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STUDIES WITH AN UNCLASSIFIED VIRUS ISOLATED FROM DIARRHEIC CALVES

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ABSTRACT

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A transmissible agent (Breda agent) was isolated from a calf with diarrhea and shown to be infectious by inoculation orally into gnotobiotic and conventionally reared calves. The "Breda" agent had the morphology of a virus and possessed a hemagglutinin. Antigenic studies showed the virus to be antigenically different from bovine coronavirus, parainfluenza 3 virus, bovine rotavirus, bovine parvovirus and bovine pestivirus (BVD). Attempts to culture the virus in cell or organ cultures or in embryonated eggs, were unsuccessful. The virus was either spherical or kidney shaped, with 7—9 nm peplomers on the surface. A few particles possessed coronavirus processes of 17—20 nm, but these were arranged irregularly and were thought to be tissue debris. Three out of eight experimental calves developed severe diarrhea and the lesions in the small and large intestines were similar to those reported for coronavirus. The virus replicated in the jejunal and ileal regions of the small intestine and in the spiral colon, as judged by immunofluorescence. The virus multiplied in all experimental calves and was excreted in the feces; excretion correlating with the onset of diarrhea or a change in the appearance of the feces. There was little or no malabsorption measured by the uptake of D-xylose and the fact that infection of both the crypt and villus epithelial cells was observed, suggests that the pathogenesis may be different from rotavirus and coronavirus. Fourteen of fortyseven calves in the outbreak were infected with the virus, virus was not identified in other farm outbreaks of the disease.

INTRODUCTION

The pathogenic role of viruses in diarrhea of human beings, calves and other animals has been established. Rotaviruses have been isolated from diarrhea occurring in all animals studied, including human beings, and their pathogenesis determined experimentally in calves, pigs and mice (Mebus et al., 1971; Woode et al. 1974, 1978; reviewed by McNulty, 1978 and Flewett and Woode, 1978). Coronaviruses commonly cause diarrhea in calves (Mebus et al. 1973; Bridger et al., 1978). Although rotavirus and coronavirus are the

viruses most frequently associated with diarrhea in young calves, recently the presence of calicivirus-like agents and astroviruses (Woode and Bridger, 1978), and parvoviruses (Storz and Bates, 1973), have become recognized. Many outbreaks of the disease are associated with a combination of different viruses and pathogenic bacteria, and as an example of this it has been possible to identify from one calf with diarrhea at two days of age: *Salmonella dublin*, coronavirus, astrovirus, and three antigenically different calicivirus-like agents; most, if not all of these agents are known to be pathogenic (Woode and Bridger, 1978; Bridger and Hall, 1979). This finding is not surprising if one accepts the hypothesis that the fecal-oral route is the main method of spread of the agents. Thus the disease is complex and a continual search is being made worldwide for new viral pathogens causing scouring in animals. As a result of these studies, a new coronavirus pathogen has been identified recently in the pig (Pensaert and De Bouck, 1978).

Although 70—80% of diarrhea outbreaks may be identified as to their etiological cause, a percentage remains undiagnosed. Some of these may be due to such agents as chemical and fungal toxins but other viral agents may be present which are yet to be identified.

This paper describes the isolation of a new transmissible agent, thought to be a virus, present in an acute epizootic of calf diarrhea which could not be diagnosed etiologically as due to any of the known viral pathogens. By use of the recently described method for determining the presence of coronavirus hemagglutinin in diarrheic feces (Van Balken et al., 1978) a number of calves from this outbreak were shown to have in their feces at high titer, a hemagglutinin which was antigenically distinct from coronavirus and a particle observed by electron microscopy with a morphology different from the other enteritis inducing viruses known to infect the bovine species.

MATERIALS AND METHODS

Source of virus

A beef herd in Iowa had experienced severe neonatal calf diarrhea for at least three years, in which the mortality rate was high during excessively cold weather. No etiological agent had been associated with the majority of affected calves, although an occasional isolation was made of rotavirus or coronavirus. During the period from 25 March—1 May, 1979, a detailed investigation was made in an attempt to identify the presence of a viral agent. Of the first 69 calves born, 39 developed diarrhea and 6 died. The remaining 30 calves remained normal. Diarrhea commenced at any age from 2—20 days, but usually at 3—5 days. The clinical syndrome was usually severe and similar to that seen with rotavirus or coronavirus infections. There was yellow to white semisolid or watery diarrhea, profuse in quantity, rapidly leading to severe dehydration. The first 69 calves born were all housed immediately after birth and 66 placed in direct or indirect contact with other

scouring calves, as diarrhea commenced with the third calf born. This practice was thought to have contributed to the rapid spread of infection and development of disease at an early age. However, from 13 April the calves were born and reared at pasture, but this practice did not eliminate the disease. From 13—19 April, 12/30 calves born developed diarrhea and three died after 3—5 days duration of diarrhea. Between 22 April and 1 May, 29 calves were born, all of which remained clinically normal. Diarrheic samples were studied by electron microscopy for the presence of viruses. One preparation (B276) had a large number of viral-like particles which were described as atypical coronavirus. This agent was shown to be different from coronavirus by serological tests, and further studies were made in order to characterize the agent as a virus, which was named "Breda" agent after the town in Iowa from which area it was isolated.

Animal inoculation

A sample of a diarrheic feces from calf B276 (the "Breda" agent) was diluted 1:3 (*v/v*) in phosphate buffered saline (PBS), pH 7.2, centrifuged at 6000 *g* and the supernatant fluid passed through a 0.45 μm membrane filter. Gnotobiotic calves (GC) were produced and maintained using a method described recently, modified by an open caesarean method (Matthews et al., 1981). Following caesarean section under deep general anesthesia, the calves were exposed briefly to the air of the operating theater, while being passed through a germicidal trap containing 10% Wescodyne (Wests Chemical Production). The calves were inoculated orally with 5 ml of the 0.45 μm filtrate at 0—8 days of age. Colostrum-deprived (CD) new born calves were placed in isolation and inoculated as above. Two calves (CF) were fed colostrum, one 8 h before (CF 2) and one 7 h after (CF 1) virus inoculation. Fecal samples and the body temperature were collected and recorded respectively twice daily. For pathological studies, the calves were killed either at the first sign of diarrhea or 24 h later (Calves CD 1, CD 2, and GC 1).

The above preparation of the "Breda" agent was inoculated orally (0.1 ml per rat) into two litters of new-born rats and intracerebrally (0.01 ml) into three litters of new-born mice. The rats and mice were observed for clinical signs and fecal samples were removed daily for testing for the presence of the agent.

D-xylose absorption test

The method used has been described previously (Woode et al., 1978). Seventeen hours after the last feed, the calves were given by mouth 25 g of D-xylose (Fisher Scientific Co.), dissolved in 500 ml of water, corrected to pH 5.5 with HCl and sterilized by autoclaving at 120°C for 60 min. Blood samples were withdrawn into tubes containing EDTA at 1 and 2 h pi. The plasmas were separated and deproteinized: 0.4 ml of plasma was diluted in

2.8 ml of water and 0.4 ml of 10% solution (*w/v*) of $ZnSO_4 \cdot 7\ H_2O$ and 0.4 ml of 0.5 N NaOH were added. One ml of the supernatant fluid was added to 5 ml of 2% (*w/v*) P-bromoaniline (anhydrous, grade 1; Sigma Chemical Co.) in glacial acetic acid and saturated with thiourea (Fisher Scientific Co.). The tubes were held at 70°C for 10 min and then cooled and held at 20°C for 70 min in the dark. The reaction was read in a spectrophotometer (Coleman Junior) at 520 nm. Control tubes included a standard that consisted of 10 mg D-xylose in 100 ml benzoic acid, a water "blank" and an unheated mixture of each plasma sample with the bromoaniline stain. The concentration of D-xylose in blood was calculated as mg/100 ml of blood. The reduction in absorption rate when calves had diarrhea was expressed as a percentage of the normal absorption rate.

Antisera

Serum was taken from the experimentally infected gnotobiotic calf, GCO, before inoculation and 27 days later. Antisera were prepared also in guinea pigs by intramuscular inoculations of 10^6 HA units of "Breda" agent in 0.1 ml volumes twice a week for three weeks. None of the preinoculation sera contained antibody to the "Breda" agent.

Antiserum to bovine coronavirus, kindly supplied by Dr. C.A. Mebus, had a titer by immunofluorescence (IF) assay of > 160 to coronavirus and neutralized 3072—6144 coronavirus HA units. Rabbit antiserum to bovine coronavirus and conjugated with fluorescein was kindly supplied by Dr. Bass of Norden Laboratories. Bovine rotavirus antiserum prepared in a gnotobiotic calf was kindly supplied by Dr. A. Torres-Medina; this antiserum had a neutralizing antibody titer of 2560 with bovine rotavirus. Antiserum to the bovine parvovirus, strain HADEN, was prepared in a rabbit and had a HI titer of 1024 with the homologous virus. Antiserum to parainfluenza 3 (PI3) virus, kindly supplied by Dr. R. Van Deusen, National Animal Disease Center (NADC), had a titer of 160 with the homologous virus.

Hemagglutination and hemagglutination inhibition

A modification of the method for cornavirus identification in calf feces (Van Balken et al., 1978), was used for the detection and identification of both bovine coronavirus and the "Breda" agent in feces. Serial two-fold dilutions of the fecal samples diluted 1:3 in PBS and centrifuged at 6000 *g* were made in 10 μl of PBS. Fetal calf serum was diluted 1:5, adsorbed with packed rat erythrocytes until all spontaneous hemagglutination was lost and 10 μl of a 1:40 dilution added to each well. Ten μl of 1% rat erythrocytes in PBS with 0.1% bovine serum albumin were added to each well and incubated for 1.5 h at 20°C. For antigenic identification of the "Breda" agent preinoculation serum samples and antisera prepared against the agent in guinea pigs and in calf GCO were absorbed with rat erythrocytes, diluted

1:40 with PBS and used in the above test in place of the fetal calf serum. A reduction in HA titer of 64 fold or greater by antisera when compared with the titer in the presence of non-immune preinoculation serum or sera prepared against bovine coronavirus, parvovirus and PI3 virus, was taken as evidence of antigenic specificity. This was called the HAHI test. The same method was used for the identification of coronavirus, with bovine coronavirus antiserum kindly supplied by Dr. C. A. Mebus. For the hemagglutination inhibition (HI) test for determining antibody in field and experimental serum samples, two-fold dilutions of sera were made in PBS in 10 μl quantities. Ten μl of eight HA units of virus were added, the mixtures agitated and incubated for 30 min at 20°C. Ten μl of 1% rat erythrocytes were then added to each well and the test incubated for 1.5 h at 20°C. All serum samples were adsorbed with rat erythrocytes prior to testing. A HA unit was defined as the highest dilution of the sample showing hemagglutination and was expressed as the reciprocal of the dilution commencing with 1:6.

Adsorption and elution of "Breda" agent with rat erythrocytes

Rat erythrocytes were washed three times with PBS at 4°C and 0.2 ml of packed cells were mixed with 0.2 ml of "Breda" agent containing approximately 200,000 HA units/10 μl. The mixture was incubated for 30 min at 20°C and 30 min at 4°C. The cells were then washed six times in 14 ml of cold PBS (4°C). The cells were finally resuspended to 0.5 ml in PBS and incubated at 36.0 ± 1°C for 90 min. The cells were pelleted at 1000 rpm and the supernatant removed.

Tissue culture of virus

Primary calf kidney (CK cells), primary bovine thyroid cells (CTh), human rectal tumor cells (HRT_{18}) and Madin Darby Bovine Kidney cells (MDBK) were prepared in tubes with flying coverslips. The growth medium was Eagle minimum essential medium (MEM) with 0.25% lactalbumin hydrolysate and 10% fetal calf serum. Prior to infection of cultures with virus, cells were washed twice with the medium devoid of serum and after inoculation maintained in this medium for 7—14 days. The virus preparation was diluted tenfold in serum-free MEM and dilutions from 10^{-0}—10^{-7} inoculated into tubes. Cultures were observed for the development of cytopathic effects (CPE) and tested for the presence of hemagglutinin. They were fixed at 24 h intervals for immunofluorescence (IF) studies. The cells were then passed serially three times with 0.2% trypsin, into both established monolayer cultures or new monolayer cultures at seven days and fourteen days post infection. Each passage was examined at seven days for the presence of virus by HA, IF and CPE. In addition, a preparation containing 200,000 HA units of the "Breda" agent was treated with 500 μg of trypsin at 37°C for 30 min, prior to inocula tion into cell culture. The media for these cultures were then supplemented

with 50 pig trypsin (Difco). The cultures were studied for the presence of viral HA, IF and CPE. The virus preparation used was shown to be infectious by inoculation into the experimental calves.

Egg inoculation

Ten-day-old chicken embryos were inoculated by the allantoic route with 0.1 ml dilutions of virus in "serum free" MEM. Allantoic fluid was harvested at daily intervals and the embryos examined for gross lesions seven days post infection. The allantoic fluids were tested for the presence of viral HA. Two further passes of the fluids were performed in 10-day-old embryos. Trypsin treated virus, as above, was inoculated also into 10-day-old chicken embryos.

Organ culture

Bovine tracheal organ cultures, prepared using the methods previously described by Bridger et al. (1978), were inoculated with ten-fold dilutions of "Breda" agent. As controls, bovine coronavirus also was cultured. Medium was removed at intervals and tested for the presence of viral HA.

Immunofluorescence

(1) *Gut ring sections*, 1—4 μm thick; frozen sections were fixed in acetone for 10 min and air dried. A 1:40 dilution of the gnotobiotic calf antiserum to the "Breda" agent (GCO) was applied for 1 h, the section washed and then reacted with fluorescein-conjugated rabbit antiserum to bovine immunoglobulins (Cappel Laboratories). As controls, sections were stained with pre-inoculation GCO calf serum and conjugate, and conjugate alone, and the fluorescence compared. Fluorescent cells, thought to be lymphocytes, were demonstrable in the lamina propria but not in the epithelial cell region. The sections also were reacted with the rotavirus antisera by the above method, with the bovine coronavirus conjugated antiserum, by the direct immunofluorescent method, and with goat antiserum to PI3 virus, and with fluorescein conjugated rabbit anti-goat IgG sera kindly supplied by Dr. Prem Paul, NADC and Cappel Laboratories.

(2) *Tissue culture coverslips*, inoculated with the "Breda" agent and with cell culture passes, were removed at 24 h intervals for up to seven days, washed in distilled water, air dried, fixed in acetone for 10 min and reacted with the antisera following the methods described in (1) above. HRT_{18} cells infected with two isolates of bovine coronavirus were fixed for immunofluorescence after 72 h.

Electron microscopy (EM) and immunoelectron microscopy (IEM)

The fecal hemagglutinin preparations were pelleted at 100,000 *g* for 1.5 h in a Beckman L5-65 ultracentrifuge. The pellet was resuspended in a few drops of water. One drop of the pelleted virus preparation was mixed with 15 drops of water, one drop of 1% bovine serum albumin and two drops of 4% potassium phosphotungstate, pH 6.8. After 5—10 min incubation, the mixture was sprayed onto carbon-collodion coated grids and examined with an electron microscope. For immunoelectron microscopy, the viral preparation was incubated with an equal volume of a 1:5 dilution of antiserum in PBS at 37°C for 60 min and overnight at 4°C. The serum—virus mixture was pelleted at 100,000 *g* for 60 min and resuspended and stained as above. The measurement of particles was obtained by mixing tobacco mosaic virus (TMV) with the fecal virus preparations. Actual magnification obtained on electron micrographs was calculated based on the 18 X 300 nm dimensions of TMV.

Pathology

Tissues for histopathological examination were removed under pentobarbitone anesthesia. A short portion of intestine from the anterior, middle and posterior (50 cm from the ileo-caecal valve) regions of the small intestine and from the spiral colon, were opened and laid flat on cardboard before plunging into 10% buffered formalin solution. Other organs fixed with formalin included abomasum, liver, kidney, lung and bladder. Two or three blocks of each fixed tissue were dehydrated and embedded in paraffin wax; sections were cut at 5 μm and stained with hematoxylin and eosin.

Field survey for the presence of "Breda" agent and coronavirus in herds

As part of the survey into the etiology of calf scours, diarrhea samples were collected from a number of herds in which severe diarrhea had occurred, and these were tested for the presence of bovine coronavirus and the "Breda" agent by hemagglutination, the specificity of the HA being determined with the specific antisera for each virus (HAHI) and by EM studies.

RESULTS

Clinical signs of infection in experimental calves

The three colostrum-deprived calves (CD 1, CD 2 and CF 1) developed diarrhea at 24, 28 and 72 h post infection respectively. The feces changed in color from dark brown to orange/yellow and shortly afterwards to a brilliant yellow, profuse in quantity and watery in consistency. CD 1 was acutely depressed, shivered constantly, and for the next 18 h refused food,

remained depressed, stood with difficulty and appeared dehydrated and weak when the autopsy was performed 48 h pi. Calf CD 2 at 23 h pi was depressed, shivered frequently and was unable to stand without assistance. The calf was anesthetized for post mortem examination at 28 h pi, at which time diarrhea was first observed. The third calf, CF 1, developed hyperpnea (112 respirations/min) 24 h pi, which declined to 80 respirations/min at 40 h pi and remained at that level for the remainder of the calf's life. The diarrhea was severe in this calf, yellow in color and watery in consistency and dehydration, as judged by the skin pinch test and sunken eyes, became apparent within 72 h pi. The calf refused one feed each day from 24 h pi and remained acutely depressed. At 72 h pi, a bilateral watery eye discharge developed and finally at 94 h pi the calf was unable to stand, refused feed and died at 96 h pi. The other colostrum-fed calf (CF 2) remained clinically normal throughout the experimental period, although the feces became yellow in color which correlated with appearance of viral hemagglutinin in the feces (Table I).

TABLE I

Experimental Calves Infected Orally with Fecal Filtrates of "Breda" Agent

Calf No.	Origin	Age Inoculated (h)	Age Diarrhea commenced[a] (h)	Clinical Severity[b]	Pyrexia	Mal absorption[c]	Days Virus excreted[d]	Age autopsied (h)	Fate
1	CD1[e]	4	24	4	–	NT	1	44	S[i]
2	CD2	6	28	?	–	NT	1	28	S
3	CF1[f]	5	36	4	+	—	4	96	D[j]
4	CF2	96	168	1	–	NT	3—4	ND	S
5	GCO[g]	192	216	2	+	—	5	ND	S
6	GC1	48	72	4	+	—	2	120	S
7	GC2	72h	96	1	–	NT	4	ND	S
8	GC3	24	72	1	+	NT	5	ND	S

a Diarrhea defined as changes in fecal color, fluid consistency and volume.
b Scale 1—4 of increasing severity based on clinical signs of dehydration and anorexia.
c Malabsorption. A reduction of D-ylose absorption of 60% or greater.
d EM and HA determination.
e Colostrum-deprived
f Colostrum-fed.
g Gnotobiotic calf.
h Inoculum: fecal filtrate from calf No. 6.
i S: survived until euthanised.
j D: Died.

The first gnotobiotic calf (GCO) inoculated at eight days of age, developed a mild scour 24 h pi, which was green in appearance and changed to a semi-solid with flecks of yellow 24 h later. This calf returned to normal within 24 h and remained in good health until autopsied 27 days later. Calf GC 1, which was inoculated at two days of age, developed a profuse scour 72 h pi, with yellow floccules floating in a whey-like fluid. At 96 h, the fecal motions changed to semi-solid in consistency and greenish yellow in appear-

ance but at 120 h watery profuse diarrhea reoccurred. This calf retained its appetite, but when autopsied at 120 h pi it had lost weight, was dehydrated as judged by a skin pinch test and the ribs were prominent and the eyes sunken. GC 2, which was inoculated at 72 h of age, did not develop diarrhea and remained in good health although the semi-solid greenish/yellow colored feces increased in volume until seven days pi at which time the fecal samples were difficult to obtain. Calf GC 3 was inoculated at 24 h of age with the virus filtrate diluted 1:100 and at 48 h pi. The feces changed from dark green to orange-yellow in color. Nine hours later the feces were very loose, mucoid and contained yellow floccules but at 72 h pi, it became semi-solid and greenish-yellow. Anorexia was not observed (Table I).

All the new-born rats and mice remained clinically normal for three weeks following inoculation. Two litters of mice and their dams developed diarrhea at three weeks together with a control litter. However, the "Breda" agent could not be detected in the feces or intestinal contents of these animals.

D-xylose absorption studies

Studies were not performed on CD 1, CD 2, CF 2, GC 2 or GC 3. Calf CF 1 was inoculated at birth and thus no normal absorption rates were obtained, but when in severe diarrhea, it had blood levels of D-xylose at 1 and 2 h post feeding of 65 and 75 mg/100 ml, respectively. These are similar to normal levels for non-diarrheic calves. Calf GCO, with mild diarrhea, showed reductions of D-xylose absorption at 1 and 2 h, when compared with pre-inoculation absorption rates of 35% and 16% respectively, and calf GC 1, with severe diarrhea, reductions of 53% and 42% from preinoculation absorption rates. Studies with rotavirus showed reductions of 60—100% in the absorption rate (Woode et al., 1978).

Hemagglutination and hemagglutination inhibition

In attempts to determine the optimal temperature for hemagglutination, the standard preparation of B276 was titrated and incubated for 1.5 h at 4° C, 20°C (room temperature) and 37°C. However, no difference was observed in the titer at the three temperatures. The hemagglutinin assay was read at 1.5 h but was stable for a further 20 h at room temperature. No agglutination occurred when the viral hemagglutinin was reacted with human group O, bovine, hamster, guinea pig, chicken, turkey or goose erythrocytes.

The HA titers of feces from experimental calves before inoculation were 3.68—6.6 $\log_2$. Fecal samples obtained from naturally infected and all the experimentally infected calves with diarrhea had HA titers which peaked in different calves between 11.6 $\log_2$ and 25.6 $\log_2$. Hemagglutinin was detectable for 3—4 days (Table I).

The guinea pig and calf antisera diluted 1:10, generally reduced the HA titer of virus to the same titer as in the preinoculation samples from the same

experimental animal, or when the HA titer was high, as with B276, neutralized 15.6—16.6 $\log_2$ HA units. In contrast, antisera prepared in gnotobiotic calves against bovine coronavirus and bovine rotavirus only neutralized 1—3 $\log_2$ HA units, guinea pig antisera to bovine parvovirus neutralized 1—2 $\log_2$ HA units and goat antiserum to PI3 virus neutralized 3—4 $\log_2$ HA units, of preparation B276. Dr. McClurkin, NADC, was unable to isolate BVD virus from the B276 inoculum, and the antiserum prepared in calf GCO did not have IF antibodies to BVD.

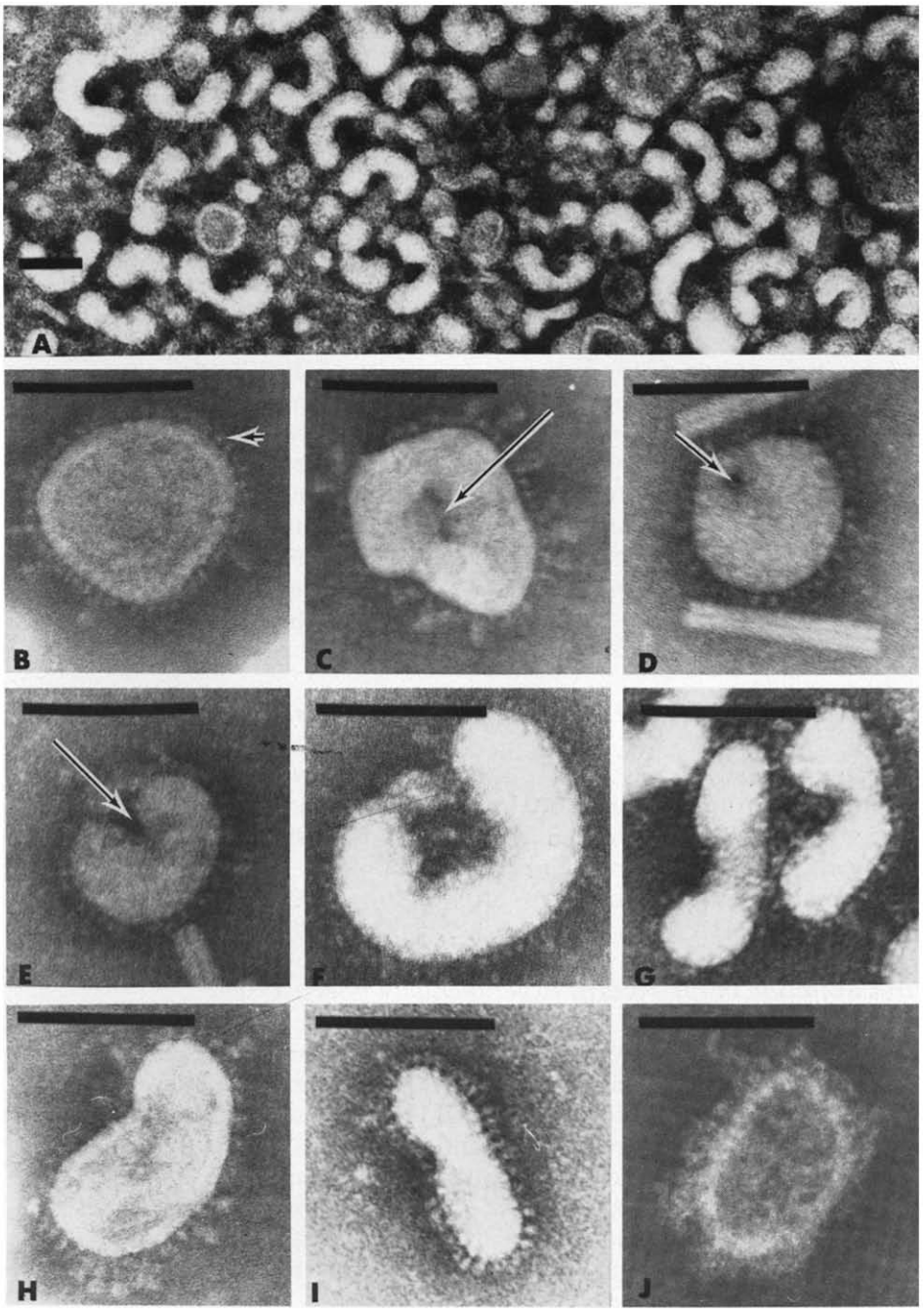
The HI titer of serum prepared in GCO against eight HA units of B276 rose from a preinoculation titer of 2.5 to 6.6 $\log_2$, 27 days pi. In contrast, most field sera had HI titers ranging from less than 2.6 to 5.6 $\log_2$. Six to eight serum samples were obtained from each of four unrelated herds and the HI titers determined. The range of titers in Herd I were 3.6—5.6 $\log_2$, Herd II 4.6—6.6 $\log_2$, Herd III 2.6—3.6 $\log_2$, and Herd IV 3.6—4.6 $\log_2$. Herd I animals were those which included calf B276 in the "Breda" herd. The convalescent serum of this animal neutralized approximately 6—7 $\log_2$ HA units of virus at a 1:10 dilution and had a titer of 3.6—4.6 $\log_2$ against eight HA units. In two adult heifers, which were inoculated orally and twice weekly subcutaneously with 17.6 $\log_2$ HA units of virus, the preinoculation HI titers were 4.6 and 5.6 $\log_2$ respectively and rose to 5.6 and 6.6 $\log_2$ respectively after 21 days.

In attempts to remove from sera the low level of "non-specific" inhibition of hemagglutination, the sera were treated with kaolin and/or heat inactivated at 56°C/30 min. Both treatments reduced the non-specific inhibitory titer two-fold but also reduced the specific HI titer by the same amount. It was considered that the HI test was an unreliable method for the determination of antibodies to the "Breda" agent in field animals, largely due to the apparently poor antigenic response, as measured by this test, to natural infection and the small difference between the HI titer of "negative" sera and the convalescent titer.

Electron microscopic studies

The agent had two distinct morphologies, approximately spherical and sausage shaped, which frequently was curved to resemble a kidney shape (Fig. 1). The spherical particle measured 89 ± 7 nm × 75 ± 9 nm with

Fig. 1. Ultrastructural morphology of the "Breda" virus in potassium phosphotungstate negative stain preparations, from calf fecal specimens. Bar = 100 nm. A. Original calf sample of "Breda" agent. Numerous kidney shaped particles are evident. B—H. Various round and kidney shaped forms of the "Breda" virus. B. Arrow indicates short projections (7.6—9.5 nm). Longer projections are occasionally seen, thought to represent adherent tissue debris. C—H. demonstrate the relationship of the two particle types. Arrows indicate cleft in which stain has penetrated in spherical particles (C,D and E) and larger clefts of kidney shaped particles (F,G,H). Rods in CDE are portions of TMV. I. Similar particle observed in the feces of one diarrheic calf from South Dakota. J. Immunoelectron microscopy of the "Breda" virus with convalescent serum of calf GCO.


A
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peplomers 7.6—9.5 nm long. The other particles measured 120 ± 15 nm × 32 ± 8 nm with similar peplomers. A minority of particles possessed poorly defined peplomers similar in size and shape to coronavirus (17—24 nm). An electron-dense indentation was frequently observed in the center, or slightly off center, of many of the spherical particles. The proportion of kidney shaped to spherical particles varied considerably between samples.

There was an excellent correlation between the presence of particles observable by EM and the presence of specific hemagglutinin in feces of both experimental and naturally infected animals, with the exception of calves CF 1 and CF 2. These had low HA titers (11.6 $\log_2$) and the characteristic particles were not readily observed.

As a confirmation of the antigenic specificity of the "Breda" virus, immunoelectron microscopy was performed on bovine coronavirus in a fecal preparation and two different preparations of "Breda" agent (from B276 and CD 1), with antisera against coronavirus and "Breda" agent. Both sera coated and agglutinated their homologous viruses, and coronavirus antiserum coated but did not agglutinate the "Breda" agent particles. In contrast, the "Breda" antiserum neither coated nor agglutinated coronavirus. A mixed preparation of the two viruses, when reacted with each antiserum, also showed these effects. The "Breda" agent coated with homologous antiserum is shown in Fig. 1.

Adsorption and elution of the "Breda" agent with rat erythrocytes

Bovine coronavirus adsorbed to the rat cells and was eluted after 90 min at 36.0 ± 1°C. Approximately 7.0 $\log_2$ HA units of coronavirus was adsorbed and 6.0 $\log_2$ HA units recovered after elution. No HA was observed in the final washing solution prior to incubation at 36.2°C. In contrast, no elution was observed by the "Breda" virus after 90 min at 36.0°C, but after 16 h at 36.0°C, 8.0 $\log_2$ HA units were released together with lysis of the red cells.

Virus excretion in the feces of experimentally infected calves

Fecal HA titers before virus inoculation were 2.6—5.6 $\log_2$ and rose after 24—48 h coinciding with the development of diarrhea or a color change of the feces. The fecal HA titers peaked at 11.6—12.6 $\log_2$ in calves GC 1, CF 1 and CF 2, at 18.6 $\log_2$ in calves GCO and GC 2 and 14.6 $\log_2$ with calf GC 3. The highest HA titer observed (25.6 $\log_2$) occurred in the feces obtained from the naturally infected calf B276. No correlation was observed between titer of HA in the feces and severity of infection, but the number of particles observed by EM corresponded approximately with the HA titer.

Virus excretion in the feces of experimentally infected rats and mice

The "Breda" agent was not detected in the feces of the new-born rats or

mice or their dams at any time post infection and the diarrhea developing at three weeks appeared to be related to other causes.

Histopathology

CD 1: Villi of the jejunum and ileum were atrophic and small patches of villous epithelium had desquamated from villous tips. Mixed cellular and fibrinous material was exuding into the lumen from the denuded areas. Most of the villous surface was covered by irregular squamous to cuboidal cells with basophilic cytoplasm. There were occasional synechiae between villi. Villous lamina propria was congested and contained abundant nuclear debris. Fresh hemorrhage was observed in occasional Peyer's patch lymph nodules most of which were hypocellular centrally. The surface epithelium of the colon was irregular and contained abundant cellular debris, some of which accumulated in the surface lamina propria. The abomasum was congested and had multifocal, small submucosal hemorrhages. Jejunum, cecum, rectum, liver, and kidney were not remarkable.

CD 2: The appearance of the villi and villous epithelium in the small intestinal sections was unremarkable. The colonic mucosa had many villous folds and dilated crypts. The lungs contained multifocal petechial hemorrhages and vesicular emphysema.

GC 1: The ileum had mild villous atrophy characterized by shortened villi covered with basophilic, cuboidal epithelium, and some areas of elongated crypts. A diffuse, low-grade neutrophilia was observed in the lamina propria and submucosa. Most Peyer's patch lymph nodules were hypocellular centrally. Dilated crypts and scattered crypt abscesses were present in all sections of the small intestine. Surface epithelium of the spiral colon had areas of necrosis. The spiral colon also had dilated crypts and diffuse neutrophilia of the lamina propria. Liver, kidney, and urinary bladder were unremarkable.

There was no evidence of bacterial colonization in any of the histologic sections.

Immunofluorescent studies

Calves CD 1, CD 2 and GC 1 were examined for specific immunofluorescence (IF) in cells of the small and large intestine. Calf CD 2, showed the least tissue damage and the most extensive evidence of infection by IF. Sections of the mid gut (jejunum) and ileum showed IF positive epithelial cells, mainly in the lower 50% of the villus and extending deeply into the crypt area. Scattered individual cells of the upper part of the villus also fluoresced specifically. No IF positive cells were observed in the epithelium of the anterior small intestine. Specific IF in cells was observed throughout the length of the fold of the spiral colon including the crypt cells, and most of the folds were involved. Calves CD 1 and GC 1 were examined 24 h after

the commencement of diarrhea and consequently there was more villus damage. Immunofluorescent epithelial cells were randomly scattered among the villi and crypt areas of the jejunum, ileum and spiral colon. No IF was observed in these sections when reacted with bovine coronavirus or bovine rotavirus antisera (Fig. 2 a, b, c).

Coronavirus infected HRT_{18} cells immunofluoresced specifically with coronavirus antiserum but no fluorescence was observed at a 1:20 dilution with GCO convalescent antiserum.

Farm survey for the presence of bovine coronavirus and "Breda" agent in feces

From the Breda herd, 47 diarrheic calves were sampled at least once and a few sampled two to four times. Fourteen were shown to be infected with the "Breda" virus by HAHI and eleven by EM, with three doubtful positives. One calf was infected with coronavirus, one with rotavirus and one possibly with all three agents.

A total of 339 fecal samples from a variety of farms other than the Breda

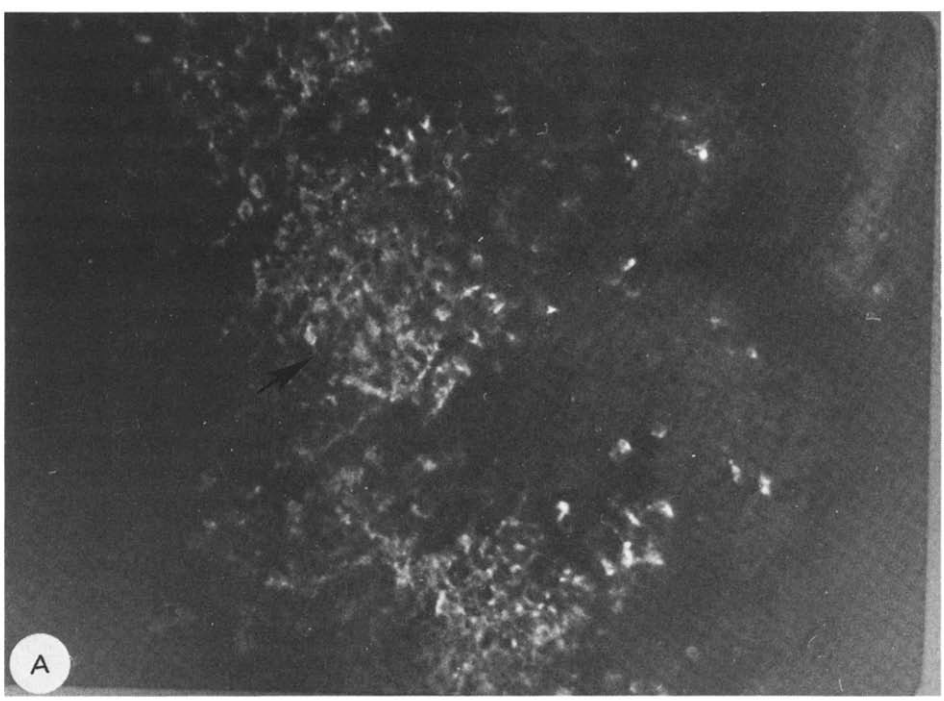
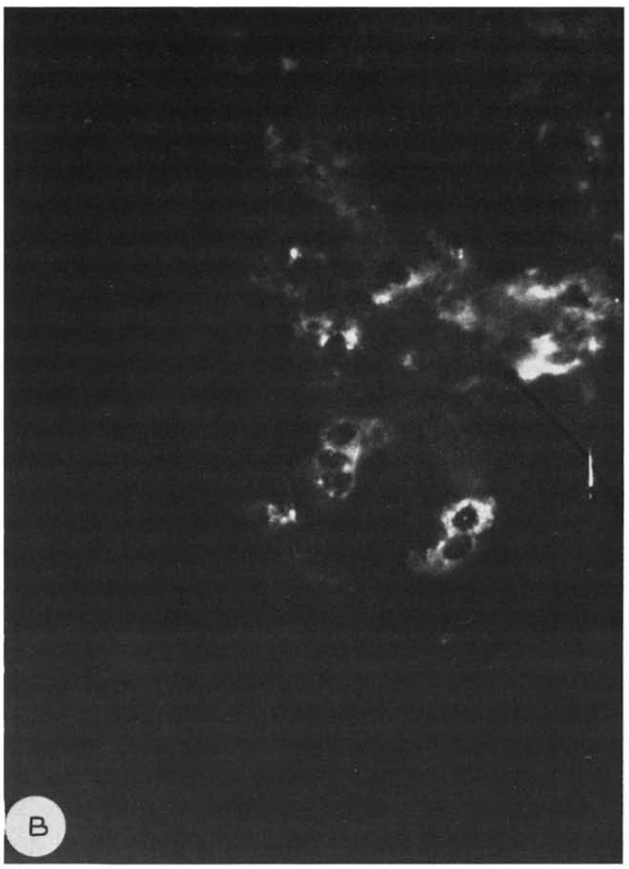


Fig. 2. Immunofluorescence of sections of (A,B) posterior small intestine and (C) of spiral colon, from calf No. 2. Note (arrow) immunofluorescence in the crypt regions and occasional cells in the upper villous epithelium of the small intestine (A) and cytoplasmic immunofluorescence (B).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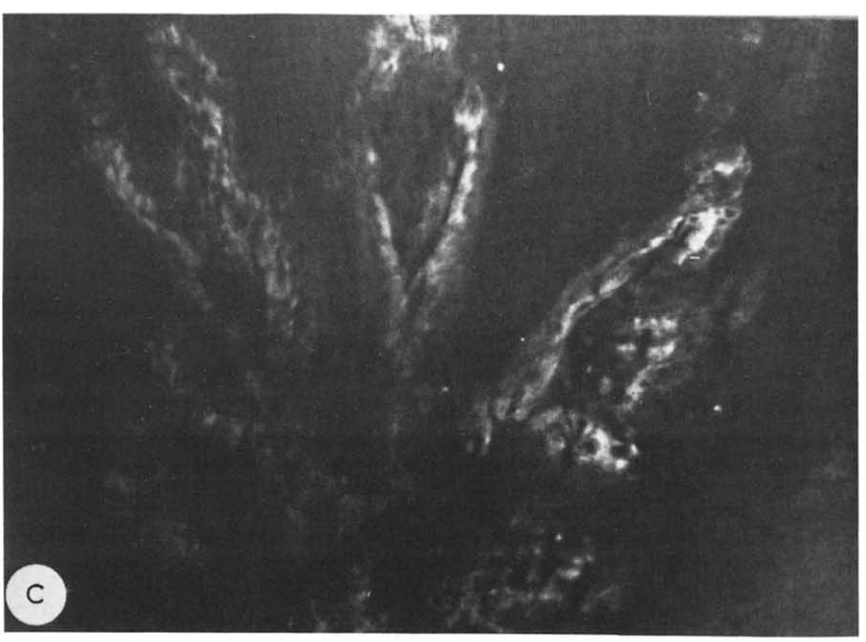
C

herd were examined. Fifty-five were positive for coronavirus by HAHI, 53 by EM, and one by EM only. None was positive for the "Breda" virus by HAHI or EM. Twelve samples had HA titers (8.6—11.6 $\log_2$) which were not related to either coronavirus or "Breda" virus antigenically and were all EM negative. With both viruses, only those samples with high titers of HA had readily observable particles with all the morphological characteristics.

The range of HA titers in feces for coronavirus in field cases was 7.6—17.6 $\log_2$, and for the "Breda" agent 7.6—25.6 $\log_2$.

Attempts to isolate virus in organ cultures, cell cultures and embryonated eggs

All attempts to isolate the virus in cell cultures, intestinal and tracheal organ cultures and embryonated eggs were unsuccessful. Cell culture preparations were taken to 3—5 passes and the preparations were examined for the presence of viral specific immunofluorescence and HA. As controls for the cell culture methods, fecal bovine coronavirus was shown to replicate in trachea organ cultures, HRT cells, MDBK cells and bovine thyroid primary cell cultures, and bovine rotavirus replicated in the primary and secondary CK cells. No detectable HA developed in the allantoic fluid of all inoculated eggs, which were sampled at day 3 and 7 pi. Eggs that died were shown to be contaminated with bacteria. Pretreatment and treatment at each pass with trypsin had no apparent influence on the infectivity of the virus for these culture systems.

DISCUSSION

The first intention of the present study was to confirm the infectious nature of the particle, as virus-like particles, the so-called pleomorphic "fringed-particles", are common in feces but these (Woode, unpublished data) are not infectious. Apart from the fecal preparation of calf B276, which contained the "Breda" agent with large numbers of similar sized and shaped virus-like particles, most of the hemagglutinin positive calf samples contained fewer particles with less well-defined morphologies and were originally dismissed as non-viral in nature or considered to be "atypical" coronavirus particles. Although all early attempts to culture the agent in vitro were unsuccessful, from precedents established with other enteritis inducing viruses, we assumed that the natural host would be the most sensitive system for demonstrating infectivity (Woode, 1979).

The "Breda" agent was shown to be infectious in both gnotobiotic and conventionally derived calves as all the experimental calves inoculated orally excreted the agent for 3—4 days in the feces and one serial passage of the agent through two calves was successful. The agent can be tentatively classified as a virus on the bais of its infectious nature in calves, the size, shape, presence of the hemagglutinin and the specific immunofluorescence in the cytoplasm of infected epithelial cells.

These studies have not confirmed that the virus is pathogenic in all non-immune calves.

It is possible that the severe clinical effect observed in 4/8 experimental calves, which is similar to the percentage suffering severe diarrhea on the farm, is controlled by individual variation in response. Similar observations have been reported for rotavirus infection in pigs (Woode and Crouch, 1978). An alternative interpretation of the data, however, is that this agent requires a microbial flora before virus infection, to demonstrate a full clinical effect, although there could be exceptions. All three colostrum-deprived calves developed severe clinical signs of disease in contrast to the gnotobiotic calves, in which only 1/4 was severely affected.

The studies have not elucidated fully the pathogenesis of the agent. The initial studies show that the pathology of the small intestine is consistent with a rotavirus (Mebus et al., 1971, Woode et al., 1974) or a coronavirus (Mebus et al., 1973, Bridger et al., 1978) infection. The immunofluorescent data of the small intestine and colon, and the pathology of the colon, shows a greater similarity with coronavirus infection rather than with rotavirus. Only with lamb rotavirus has infection of the colon been demonstrated (Snodgrass et al., 1977). The watery nature of the diarrhea which is commonly observed with gnotobiotic calves infected with coronavirus and with calf GC 1 in this study, is probably due to the colonic lesion and consequently, reduction in the ability to absorb water. However, the immunofluorescent studies suggest that the pathogenesis may differ from coronavirus in that most immunofluorescent cells were observed in the lower 50% of the small intestinal villus and throughout the crypt region, and in the colon throughout the length of the villus and crypt. This would imply that the primary site of infection is in the crypt cells and infected cells migrate up the villus before being shed as the viral cytopathic effect develops.

The minimal malabsorption effect as measured by D-xylose also suggested that the pathogenesis may differ from rotavirus (Woode et al., 1978) and from the calicivirus-like agents (Woode and Bridger, 1978). Further studies are planned to determine the pathogenesis of the agent.

The hemagglutination method proved to be the most useful method for identifying antigenically the agent in fecal samples. From these data and from data obtained from immunofluorescent studies of the agent in gut sections, and coronavirus in gut sections and tissue cultures, the "Breda" virus appears to be antigenically distinct from other bovine hemagglutinating viruses. These include bovine coronavirus (as characterized by Sharpee et al., 1976), parvovirus, and rotavirus, parainfluenza 3 virus in this study, and reovirus 3 (Woode, unpublished data). In addition, bovine pestivirus (BVD) could not be isolated from preparations containing the "Breda" virus, and antiserum to the "Breda" virus did not possess antibodies to BVD. The HI test for studying sera for the presence of antibody was considered to be unreliable and thus serological data suggestive of widespread distribution of the agent on farms should be treated with reservation. The virus may be common in the U.S. as a similar agent has been observed in one calf in South Dakota and in calves in Ohio, and antiserum to the "Breda" virus reacts

specifically with the Ohio isolate (Saif, personal communication, 1981), however, it appears that these two latter viruses are different serotypes.

The failure to demonstrate "Breda" virus elution from erythrocytes, in contrast to the elution obtained with bovine coronavirus, demonstrates another dissimilarity between these two viruses.

The morphological data indicate both similarities and differences when "Breda" virus is compared with coronaviruses in general and bovine coronavirus in particular. The morphological description of bovine coronavirus as observed by the authors and others (Sharpee et al., 1976; Bridger et al., 1978) is: generally round, 110—180 nm in diameter with peplomers (17—24 nm) and a centrally located electron-dense area. An inner layer of short peplomers (10 nm) is seen on some particles. In contrast, the "Breda" agent is more pleomorphic with at least two distinct shapes: ovoid (89 × 75 nm) and sausage or kidney shaped (120 × 32 nm). The majority of particles possess peplomers (7.6—9.5 nm). Like coronaviruses, the "Breda" virus frequently shows an electron-dense region. This appeared to represent the cleft region seen in the kidney particles and may be similar to the internal component reported in avian infectious bronchitis virus, a coronavirus (Bingham and Almeida, 1977). In the authors' experience, the short peplomers are not visible on bovine coronavirus particles, when they lack the 17—24 nm peplomers. The short peplomers seen on the "Breda" agent are similar to those of many enveloped viruses from a variety of families (Dalton and Haguenau, 1973).

Definitive taxonomic classification of this agent will require characterization of its physical and chemical properties. Antiserum to the "Breda" virus is being examined for the presence of immunofluorescent antibodies to a number of animal coronaviruses.

The histopathological lesions of villous atrophy with basophilic cuboidal to squamous epithelium were similar to alterations attributed to intestinal infections with bovine rotavirus and bovine coronaviruses (Mebus et al., 1971, 1973). The absence of such changes in calf CD 2 was presumably due to the early time of autopsy of this calf, as the intact villi demonstrated extensive virus-specific immunofluorescence. The epithelial irregularity and necrosis seen in the colon of calves CD 1 and GC 1 indicate a role of colonic dysfunction in the pathogenesis of diarrhea produced by infection with this virus.

The inability, at this stage of the research, to culture the virus by in vitro techniques is similar to the experiences of most workers with other enteritis inducing viruses. The calicivirus-like agent of the calf and the Norwalk virus of human beings (Wyatt et al., 1979) have not yet been cultured, and until the trypsin method for the culture of rotavirus (Theil et al., 1977) and the use of HRT 18 cells for coronavirus (LaPorte et al., 1979) were developed, these agents could not be cultured readily. Fortunately, modern virology methods do not require the in vitro cultivation of viruses for antigenic and nucleic acid characterization of viruses and the large quantities of virus obtained from feces permit us to use this source of virus for most studies, including the development of vaccines.

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ADDENDUM

Three additional gnotobiotic calves have been inoculated orally 1—2 h after cesarean section, with the "Breda" virus obtained from the colonic contents of calf GC1. All three calves developed diarrhea with clinical signs as previously described, possessed villous atrophy in the posterior jejunum and ileum, and had "Breda" virus specific IF epithelial cells at these sites and in the large intestine.