Cultivation in Tissue-Culture of Cytopathogenic Agent from Bovine Mucosal Disease.* (23091)

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Since 1951 bovine mucosal disease has been reported from 20 states and Canada. Because the symptoms of mucosal disease are similar to those of other virus diseases it is believed that many cases are not diagnosed. The disease is characterized by ulceration of mucous membranes of oral cavity and of the digestive tract, increased temperature, nasal and lachrymal discharges, and diarrhea. Mucosal disease as seen in field cases has a characteristic morbidity of 2-10% with case mortality of 95%. The etiology of the disease is unknown. Ramsey(1) and Pritchard(2) showed that transmission studies made in cattle using macerated tissues and whole blood were inconclusive. Preliminary studies here presented suggest the difficulty in finding experimental animals without some degree of immunity.

To date nothing has been reported in the literature on tissue-culture isolations of agents from cattle with mucosal disease. This is a report of 2 isolations from material obtained from tissues from cases of bovine mucosal disease and a study of some characteristics of these viruses.

Materials and methods. Bovine kidney cells used for tissue-cultures were obtained from fetuses 12-15 inches long. Kidney tissues were trypsinized and prepared according to the method of Young *et al.*(3). The cells were started on medium containing 20% adult bovine serum (BoA) and 0.5% lactalbumin hydrolysate (LA) in Hanks'(6)(H) balanced salt solution. Penicillin (100 units) and streptomycin (100 μg)/ml were added to all basal media. Tissues were cultivated in Pyrex tubes (12 x 75 mm) each containing a coverslip (Corning 8 x 22 mm), and sealed with No. 00 silicone stopper. The tubes were seeded with approximately one million kidney cells in one ml of medium (BoA 20% + LA + H), placed in horizontal rack at 12° angle, and incubated at 37°C. Tissue cultures were inoculated with the virus material after incubating one week. To remove any antibodies present from the adult bovine serum used for initial growth, the tissue cultures were washed with medium (BoN 5% + LA + H) prepared with antibody-free serum. This serum was obtained from a newborn calf which had never nursed. After the second wash the medium was removed and replaced with one ml of BoN 5% + LA + H in which the appropriate dilution of virus was suspended. The cultures were again incubated at 12° angle and 37°C. *Virus materials* were obtained from 2 sources: M-833, a pool of tissues from a mucosal disease outbreak in central Nebraska herd(4) and ISC-1, a pool of lymph nodes obtained from animal with mucosal disease from Iowa herd(5). In preparation for inoculation, tissues were minced with washed ground glass (Pyrex) in mortar and diluted 1:10 with BoN 5% + LA + H. This material was then centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant was then withdrawn and treated with 1000 units of penicillin and 1000 μg streptomycin/ml for one hour at 4°C. Following this treatment a final dilution of 1:100 with BoN 5% + LA + H was prepared for inoculating the washed tissue cultures. In preparation for transfer of the virus from the tissue cultures (5th-7th day), approximately 0.5 g of sterile ground glass was added to each tube. The cultures were then shaken for 10 minutes to disrupt the cells. The supernatant was removed with capillary pipette, treated with 1000 units of penicillin and 1000 μg streptomycin, and centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant ma-

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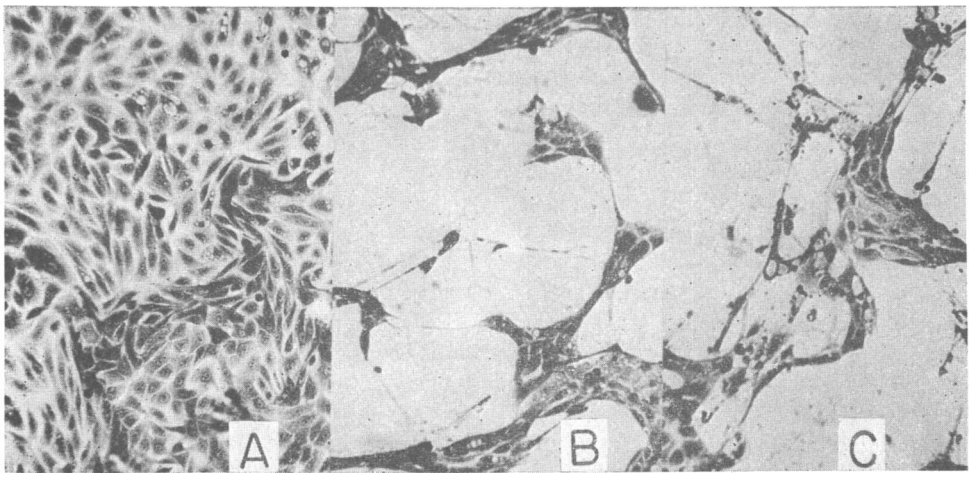

FIG. 1. Photomicrographs of May-Grünwald-Giemsa stained bovine kidney tissues. (A) Normal tissue 12 day control, (B) tissue 12 days following inoculation with virus strain M-833, (C) tissue 13 days following inoculation with virus strain ISC-1.

terial was either quick frozen and stored at -20°C or diluted 1:100 with BoN 5% + LA + H for reinoculation into washed tissue cultures. The cultures were examined microscopically for cytopathogenic changes by viewing living cells on tube wall and coverslip. When the cell changes were noted the coverslip was removed aseptically with the split end of a sterile applicator stick, stained by the May-Grünwald-Giemsa method(6), and permanently mounted on slide.

Results. Following 7-10 days incubation of virus and tissue, cytopathogenic changes were apparent. These changes started with small vacuoles forming within the cells and increasing in size until the cell disintegrated on about 10th-14th day. Fig. 1 shows photomicrographs of May-Grünwald-Giemsa stained tissues. Identity is as follows: (A) Normal tissue control stained 12 days after washing and feeding with BoN 5% + LA + H. This layer, composed mainly of epithelial cells, covered entire coverslip and was typical of growth obtained with bovine embryonic kidney cells; (B) and (C) Bovine kidney cells inoculated with virus strains M-833 and ISC-1. Both of these virus strains have the same general pattern of attack. These typical photographs show large areas of cell destruction which is followed by further disintegration until all of the tissue is completely destroyed.

Preliminary studies with virus strain M-833 show that it has an 11th passage titer of 10^{-5} (total accumulated dilution titer 10^{-22}), is inactivated over 50°C for 15 minutes, and will pass 03 Selas filter. Serial passages in an attempt to adapt the virus to 7-day embryonated chicken eggs by yolk sac inoculations were negative. The virus strain ISC-1 was also inactivated by temperatures over 50°C for 15 minutes and passed an 03 Selas filter. During primary isolation of strain M-833, 3 unrelated viruses and a tissue control were also serially passed in kidney tissue cultures from the same fetus. None of these developed cytopathogenic changes, alleviating the possibility that the agent was isolated from the kidney tissue. Strain ISC-1 was isolated later using kidney cells from a different fetus.

Serological studies were done in sheep with virus strain M-833 sixth passage material. Two sheep were each given one ml of the virus tissue culture supernatant material intravenously at weekly intervals for 3 weeks. The sheep were bled prior to initial inoculation with virus and 3 weeks following final inoculation. The results are presented in Table I and indicate that virus strain M-833 stimulated formation of neutralizing antibodies. In cross-neutralizing tests the antibodies stimulated by M-833 were also capable of neutralizing virus strain ISC-1. This suggests a relationship between the 2 strains.

TABLE I. Neutralization of M-833 and ISC-1 Viruses by Serum from Sheep Immunized with M-833 Virus.

	Virus M-833		Virus ISC-1	
	Pre-inoculation*	Convalescent*	Pre-inoculation*	Convalescent*
Sheep 145	—	+	—	+
160	—	+	—	+

* All serums diluted 1:10.
— = Antibody absent; + = Antibody in sufficient amounts to neutralize approximately 1000 infective doses of virus.

A preliminary study of bovine serum samples from 6 widely separated herds indicates that antibodies capable of neutralizing this agent are common in herds having no history or symptoms of mucosal disease. Formation of these neutralizing antibodies resulted from an infection of unknown etiology. Neutralizing antibodies were generally not found in serum samples from younger range cattle.

Although this viral agent was isolated from lesions of cattle having mucosal disease, the evidence presented does not prove it to be the causative agent of this disease. This proof can only be obtained by establishing the disease with this virus in a susceptible calf. The presence of neutralizing antibodies in the calves available for experimentation makes it impossible to conduct a study of this type at the present time. Experimental work along these lines would, of necessity, require disease-free, antibody-devoid calves taken by caesarotomy or hysterectomy and raised in strict isolation or preferable in Horsfall-Bauer type units designed for calves. These experimental studies will be undertaken as soon as these conditions can be provided.

Summary. Isolation and cultivation in tissue culture of a cytopathogenic agent recovered from tissues of cattle with mucosal disease from 2 separated herds is reported. A homologous tissue culture system was used with bovine embryonic kidney cells and newborn, antibody-free calf serum. The virus was inactivated by temperatures above 50°C for 15 minutes, would pass an 03 Selas filter and had an 11th passage titer of 10^{-5}. In sheep, the virus stimulated formation of neutralizing antibodies. A preliminary neutralization study indicated that antibodies are common in animals from herds having no previous history of mucosal disease.

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