1	0	0
5	7,310,000	6,000	35	62	2	1	0
6	7,290,000	4,300	37	61	2	0	0
7	7,500,000	3,050	30	64	4	2	0
8	6,400,000	5,800	31	65	3	1	0
9	8,210,000	8,250	40	58	1	1	0
10	7,950,000	8,750	41	59	0	0	0

Data of calf 18 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
14	8,300,000	10,250	36	61	3	0	0
21	7,900,000	9,700	42	57	1	0	0
28	8,000,000	9,400	42	55	2	1	0
35	9,540,000	9,700	40	53	5	2	0
42	8,570,000	9,500	40	52	6	2	0
49	7,900,000	8,700	47	47	2	4	0
56	8,200,000	9,500	33	59	6	2	0

Data of calf 19

0	7,850,000	9,900	--	--	-	-	-
1	7,320,000	11,900	44	51	5	0	0
2	7,150,000	8,550	40	55	4	1	0
3	7,450,000	7,300	42	53	3	2	0
4	7,900,000	6,500	30	63	5	2	0
5	7,380,000	9,950	32	62	5	1	0
6	7,650,000	9,100	32	64	3	1	0
7	7,800,000	8,950	41	55	2	2	0
8	8,300,000	4,150	30	70	0	0	0
9	7,950,000	4,000	30	66	2	2	0
10	7,300,000	4,500	32	65	3	0	0
14	6,980,000	8,300	31	68	1	0	0
21	6,720,000	15,500	34	60	4	2	0
28	7,800,000	13,500	31	62	6	1	0
35	8,250,000	11,000	49	40	3	8	0
42	7,300,000	9,150	38	55	6	1	0
49	7,960,000	8,000	37	57	5	1	0
56	8,750,000	6,350	37	59	4	0	0

Data of calf 20

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
0	6,500,000	10,050	31	69	0	0	0
1	6,840,000	9,000	39	60	1	0	0
2	6,320,000	9,400	33	66	0	1	0
3	7,200,000	11,350	11	87	2	0	0
4	7,540,000	12,000	18	82	0	0	0
5	7,100,000	11,350	14	86	0	0	0
6	7,600,000	10,600	7	93	0	0	0
7	8,000,000	10,750	12	88	0	0	0
8	11,280,000	8,750	13	85	1	1	0
9	9,150,000	5,700	18	78	4	0	0
10	11,800,000	5,900	20	76	4	0	0
14	9,750,000	8,350	24	76	0	0	0
21	7,700,000	5,950	17	81	2	0	0
28	8,800,000	13,450	20	71	7	2	0
35	12,400,000	16,200	17	79	4	0	0
42	10,250,000	13,750	18	76	6	0	0
49	9,950,000	9,450	24	72	4	0	0
56	11,850,000	13,350	23	74	3	0	0

Data of calf 21

0	8,500,000	10,300	34	63	2	1	0
1	6,850,000	9,100	--	--	-	-	-
2	7,150,000	8,300	24	72	5	0	0
3	7,350,000	4,000	10	85	5	0	0
4	7,500,000	3,950	26	69	5	0	0
5	8,550,000	4,050	10	81	9	0	0
6	9,400,000	4,500	15	82	2	1	0
7	8,350,000	4,900	21	74	4	1	0
8	8,100,000	5,100	34	61	5	0	0

Data of cow 21 (continued)

Post-exposure days	Erythro-cytes	Leuko-cytes	Neutro-phils	Lympho-cytes	Mono-cytes	Eosino-phils	Baso-phils
9	8,570,000	6,500	21	77	2	0	0
10	8,400,000	6,050	24	71	2	3	0
14	9,800,000	6,400	30	70	0	0	0
21	10,200,000	8,100	23	74	2	1	0
28	8,000,000	10,350	21	75	2	2	0
35	11,500,000	7,100	23	72	3	2	0
42	7,600,000	8,550	24	74	2	0	0
49	8,150,000	7,150	22	73	1	4	0
56	8,500,000	6,600	21	75	4	0	0

Body Temperature Records of Three Cows

Post exposure days	Cow 1 AM	Cow 1 PM	Cow 2 AM	Cow 2 PM	Cow 3 AM	Cow 3 PM
0	101.8	101.6	101.8	101.2	101.4	101.4
1	102.0	101.8	101.6	101.6	101.0	101.4
2	101.6	102.2	101.6	102.0	102.2	101.8
3	101.8	102.6	102.0	101.0	102.0	102.4
4	102.2	102.6	102.0	102.0	102.0	101.6
5	102.4	102.4	102.0	101.8	101.4	102.0
6	105.2	102.0	102.2	102.2	101.8	102.2
7	102.4	101.8	102.4	101.6	101.6	101.8
8	102.2	103.2	102.0	102.2	102.0	102.0
9	102.0	101.8	102.0	102.2	102.2	101.4
10	102.0	101.6	102.2	102.0	101.4	101.4
11	101.6	101.8	102.4	102.0		
12			102.6	102.0		
13			102.2			
14			101.8			

Record of Body Temperatures of Calves

Post exposure days	Calf 1		Calf 2		Calf 3	
	AM	PM	AM	PM	AM	PM
0	101.8	102.6	102.0	102.0	101.6	101.2
1	103.6	103.6	103.4	103.6	101.6	101.6
2	102.4	103.2	103.4	104.0	102.8	102.8
3	102.4	103.4	103.6	104.0	102.6	102.8
4	103.6	103.4	104.0	103.6	102.8	102.8
5	104.0	103.8	103.0	104.0	103.1	102.8
6	102.2	102.6	103.4	103.6	103.0	102.2
7	104.2	104.0	103.6	103.4	102.0	102.2
8	103.6	103.6	103.6	103.4	102.8	102.6
9	103.8	103.8	103.6	103.4	102.8	102.8
10	104.0	104.2	103.4	103.2	102.8	102.8

Post exposure days	Calf 4		Calf 5		Calf 6	
	AM	PM	AM	PM	AM	PM
0	102.2	103.2	102.0	102.2	102.4	102.0
1	103.8		101.2	102.0	102.8	102.8
2	Death		103.2	103.2	103.0	103.6
3			102.2	102.4	103.4	103.8
4			102.4	102.4	104.0	104.6
5			101.4	102.0	103.6	104.0
6			101.8	101.4	103.4	103.6
7			101.8	101.0	103.4	103.6
8			102.0	102.0	102.6	103.0
9			102.4	102.0	102.4	102.8
10			102.2	102.0	102.2	102.8

Record of Body Temperatures of Calves (Continued)

Post exposure days	Calf 7		Calf 8		Calf 9	
	AM	PM	AM	PM	AM	PM
0	103.0	102.0	101.2	101.8	101.0	101.4
1	102.6	102.2	101.2	101.4	102.4	102.0
2	Death		102.6	102.6	103.2	102.6
3			103.0	102.4	102.8	103.6
4			101.4	102.0	102.4	102.0
5			102.6	102.0	102.6	102.0
6			101.6	101.8	102.8	101.6
7			101.6	102.4	102.6	102.4
8			102.2	102.2	102.0	102.0
9			102.6	102.0	101.8	101.8
10					102.2	102.0

Post exposure days	Calf 10		Calf 11		Calf 12	
	AM	PM	AM	PM	AM	PM
0	101.8	101.8	101.2	102.2	100.6	100.8
1	102.0	101.8	101.2	102.0	100.2	102.4
2	103.4	103.2	102.0	102.8	103.6	102.8
3	103.6	104.6	101.2	101.0	102.6	102.2
4	104.4	105.0	100.0	101.4	102.2	103.2
5	103.0	104.6	101.2	101.2	103.2	103.2
6	103.0	105.0	101.0	101.8	102.6	102.0
7	104.2	104.2	104.0	102.0	102.8	102.6
8	104.4	104.0	101.2	102.4	102.6	103.2
9	102.4	102.6	102.8	102.6	103.4	101.8
10	102.4	102.6	101.6	102.4	103.2	102.2

Record of Body Temperatures of Calves (Continued)

Post exposure days	Calf 13		Calf 14		Calf 15	
	AM	PM	AM	PM	AM	PM
0	100.4	102.6	102.2	101.2	102.4	101.4
1	102.2	101.4	101.8	102.0	101.2	101.6
2	101.8	102.2	102.4	102.6	102.6	102.8
3	101.8	101.4	102.8	102.0	102.0	101.8
4	102.0	103.4	102.0	102.2	101.8	101.6
5	102.2		102.4	101.8	104.6	101.6
6	Death		102.4	103.0	101.0	101.4
7			103.6	103.0	101.2	102.0
8			101.6	101.6	101.8	103.4
9			101.2	102.6	101.6	102.4
10			101.4	102.2	102.0	102.0

Post exposure days	Calf 16		Calf 17		Calf 18	
	AM	PM	AM	PM	AM	PM
0	102.0	101.4	101.0	101.4	102.0	102.2
1	101.4	101.4	103.0	103.0	101.8	102.0
2	103.0	103.2	103.4	103.4	103.4	103.2
3	104.4	103.8	103.4	103.4	103.6	102.8
4	103.6	104.6	103.0	102.6	102.0	103.4
5	104.4	103.8	102.4	102.4	102.2	102.0
6	105.2	105.0	102.2	103.4	102.0	102.0
7	104.2	104.6	103.6	103.6	102.0	102.0
8	105.4	103.6	102.0	102.4	102.0	102.8
9	101.6	102.6	103.0	103.6	102.4	102.8
10	102.0	102.0	102.0	102.0	103.0	102.6

Record of Body Temperatures of Calves (Continued)

Post exposure days	Calf 19		Calf 20		Calf 21	
	AM	PM	AM	PM	AM	PM
0	102.2	103.0	101.6	102.0	101.8	103.0
1	101.2	102.0	101.4	102.0	101.6	102.0
2	103.0	103.4	102.8	101.2	100.2	100.2
3	103.8	103.8	102.4	101.4	100.8	101.0
4	103.2	102.8	104.4	102.4	100.6	101.8
5	102.2	101.4	101.8	101.2	101.4	102.0
6	101.8	101.8	102.4	102.0	101.0	102.4
7	102.0	102.2	103.4	101.4	100.6	102.2
8	101.8	101.6	102.4	102.4	101.2	100.8
9	102.4	103.0	101.2	102.6	102.2	102.6
10	102.2	102.8	101.6	101.2	100.4	101.4