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ISOLATION OF A VIRUS FROM THE VIRUS DIARRHEA — BOVINE MUCOSAL DISEASE

By

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Cattle are susceptible to a number of infectious agents — viruses — which have a peculiar affinity for the mucous membranes of the respiratory or alimentary tract. The resultant diseases include the virus diarrhea-mucosal disease complex (VD & MD), malignant catarrhal fever (MCF), infectious bovine rhinotracheitis (IBR), rinderpest and others.

They are all contagious, febrile diseases characterized by nasal and lachrymal discharges, salivation, coughing and/or diarrhea. In some cases, ulcers are seen on the mucous membranes of the digestive tract and in the interdigital clefts. Exanthema may also occur. Thus, the clinical picture varies from case to case, and any symptom may be predominant. Morbidity and mortality vary in the different conditions.

Isolation of viruses from cattle with VD & MD has been reported in many recent papers (1—3, 6, 12—17, 21, 22). It should especially be noticed that the human pathogenic myxovirus para-influenza 3 has been isolated in Sweden (3) from cattle with mucosal disease, and that a virus with properties similar to those of para-influenza 3 virus has been recovered in the U.S.A. from cattle with shipping fever (1,17). Virus strains belonging to the adenovirus group (10, 11) and reo-viruses (20) have also been isolated from cattle.

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It is the purpose of this report to describe the isolation of a virus — designated KO-23 — from a cow with VD & MD. The virus is pathogenic for baby mice and antibodies against this or closely related viruses seem to occur with a high frequency in man (18).

MATERIALS AND METHODS

Tissue cultures. The cell cultures were prepared from kidneys of baby calves killed within the first 48 hours of life. The kidneys were removed at once and freed from fatty and fibrous membranes. The cortex was cut in suitable pieces and stored in T. C. Medium 199 (Difco) at 4°C overnight. Before trypsinization the medium was decanted, and the cortical tissue was minced with a pair of scissors, after which the tissue was transferred to phosphate-buffered saline (pH 7.3) containing 0.25 per cent trypsin (Difco 1:250). It was not necessary to wash the trypsinized cells which, after centrifugation, were suspended in T. C. Medium 199 containing 10 per cent calf serum. The cells were counted in a hemocytometer and the suspension diluted to give 500.000 cells per ml. Test tubes were seeded with 1 ml. of this suspension and incubated at 37°C in a stationary, inclined position. After 2—4 days of incubation, the medium was changed, and just before inoculation on the 6th—8th day replaced by Eagle's medium (5) with 10 per cent horse serum.

Cultures of human amniotic cells, human fibroblasts (skin-muscle), minced chick embryo cells, etc. were prepared in the same way with minor variations in the composition of the nutrient medium. The maintenance medium was the same as that used for calf-kidney cultures.

Infectivity titrations. Serial ten-fold dilutions of infected fluid were inoculated in 0.1 ml. amounts into each of three calf-kidney cultures. After incubation at 37°C the tubes were examined every second day for cytopathic changes. The cultures were observed for 14 days, and the 50 per cent infectivity end point was calculated by the method of *Kärber* (9). Titers are expressed as $-\log 10\ TCD_{50}$ per 0.1 ml. of undiluted test material.

Neutralization tests. In an attempt to identify virus KO-23 a number of immune sera were tested by the constant virus-varying serum method. Serum inactivated by heating at 56°C for 30 minutes was diluted two-fold from 1/2 to 1/1024 in Eagle's medium, and to each dilution was added an equal volume of a

virus dilution containing approximately 50 infective doses per 0.1 ml. The exact dose was calculated in a simultaneous titration. The serum-virus mixtures were incubated at 37°C for 30 minutes and then inoculated in 0.1 ml. amounts into each of three tissue culture tubes. Readings were made every second day for two weeks.

Specimens were collected as nasal and/or rectal swabs. The cotton swab was kept in 2 ml. of Eagles medium containing 10 per cent horse serum plus 100 units of penicillin, 100 units of streptomycin and 100 units of mycostatin (Squibb) per ml. The specimens were stored at —20°C as soon as possible. Before inoculation, the specimen was thawed and centrifuged. 0.1 ml. of fluid was then inoculated into each of 3—5 tissue culture tubes. Irrespective of the results, isolation attempts were repeated with the same specimen.

Animal experiments. Inbred AKA mice aged either 1—2 days or 6—7 weeks were used for inoculation with virus from the tissue cultures. Adult mice received 0.03 ml. fluid intracerebrally, 0.03 ml. subcutaneously, or 0.05 ml. intranasally. Intracerebral and intranasal inoculations were carried out under light ether anesthesia. The baby mice were injected by the same routes as the adults but the doses were 0.01 ml., 0.01 ml. and a minimal drop.

Serial ten-fold dilutions of infected tissue culture fluid were titrated in baby mice by the intracerebral route. Each dilution was inoculated into a litter of mice, using the same number of animals from each litter. LD_{50} was calculated according to the method of Kärber.

On continued passage the mice were killed when symptoms appeared, and the brain was removed aseptically. Pooled brain tissue was ground in a mortar and diluted with Hank's balanced salt solution to a 10 per cent suspension. After centrifugation at 1500 r. p. m. for 10 minutes the supernatant fluid was inoculated into baby mice.

Hemagglutination. Red cells from guinea pigs, cattle, chickens, sheep and humans were collected in citrate solution. They were then washed several times in 0.85 per cent saline and preserved as a 10 per cent suspension in phosphate-buffered saline. For the experiments an 0.25 per cent suspension was used.

Serial two-fold dilutions of tissue culture fluid were prepared, and to each tube was added an equal amount (0.5 ml.) of the

0.25 per cent red cell suspension. The tubes were incubated overnight at + 4°C and read by the pattern method. The highest dilution of virus fluid giving full hemagglutination was taken as the end point.

CASE HISTORY

In inoculation experiments in January 1961 — with material from a number of herds with present or previous experiences of mucosal disease — isolation was made of virus KO-23 from one animal in a small herd (E. T., Feldballe). The herd consisted of one cow and 2 calves. It was stated that the cow had been ill for a couple of days. The animal was depressed and refused to eat, and the milk yield was heavily reduced. The calves did not show clinical signs of disease at the first examination. Physical examination revealed the following findings: The cow, a 4-year-old Red Danish Milkbreed was standing in her stall. The general condition was distinctly affected, the carriage normal, and the cow well nourished showed distinct signs of fever. It was sweating, breathing heavily and was inattentive to the surroundings. There was pronounced depression and anorexia. The rectal temperature was 41.7°C, respiration 40 and pulse 94. The eyes were somewhat blurred, the conjunctivae reddish, moist, slightly swollen, and there was plenty of serous lacrimation. The nasal mucosa was very red, congested and moist, and ample amounts of serous fluid were running from the nostrils. The oral mucosa was more diffusely red than normal, and salivation rather strong. The vaginal mucosa was more moist and more congested than normal. Now and then the cow had coughing attacks with a moist, slightly suppressed cough. The borders of the lungs were normal, no dullness on percussion, but over the lung area rales were heard. The heart sounds were normal. All accessible lymph nodes were of normal size and consistency, and the skin was of normal appearance.

The cow drank plenty of water. Rumination had ceased, and the gastro-intestinal functions were diminished. Feces were somewhat thin, but not of a watery nature. The udder appeared normal. The gait was normal, and the musculatory and nervous systems appeared unaffected.

The urine yielded a positive Heller's reaction, and examination of the blood showed leukopenia. The formol gel test was negative.

At a second examination 3 days later, the general condition had improved to some extent. The temperature was now 39.4°C, respiration 34 and pulse 84. The conjunctivae were more edematous, and edema of the eyelids was also present. The lacrimation was more pronounced, more mucous and purulent, and the nasal discharge had also changed character. It was more viscous, darker and contained purulent lumps. The nostrils were coated with crusts, the mucous membrane heavily red, darker and with several small erosive changes. The oral mucosa was still very red and showed some small buccal erosions. Salivation was intensive and foul-smelling. The coughing had become more frequent and tougher. The lung area was normal.

Considerable enlargement of the retropharyngeal lymph nodes was noted. Feces were thinner, but still not watery. There was no exanthema of the skin.

After another 4 days the cow was free from fever (38.5°C). The appetite was normal and the mucosal changes were less pronounced. The interdigital clefts remained normal; there was no sore gait and no abortion.

About 4 days later the two calves showed slight illness for a couple of days, but they received no treatment.

RESULTS

Material from rectal and nasal swabs from the cow was inoculated in calf kidney tissue cultures. After 4 days a cytopathogenic effect (CPE) was observed in the cultures inoculated with material from the nose, whereas cultures from the rectal swab remained negative after an observation period of 2 weeks. Re-isolation gave the same result.

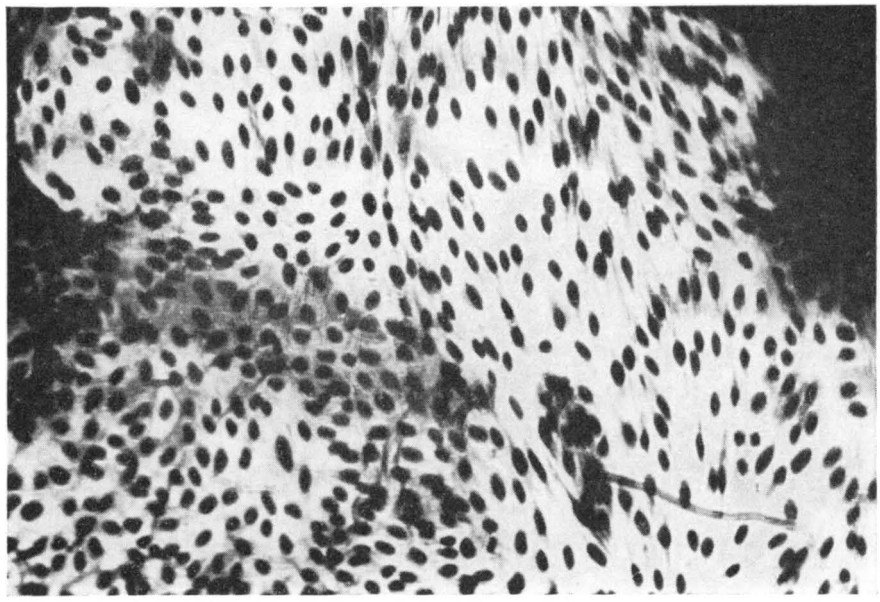
Cytophatogenic effects. Using a small inoculum, the CPE in calf kidney cultures inoculated with virus KO-23 showed scattered plaques with degenerated, round, dense cells and a lace-like structure of the tissue (Fig. 1).

The cultures soon became characterized by dislodgement of destroyed cells, which had a slight tendency to agglutination. In Giemsa-stained preparations a number of syncytial, multinucleated cells were visible (Fig. 2). Vacuoles in the cytoplasm or intranuclear or intracytoplasmic inclusion bodies were not observed. No CPE was noted in repeated passages to tubes containing the following cell types: human amnion cells, human fibroblasts (skin-muscle preparations), L-cells or minced chick-embryo tissue.

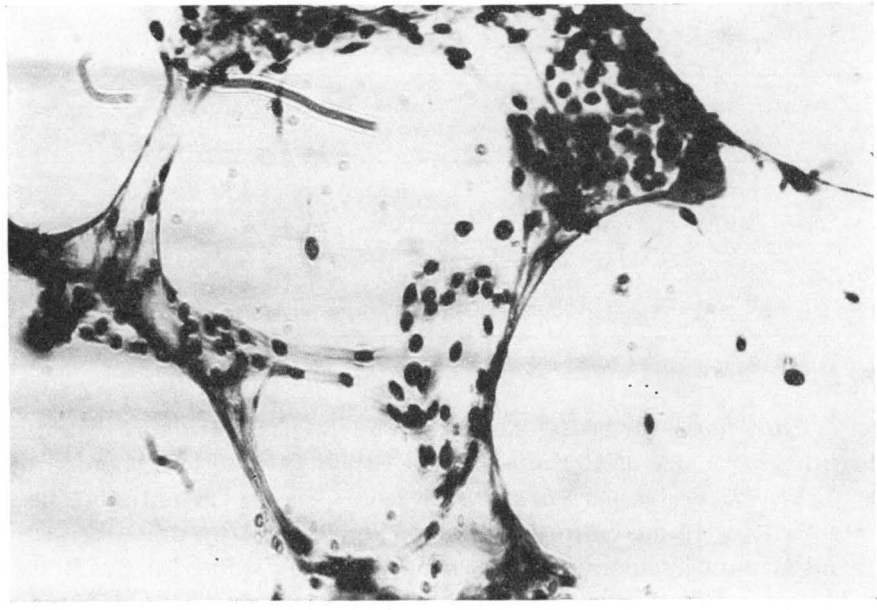
Embryonated eggs. Attempts to pass the virus by the allantoic cavity route on 10-day-old embryonated eggs have so far remained negative.

Infectivity titrations. Titration of infected fluid from the third passage of virus KO-23 gave a titer of 5.5 and that of the sixth passage was 7.3. In addition to calf kidney cells the virus can be grown in guinea pig kidney cells although with a lower titer: 6.0 as compared to 4.5 in parallel titrations.

Effects of chemicals. Only the sensitivity to ether has been studied. The infectivity of virus KO-23 is destroyed by exposure to 20 per cent anesthetic ether for 24 hours at +4°C.



a.



b.

Fig. 1. Cultures of calf kidney cells, (a) normal, (b) nine days after inoculation with virus KO-23.

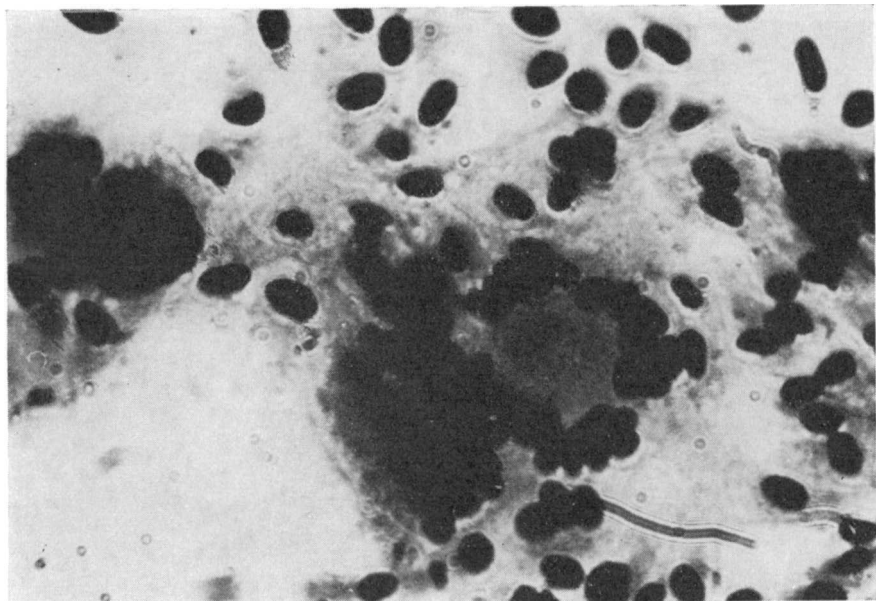


Fig. 2. Giant cells in calf kidney culture infected by virus KO-23.

Table 1.
Effects of Temperature on Infectivity of Virus KO-23.

After storage for	Temperature centigrades + 37°	+ 20°	+ 4°	— 20°
1 day	3.5[1])	5.8	4.8	5.5
6 days	0	5.1	3.8	5.5
11 days	0	4.1	2.1	5.1
23 days	0	0	0	5.8
11 weeks	–	–	–	5.5

[1]) — log 10 TCD_{50}

Filtration. Virus KO-23 was found to pass Millipore filters with a pore size of 450 mμ, but not filters with 100 mμ a.p.d.

Effects of temperature. As can be seen in Table 1. the infectivity of tissue culture fluid was rapidly destroyed at +37°C, and at room temperature the virus was fairly stable for the first 11 days. In other experiments it has been shown that virus KO-23 retained its infectivity for more than 11 weeks when kept at —20°C.

Hemagglutination. The capacity of the virus to agglutinate

red cells from various animals was investigated, and the results are summarized in Table 2.

Table 2.
Hemagglutination of Red Blood Cells from Various Species caused by Virus KO-23.

Cow	Sheep	Human type 0	Chicken	Guinea pig
1/256	1/4	1/64	1/4	1/16

Highest dilution of virus giving full agglutination.

Relationship of virus KO-23 to other viruses. In order to determine the possible serologic relationship between virus KO-23 and other viruses, serum neutralization tests were performed. The sera used were: ECHO-10 (Lang) antiserum (monkey serum) obtained from the National Foundation for Infantile Paralysis Inc., USA and 4 sera provided through the courtesy of James H. Gillespie VMD Cornell University, Ithaca N.Y. These sera were marked: VD-antiserum (cow) No. C583R, 15/8/61, 33 days postimmune serum and a corresponding serum No. C583R, 26/6/61 pre immune serum. Furthermore, an IBR-antiserum (rabbit) No. RT25BK, 10/10/61 3 weeks postimmune serum and a pneumoenteritis virus antiserum (cow) No. C577R, 28/8/61 120 days postimmune serum.

The results of the neutralization are given in Table 3.

Table 3.
Neutralization Test on Virus KO-23 with known Antisera.

Immune serum	Serum dilution									Virus
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	TCD_{50}
ECHO-10 (Lang)	0/3[1]	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1000
„	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	50
VD pre-immune	nt[2]	nt	3/3	3/3	2/3	0/3	0/3	0/3	0/3	63
VD postimmune	nt	nt	3/3	3/3	0/3	0/3	0/3	0/3	0/3	63
IBR	nt	nt	0/3	0/3	0/3	0/3	0/3	0/3	0/3	63
Pneumo-enteritis	nt	nt	3/3	3/3	1/3	0/3	0/3	0/3	0/3	63
Cow 23	3/3	3/3	3/3	3/3	3/3	2/3	0/3	0/3	0/3	31

[1] Numerator, number of tubes with neutralization. Denominator, total number of tubes.

[2] nt. Not tested.

Complement fixation test with virus KO-23 as antigen and an adenovirus type 11 antiserum was negative.

Animal experiments. Six-week-old mice were inoculated intracerebrally, intraperitoneally and subcutaneously with undiluted tissue culture fluid containing $10^{5.8}$ TCD_{50} of virus per 0.1 ml. In no case were symptoms of infection observed during an observation period of 4 weeks.

Intracerebral injection of the same material into baby mice resulted in infection 6—7 days later. The mice were apathic and looked thinner and smaller than the animals of the control group. The gait was considerably more reeling and shaky than normal, and when a mouse rolled over, it did not try to turn back into the normal position. Paralysis, as seen in polio virus or coxsackie virus infections of mice, did not occur. In a few cases. however, convulsions were noted. Infantile virus diarrhea of mice did not develop. All animals died 1—3 days after the onset of symptoms.

Table 4.
Intracerebral Titration of Virus KO-23 on Newborn Mice.

Virus dilution	No. of mice per litter	Number of deaths day																			Survivors 28th day
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Undiluted	7						2	2		3												
10^{-1}	7									6	1											
10^{-2}	7								1	1	2	1	1	1								
10^{-3}	7										2	2	2	1								
10^{-4}	7										1	1	1					1		1		2
10^{-5}	7												1									6

Titration on baby mice by the intracerebral route of virus infected tissue culture fluid containing $10^{6.3}$ TCD_{50} gave a negative log 10 LD_{50} of 4.3. Table 4 shows the survival time in days for baby mice after intracerebral inoculation of virus KO-23.

Several attempts were made to pass the virus by intracerebral inoculation of brain material from sick animals. In the second passage only 50 per cent of the mice died, and in third passage no symptoms at all developed. The virus could be detected in brain suspensions from sick animals in the second passage by inoculation of the brain suspension on tissue culture tubes. No quantitative estimations were performed.

DISCUSSION

The virus which was isolated from a cow with mucosal disease has a size of more than 100 mμ and less than 450 mμ. It is destroyed by exposure to 20 per cent ether at 4°C for 18 hours, and is pathogenic for baby mice after intracerebral inoculation although the virus disappears after the second passage. The virus seems to be relatively common in Danish cattle as appears from a survey of antibodies in serum from cattle (19). Sera from healthy human beings also reveal antibodies in a large proportion of cases (18).

Only one serum specimen was examined from the cow in which the virus was isolated. It was taken 25 days after the infection; diluted to 1/64 it was able to neutralize 31 TCD_{50} (Table 3).

Virus KO-23 does not seem to be identical with the American VD virus since the neutralizing capacity of the pre- and post-immune serum is almost identical. The infectivity of VD virus is destroyed by ether, but it is not pathogenic for baby mice (7). From the experiments listed in Table 3 virus KO-23 does not seem to be related to IBR virus or pneumo-enteritis virus.

Virus KO-23 differs from the ECHO viruses — with the exception of ECHO-10 (Reo virus) — in the size of the particle and in that the ECHO viruses are resistant to ether. In stained preparations of tissue cultures infected with virus KO-23 none of the typical inclusion bodies seen in ECHO-10 infections (20) were observed. As already mentioned, intranuclear inclusion bodies were not observed, and these should be typical of adeno-virus, respiratory syncytial and IBR virus infections.

Coxsackie viruses are pathogenic for baby mice, but the symptoms observed after intracerebral inoculation of these viruses differ from what is seen with KO-23. Furthermore, coxsackie viruses are smaller than virus KO-23, and they are resistent to ether.

The size of virus KO-23, the sensitivity to ether, the hemagglutination capacity and the histological changes seen in tissue cultures render it likely that the virus is related to the para-influenza group of viruses. In pathogenicity for baby mice it is reminiscent of para-influenza type 1 virus (8).

It does not appear from the literature whether para-influenza type 1 is pathogenic for cattle or not, or if it can grow with CPE in calf kidney tissue culture. According to our experiments, para-

influenza type 1 (Sendai) multiplies in calf kidney cultures with a CPE similar to that caused by virus KO-23. The possible relationship between virus KO-23 and the para-influenza group of viruses will be investigated.

Addendum

Since the present manuscript was submitted virus KO-23 has been identified as the bovine type of myxovirus para-influenza type 3. (*Rindom Schiøtt, C. & C. Hyldgaard Jensen:* A mouse pathogenic strain of para-influenza virus type 3 isolated from cattle. Acta Path. et Microbiol. Scandinav. In press).

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SUMMARY

The isolation of a virus from a cow with mucosal disease is described. The character of the virus and its possible relationship to other viruses is discussed. It is concluded that the virus is related to the para-influenza group of viruses although it is pathogenic for baby mice.

ZUSAMMENFASSUNG

Isolierung eines Virus aus der Virus-Diarrhöe — dem Mucosal disease-Komplex.

Es wird über die Isolierung eines Virus aus einer Kuh mit Mucosal disease berichtet. Die Eigenschaften des Virus und seine Beziehung zu gewissen anderen bovinen und humanen Virusarten werden diskutiert. Es wird die Schlussfolgerung gezogen, dass das isolierte Virus zur Myxovirus-Parainfluenzagruppe gehören dürfte, selbst wenn das Virus für neugeborene Mäuse nach zerebraler Injektion pathogen ist.

RESUMÉ

Isolering af et virus fra virus diarrhoe — mucosal disease komplexet.

Der omtales isolering af et virus fra en ko med mucosal disease. Virus egenskaber og relation til visse andre bovine og humane virus diskuteres. Man konkluderer, at det isolerede virus må høre til i myxovirus para-influenza gruppen, selv om virus er patogent for nyfødte mus efter intracerebral injektion.

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