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MALIGNANT CATARRHAL FEVER VIRUS IN NASAL SECRETIONS OF WILDEBEEST: A PROBABLE MECHANISM FOR VIRUS TRANSMISSION[1]

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Abstract: The virus of malignant catarrhal fever (MCFV) was isolated from the nasal secretions of 6 of 66 recently captured blue wildebeest (*Connochaetes taurinus*). Ten MCFV isolates were made from 131 nasal swab specimens but only one isolate was obtained from 168 blood samples. All MCFV isolates from nasal secretions were from wildebeest in captivity, under the stresses of confinement, changes in nutrition, or after injections of a corticosteroid drug, betamethasone. One isolate of MCFV was made from the tonsils of a pregnant wildebeest. It is postulated that nasal shedding of MCFV may be a mechanism for transmission of virus among wildebeest and from wildebeest to cattle.

INTRODUCTION

Infection of blue wildebeest with a virus which causes bovine malignant catarrhal fever (MCFV) was demonstrated by Plowright and his associates.[10] Viremia in wildebeest was detected by cattle inoculation and also by isolation of the virus in bovine thyroid (BTh) cell cultures.[5,6] In the blood of both wildebeest and cattle, virus was found to be cell-associated in the buffy-coat fraction of the blood. MCFV was characterized as a herpesvirus.[12] Virus transmission occurred among wildebeest calves in captivity, also from wildebeest to domestic bovine calves in pen contact.[6] The mode of transmission was not detected. Cattle-to cattle transmission did not occur.

In order to get more information about the carrier state in wildebeest and possible means of virus transmission, nasal secretions and body tissues of wildebeest were examined for MCFV.

MATERIALS AND METHODS

Wildebeest

Wildebeest were captured by drug immobilization or trapping in several locations on the Kajiado Plains of Kenya's Masailand. They were held for variable lengths of time at the capture sites in portable wooden pens, one or two animals per 4m x 4m pen. After a period of 2-3 weeks in field quarantine, some of

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the wildebeest were moved by truck to a paddock at EAVRO.

Drug immobilization was accomplished with a mixture of etorphine (0.008-0.015 mg/kg), acepromazine (0.03-0.06 mg/kg)[5] and xylazine (0.2-0.3 mg/kg)[6] administered via projectile syringes. The effects of etorphine were reversed with diprenorphine.[7] Xylazine alone (0.4 mg/kg) was given to penned wildebeest to facilitate handling and transportation. A few wildebeest were killed by shooting for collection of tissue specimens.

Specimen Collection

For virus isolation 14 ml of blood were collected from the jugular vein into 7 ml of 1.5% EDTA (ethylene-diamine-tetra-acetic acid) in 0.7% NaCl. Nasal secretions were obtained by gently swabbing the nasal mucosa as deeply as possible with a 20 cm cotton-tipped wooden applicator stick. Vaginal secretions were swabbed in a few wildebeest, after abortion or parturition or when lesions of any kind were present in the vagina. Tissues for virus isolation were taken from animals which were shot or which died in captivity. All specimens were transported to the laboratory on ice and were processed within 24 hours of collection.

Cell cultures

Trypsin dispersed BTh monolayers were prepared according to the methods of Plowright and Ferris[9] except that HSLS medium of Johnson and Smith[3] was used supplemented with 10% serum for cell growth and only 5% for maintenance. Secondary monolayers were prepared by dispersal of primary cultures with a mixture of 0.2% versene and 0.25% trypsin (VT) and allowed to form either in tubes or flasks. These monolayers were used routinely for virus isolation attempts from wildebeest and cattle specimens. Occasionally cell cultures were prepared from wildebeest kidney or thyroids using the same technique in an attempt to "unmask" latent virus in wildebeest tissues.

Virus Isolation

The method used for isolating MCFV from whole blood and from tissue specimens of wildebeest and cattle was as described by Plowright *et al.*[11] After collection of nasal and vaginal secretions, the swabs were immediately broken into universal bottles containing 3 ml Earle's saline solution supplemented with 10% normal calf serum and antibiotics (penicillin 200 i.u./ml; streptomycin 200 mg/ml; and nystatin 50 units/ml). Fluid into which the swabs had been immersed was inoculated onto confluent BTh monolayers. In early attempts, the fluids were inoculated directly into culture tubes without any clarification. Later, nasal swab fluids were clarified either by light centrifugation or centrifugation followed by filtration of the supernate through a 450 nm membrane filter. The supernate or filtrate was inoculated onto BTh monolayers, 0.2 ml for each of five tubes per sample. The sediments were resuspended in 1.2 ml of medium and inoculated onto BTh cells.

Inoculated BTh monolayers and uninoculated controls were examined for cytopathic effect (CPE) on the 4th day post inoculation (p.i.) and thereafter every 2nd day. Medium was changed every 4 days. Cultures were scored negative if after 21 days observation they had shown no CPE.

Cell cultures showing some CPE were dispersed using VT and the resultant cell suspension from a set of five tubes was pooled into 1 ml of HSLS medium and inoculated onto two confluent BTh secondary monolayers in 'Falcon'[8] bottles.

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[5] Etorphine combined with acepromazine, marketed as Immobilon, Reckitt and Colman, Hull, England.

[6] Xylazine as Rompun, Bayer, Leverkusen, Germany.

[7] Diprenorphine as Revivon, Reckitt and Colman.

[8] Falcon Plastics Ltd., Oxnard California 93030, U.S.A.

The monolayers were examined every 2 days. On the 7th day p.i., if no CPE had been observed, one 'Falcon' bottle monolayer was passaged into two bottles to form new subcultures and the remaining bottle was examined for 21 days. The subcultures were passaged every 7 days up to 3 times. At each passage level only half the number of bottles were subcultured, the rest being left for extended examination. Cultures showing CPE were subcultured until reproducible CPE was maximum within 5 days.

A further method of virus isolation was to prepare monolayer cell cultures from thyroids and kidneys of freshly aborted wildebeest foetuses or just-dead wildebeest. Such monolayers were subcultured up to 10 times, after which they were discarded as negative if CPE had not been demonstrated.

A viral agent was identified as MCFV by its CPE,[6,11] infectivity for cattle, producing a characteristic MCF syndrome,[2] and re-isolation of the virus from the blood or tissue of inoculated cattle. MCFV CPE appeared as foci of syncytia which eventually detached without spreading to involve the whole monolayer. In coverslip culture preparations fixed with Bouin's solution and stained with haematoxylin and eosin, the syncytia were observed to contain nuclei with large, somewhat basophilic inclusions.

MCFV isolates were stored at —70 C in the form of infected cells suspended in HSLS medium with 20% bovine serum and 10% glycerol.

Antibody Assay

Serum neutralization tests were carried out as described by Plowright[7] with minor modifications. The high passage cell-free WC11 strain of MCFV[12] was diluted to give approximately 100 $TCID_{50}$ per 0.1 ml and sera were titrated by doubling dilution series. Neutralization was effected by incubating mixtures of equal volumes of serum dilutions and virus at 4 C overnight, before inoculating five replicate roller tube secondary BTh cultures with each serum-virus mixture.

RESULTS

Viremia, Antibody and Excretion of Virus by Captive Wildebeest

Nine pregnant wildebeest cows were captured on the Kajiado plains of Kenya on 30th January, 1973. They were held under quarantine for 4 weeks in portable holding pens at the capture site. At the end of the quarantine period they were transported under sedation to the laboratory where they were released into a 2 ha paddock which was doubly fenced with inner chain-link and outer barbed wire fencing. They were allowed to graze freely with five steers in the paddock (Fig. 1).

During transportation three of the nine wildebeest reacted badly to the xylazine, acepromazine, and etorphine combination which had been used successfully on these animals at the time of capture. These three animals (W207, W208, W212) died. They were all in advanced pregnancy. Of the remaining six wildebeest cows, one (W209) died suddenly 2 days after arrival at the laboratory; another (W211) aborted 3 days after arrival, became recumbent and died 2 days later. A third wildebeest (W210) also aborted 5 days after arrival at the laboratory but did not die. There remained three pregnant wildebeest plus W210 for extended observations. The three pregnant wildebeest all calved normally 8-11 weeks after arrival at the laboratory. The calf from W214 died following an accident the day after birth. Wildebeest cow W210 was also injured and was killed, *in extremis* on 11th May. The nasal secretions and blood of the wildebeest were sampled at the time of transportation to the laboratory paddock, and then weekly for the first 8 weeks. Specimens were taken also on the day of calving, at which time the calves were also tested for viremia and nasal virus excretion. At the time intervals shown in Table 1, serum samples were collected for antibody assay. All wildebeest cows had high levels of neutralizing antibody (Table 1). Contact cattle were bled weekly for viremia testing.

No virus was isolated from any of the blood samples, vaginal swabs, tissues of

wildebeest which died, nor monolayers from aborted foetuses; nor from the calf of cow W214. However, monolayers prepared from the thyroids of wildebeest cow W210, which was killed after it had accidentally injured its neck, yielded a cytopathogenic agent with a CPE typical of MCFV. Syncytial foci were observed at the second blind passage at 6 days (Fig. 2). Syncytia were easily discernible on further subculture of the monolayer. The agent thus isolated produced typical MCFV CPE in bovine thyroid monolayers, lamb testis and lamb kidney monolayers. The CPE was characterized by formation of smooth syncytial masses. Two steers which were inoculated subcutaneously with the 4th subculture of wildebeest thyroid cells and fluids developed the fatal 'head and eye' form of MCF[2] after an incubation period of 20 days. The illness lasted 6 and 8 days, respectively. Viremia was first detected 14 days p.i. and lasted to death. The gross post-mortem lesions also were characteristic of MCF[2].

All but one of the wildebeest nasal swab samples in the first collection yielded negative results. The only isolation of MCFV was made from wildebeest cow W212, from unclarified nasal swab fluid collected on the day of transportation from the quarantine pens to the laboratory paddock. This wildebeest unfortunately died through an adverse reaction to the immobilizing drugs. Nasal swab fluids inoculated onto BTh roller tube monolayers produced typical MCFV CPE by the 10th day p.i. The agent thus isolated was passaged successfully in BTh cultures and at the 6th passage level it was inoculated into two steers in which it caused typical MCF. MCFV was isolated from nasal secretions from one of three wildebeest (W30) captured and introduced into the paddock on 18th July, 1973. This animal was tested for MCFV in blood and nasal secretions 8 times at approximately weekly intervals. MCFV was isolated only from the last sample of nasal secretions taken on 20th September, 1973.

FIGURE 1. Captive wildebeest in close association with cattle.

TABLE 1. Persistence of MCFV Neutralizing Antibody in Sera of Captured Wildebeest.

Date Animal No.	Feb. 1	Feb. 26-27	March 8	April 25	May 10-17	July 18	Aug. 8-17	Sept. 11
W207	2.0**	2.0						
W208	2.0	1.6						
W209	1.5	≥2.0						
W210*	0.8	1.4	1.1	1.6				
W211	1.2	1.5						
W212*	1.2	1.5						
W213	2.3	2.5			2.5***	2.8	2.4	2.6
Calf WC21								
ex 213					2.7***	1.0	2.0	
W214	1.0	1.6		1.2***	1.4	0.8		
Calf								
ex 214				<0.5***				
W263	1.6	1.9			1.6***		1.8	2.8
Calf WC25*								
ex 263					<0.5***	1.8	2.0	

* MCFV isolated

** $Log_{10}SN_{50}$ MCFV neutralized by serum drawn on the dates specified

*** Each calf and its dam were bled for serum within 1 day after birth

MCFV was isolated also from nasal secretions of an orphaned wildebeest calf which was captured by motor vehicle chase when it was about 2 weeks old. An attempt was made to rear it by bottle feeding but it died on the 18th day of captivity, having become weak and lethargic. MCFV was isolated from a nasal swab specimen taken when the calf was moribund but no virus was found in a blood specimen taken at the same time. In this case the nasal swab fluid had been clarified by centrifugation. MCFV was isolated from both the supernate and deposit.

Effect of a Corticosteroid on Virus excretion

Two experiments were carried out in order to assess the effect of a corticosteroid drug on MCFV excretion. In the first experiment, the three surviving wildebeest from the first group in the wildebeest-cattle contact experiment (W213, W214, W263) and the two wildebeest calves (WC21, WC25) were transferred from the paddock to holding pens; each dam was housed together with her calf. These animals had not shown viremia nor nasal virus excretion during an observation period of 5½ months. The wildebeest cows were each given a course of 40 mg betamethasone daily by intramuscular injection for 7 days. Each cow was also given a daily intramuscular injection of 2 x 10^6 i.u. penicillin and 2 g streptomycin. Handling was facilitated by xylazine sedation. All of the wildebeest cows and calves were each tested daily for viremia and nasal virus excretion for 9 days, at which time they were returned to the paddock where they continued to graze with contact steers and three other freshly introduced wildebeest. Sampling was continued on days 14, 21 and 40 after the first betamethasone injection.

Viremia was not demonstrated in any of the animals but MCVF was isolated from the nasal swab sample collected from cow W263 on the 14th day after

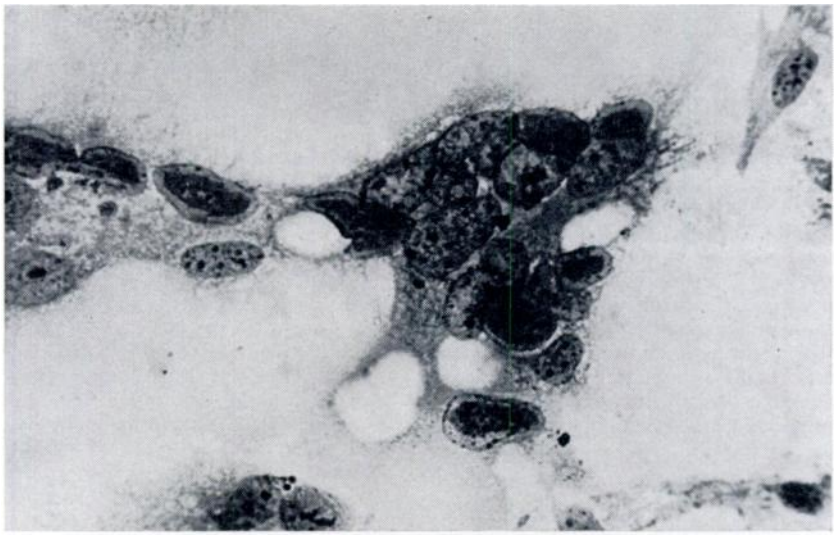

FIGURE 2. Syncytium produced in bovine thyroid cell monolayer by a strain of malignant catarrhal fever virus isolated from wildebeest nasal secretions. Note prominent inclusions in some of the nuclei. Haematoxylin-eosin x 800.

the initial betamethasone injection (i.e. 7 days from the last injection). Virus was also isolated from her calf, WC25, persistently from the 14th up to the 56th day, when it died. Altogether there were six nasal MCFV isolations made from this calf by weekly samplings. Before it died calf WC25 had been thin and unthrifty, presumably because of early cessation of lactation by its dam, caused probably by the betamethasone injections. It died after immobilization and handling. Necropsy revealed no lesions suggestive of MCF. No virus was isolated from spleen and lymph nodes but MCFV was isolated from the buffy coat of the terminal blood sample.

In the second experiment, eight wildebeest cows were immobilized in the field and held in pens at the capture site. They were given a daily course of betamethasone by intramuscular injection for 7 days and blood and nasal secretions were tested as in the first experiment, except that vaginal swabs also were taken. From one wildebeest cow MCF virus was isolated from nasal secretions collected on the 13th day after the beginning of the 7 day course of betamethasone injections.

All eight of these wildebeest had MCFV neutralizing antibody at the beginning of the experiment, with titres ranging from 1.8-2.2 $\log_{10}$ SN_{50}. Sera taken 11 to 16 days later, after the betamethasone injections but tested at the same time as the pre-injection samples, had titres reduced in six of seven of the animals, the mean reduction being 0.4 $\log_{10}$ SN_{50}. One of the post-injection sera was lost. The one wildebeest which did not show a reduction in antibody titre following betamethasone treatment was the one from which MCFV was isolated from nasal secretions. This wildebeest showed a titre of 1.8 $\log_{10}$ SN_{50} in both the pre-and post-injection serum samples.

From the 7th to the 9th days, all eight wildebeest in this experiment developed pustular vulvo-vaginitis. Infectious bovine rhinotracheitis (IBR) virus was isolated from vaginal swab specimens from seven of the eight wildebeest. This disease outbreak is described in another publication.[4]

Wildebeest—Cattle Contact

Altogether 17 steers were used in the contact experiment in the paddock at Muguga. Initially three groups of four animals each were serially introduced into the paddock. The first group was removed from the paddock at the time of the first wildebeest abortion. The second group was introduced then and removed on the day of the second abortion and the third group which was introduced at that time was removed when the first wildebeest calved normally, at which time a fourth group of five steers was introduced and remained in contact with the wildebeest for 190 days. The first 12 cattle have remained healthy to date (12 months). The last group of five cattle was housed after having been in contact with wildebeest for 190 days without any clinical response. They were then challenged by subcutaneous inoculation with the nasal virus isolate from W30 to determine their susceptibility to MCF. Before challenge they were blood sampled daily for 14 days for evidence of viremia. Only three cattle were challenged and the other two were housed with them in one loose box. Rectal temperatures were recorded daily and whole blood samples were taken at 2 day intervals until the first animal showed a clinical response, after which blood samples were collected daily up to the time of death. On the 6th day p.i. one of the inoculated cattle and one uninoculated control animal were viraemic. This control animal became febrile on day 12 and the pyrexia together with viraemia continued until death on the 21st day p.i. The inoculated cattle became febrile on days 16, 19, and 20, respectively, and died on days 26, 27 and 30 p.i. Viraemia was demonstrated in all animals. The CPE in cell cultures inoculated with blood samples and the clinical and pathological signs in the three inoculated and the control were all typical of MCFV.

Isolation of MCFV from Wildebeest Tonsil

Blood and nasal swab specimens were tested for MCFV form 43 free-ranging wildebeest comprising 36 adult females,

two adult males and five calves. No virus isolations were made. MCFV was isolated, however, from the tonsil of a pregnant wildebeest shot on 20th November 1973, about 4 months before the normal calving period.

Other numbers of wildebeest tissues processed but found negative for MCFV were the following: lymph node 9, thyroid 6, foetus 4, trachea 3, pharynx 3, nasal mucosa 2, brain 1, vagina 1, and placenta 1.

The wildebeest tonsil MCFV isolate was inoculated subcutaneously into four 3 year old steers, as 10 ml each of infected tissue culture medium and cells from the third passage in BTh cells. All four steers developed typical MCF. Viraemia was first demonstrated on days 12, 14, 14 and 24 p.i., respectively, and fever commenced on p.i. days 20, 18, 20 and 29, i.e. 4 to 8 days after the beginning of viraemia. Viraemia was detected intermittently until the animals died, on days 27, 28, 30 and 58 p.i. respectively. Nasal secretions were swabbed and inoculated onto BTh cells at 2 day intervals until the first animal became febrile, then daily until death. MCFV was not isolated from any of the nasal swab samples.

Summary of MCFV Isolations from Wildebeest

Blood and nasal swab specimens and various body tissues were tested for MCFV from 66 wildebeest, comprising 55 adult females, three adult males, and eight calves. Only one isolate of MCFV was made from 43 free-ranging wildebeest when specimens were taken at the time they were shot or captured; this isolate was from tonsil tissues. In contrast, isolates of MCFV were made from nasal secretions of six of 23 captive wildebeest, four from 19 adult females and two from three wildebeest calves. MCFV was isolated repeatedly from nasal secretions of one of the captive calves. One isolation of MCFV was also made from thyroid tissues of a captive wildebeest cow.

DISCUSSION

In the studies reported here, MCFV was demonstrated in the nasal secretions of six of the 66 wildebeest (i.e. 9%) and altogether, virus was demonstrated in 8% of 131 nasal swabs tested. In contrast only one of the 168 wildebeest blood specimens yielded MCFV, in spite of the fact that a much greater amount of cellular material was inoculated from buffy coats than from nasal swabs. Hence, the quantity of virus in nasal secretions seems to have been higher than in blood. Also this nasal virus excretion in the absence of a detectable viraemia seems to suggest the source of such virus to have been located either in the upper respiratory or the pharyngeal-tonsilar regions. Our isolation of MCFV from tonsilar tissue lends weight to this argument. Besides the kidney and thyroids, MCFV may also persist in the tonsilar or pharyngeal tissues of carrier wildebeest. Further studies are required to determine whether persistence of MCFV in the tonsil occurs only in wildebeest, which could explain why this species transmits MCFV readily to cattle, and horizontally within the wildebeest population.

Our results indicate that shedding of MCFV in nasal secretions is probably the principal mechanism for transmission of infection among wildebeest and from wildebeest to cattle, at least during close contact, as shown previously by Plowright.[5,6] We have not been able so far to demonstrate unequivocally whether wildebeest excrete virus nasally in a stable infectious cell-free state. All nasal virus isolates except one were made from unclarified fluid. In one instance in which we isolated virus after attempts to separate out cell-associated virus by light centrifugation, virus was isolated from both the supernate and precipitate. However, until further evidence is obtained, these results do not constitute sufficient grounds for claiming that wildebeest excrete MCFV nasally in a cell-free stable infectious state. Our isolate of virus from the supernate might be a reflection of poor cell packing by centrifugation. The progeny of all the isolates in our studies were cell-associated. In this context, it

should be observed that infectious cell-free virus has been demonstrated in the feather follicle epithelium in Marek's disease, another typically cell-associated herpesvirus infection.[1]

Plowright detected higher rates of viraemia in wildebeest calves than in adults, and among adults, viraemia was most common during late pregnancy.[5,6] Epizootiologically, MCF in cattle is usually associated with calving in the contact wildebeest population. The stresses of late pregnancy and calving probably lead to a reactivation of latent virus in wildebeest, to transplacental infection and wildebeest-to-wildebeest transmission, in addition to dissemination of the virus by neonatal calves, as demonstrated by Plowright.[5,6] In our studies, we isolated MCFV from nasal secretions from animals which had been held in confinement in pens for a period, under the stress of artificial feeding, or after betamethasone administration. Although the numbers studied are small, our results indicate that betamethasone reactivated latent MCF virus infection in wildebeest, in the same manner as Sheffy and Davies[12] demonstrated reactivation of IBR virus in cattle. Incidentally, we also obtained evidence of reactivation of IBR in the wildebeest.[4]

Grazing of wildebeest in a paddock does not seem to have constituted sufficient stress to the wildebeest to result in carrier breakdown. That the majority of the wildebeest in the paddock probably carried MCF virus is supported by the isolation of MCFV from W263 after betamethasone injections and in cell cultures derived from the thyroid of W210. The virus isolated from the nasal secretion of wildebeest cow W30 may well have originated from W263 or calf WC25, since these two animals had been excreting virus for at least 7 weeks whilst in contact with W30 and before virus was detected in W30. The apparent spontaneous development of MCF in one of the five cattle is most likely explicable on the steer having acquired infection from contact with wildebeest in the paddock. These cattle were probably challenged when some of them were incubating the infection. It is unlikely that the control steer which developed apparently spontaneous MCF could have acquired infection from the inoculated pen-mates, as it developed viraemia and disease before the inoculated animals. Also, retrospectively, we have realized that this animal developed MCF viraemia 61 days after our last isolation of MCFV from nasal secretions of wildebeest W30 in the paddock. Such a period is within the limits of the incubation period of MCF in cattle.[2,6]

These experiments have provided additional evidence on the carrier status of MCFV in wildebeest and have demonstrated that nasal excretion may be the principal route of transmission of MCFV from wildebeest to cattle and within the wildebeest population.

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