Contamination of Primary Embryonic Bovine Kidney Cell Cultures with Parainfluenza Type 2 Simian Virus 5 and Infectious Bovine Rhinotracheitis Virus

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Two distinct viruses were isolated from bovine embryonic cell cultures after two subcultures from the primary cells. One virus was identified as parainfluenza type 2 simian virus 5 (SV-5), and the other was identified as infectious bovine rhinotracheitis virus. Six months later, stock cultures of pig kidney (PK-15) cells were found to be contaminated with SV-5 virus. We believe that the source of the SV-5 virus in the bovine cells was a cross-contamination from monkey kidneys during preparation of the cell cultures. The infectious bovine rhinotracheitis contamination was probably of endogenous origin. The bovine embryonic cell cultures were the probable source of contamination of the PK-15 cells with SV-5 virus.

The hemadsorption (HAD) technique is routinely used in this laboratory for the detection of bovine parainfluenza (PI-3) virus in cell cultures inoculated with clinical specimens. During the summer of 1975, HAD was demonstrated in several lots of uninoculated, commercially prepared primary bovine embryonic kidney (PBEK) cells grown in culture tubes. Attempts to isolate a virus from positive HAD cultures by passing the cells and fluid after one freeze/thaw cycle were unsuccessful. However, the procedure of subculturing viable cells yielded two distinct viruses.

Because endogenous bovine viruses are known to occur in commercially produced fetal bovine serum (8, 10, 13) and in primary bovine cell cultures (1, 9), it was postulated that both viruses were of bovine origin. The two viruses were later identified as a bovine herpesvirus and a myxovirus probably of simian origin.

This report describes the isolation and identification of infectious bovine rhinotracheitis (IBR) and parainfluenza type 2 simian virus 5 (SV-5) viruses from bovine embryonic kidney cells and discusses the probable sources of contamination.

MATERIALS AND METHODS

Cell cultures. Pig kidney (PK-15) cells were grown in tubes and flasks with Eagle minimal essential medium with nonessential amino acids, glutamine, 0.5% lactalbumin hydrolysate plus 10% fetal bovine serum, and antibiotics. PBEK cells in tubes and bottles were obtained from a commercial source. Minimal essential

medium with 2% fetal bovine serum was used as maintenance medium. All cell culture media contain penicillin (200 U/ml), streptomycin (200 µg/ml), and amphotericin B (2.5 µg/ml). PK-15 and PBEK cell cultures were serially passed by splitting 1:3 with trypsin-ethylenediaminetetraacetic acid. Primary human embryonic kidney (HEK) and primary rhesus monkey kidney (MK) cell cultures were handled and passaged as described elsewhere (6).

Processing of serum for virus isolation. Stock fetal bovine serum was tested for endogenous viruses by incorporating 6 ml of serum into the growth medium of cells grown in 75-cm² flasks. When confluent, the cells were passed into Leighton tubes containing cover slips. The cover slip preparations were examined by HAD and by fluorescent microscopy after staining with specific IBR conjugate.

Egg inoculation. Embryonated chicken eggs were inoculated via the amniotic and allantoic routes and blind-passed twice as previously described (11).

Animal inoculation. The virus used for animal inoculations was the seventh cell culture passage of the BEK-22 isolate. After purification by three terminal dilutions, the inoculum was prepared in a flask culture of PBEK cells. The virus harvest was centrifuged at 4,300 × g for 20 min to clarify. The supernatant fluid was stored at -68°C until used.

Guinea pigs were immunized by intranasal instillation of 0.1 ml of undiluted virus under light anesthesia, followed 21 days later by 1.0 ml of virus given intraperitoneally. Serum was obtained before inoculation and at 21 and 42 days after the initial inoculation. Two 2-week-old seronegative calves of mixed breed were used in transmission studies. One calf was inoculated intranasally and the other was inoculated intratracheally with 10 ml of undiluted virus containing 10^{6.5} mean tissue culture infective doses per 0.1 ml. Nasal

swabs were obtained before and after virus exposure for virus isolation. Blood was collected before and 21 days after inoculation for serological testing. Hemagglutination (HA) and hemagglutination inhibition (HI) tests with the guinea pig and calf sera were performed with guinea pig erythrocytes in microtiter plates as previously described (5).

Chloroform sensitivity. Sensitivity to chloroform was determined by the method previously described (4).

Histological preparations. Infected cell cultures grown on cover slips were fixed with Bouin fluid and stained with hematoxylin and eosin.

Electron microscopy. Electron microscopy was carried out on unconcentrated cell culture fluids harvested when nearly 100% of the cells were showing cytopathic changes as described (6).

Serology. Complement-fixation (CF) tests were carried out by the standard CF method with overnight fixation of 5 units of complement (2). HA and HI tests to identify the myxovirus isolates were performed by the standardized methods with 0.4% guinea pig erythrocytes and 0.01 M phosphate-buffered saline diluent (7). HAD and HAD inhibition were performed in PBEK tubes with guinea pig cells as described by Chanock et al. (3). Bovine serum samples submitted to the diagnostic laboratory for routine serology were randomly selected and tested for antibodies to the myxovirus isolate by the HI test (5).

RESULTS

After positive HAD, all direct attempts to isolate an agent from the fluids and cells from primary cultures of bovine embryonic cells failed; therefore, the cells were subcultured. On day 2 after the second subculture of the bovine kidney cells grown on cover slips, syncytial formations were observed (Fig. 1). In hematoxylin and eosin-stained cell culture preparations, two types of cytopathic effects were recognized. One was characterized by syncytial cells containing eosinophilic inclusion bodies in the cytoplasm (Fig. 2), and the other was characterized by cells containing nuclear inclusion bodies suggestive of a herpesvirus (Fig. 3). Both viruses were completely inactivated after chloroform treatment.

The two viruses were separated by two serial passages in the presence of IBR immune serum. The syncytium-producing virus referred to as BEK-22 was further passed in PBEK, MK, and HEK cells after three terminal dilutions in PBEK cells. No additional studies were done with the IBR isolate.

The BEK-22 isolate replicated well in PBEK and MK cells with myxovirus-like cytopathology but to a lesser degree in HEK cells. All positive cultures hemadsorbed guinea pig erythrocytes. No virus was demonstrated after three passages in embryonated eggs inoculated by the amniotic and allantoic routes. Likewise, virus was not demonstrated in cell cultures grown in the pres-

FIG. 1. Multinucleated giant cells in second subculture of bovine embryonic kidney cells infected with BEK-22 virus. Hematoxylin and eosin stain; $\times 100$.

FIG. 2. Giant cell with multiple cytoplasmic inclusion bodies in second subculture of bovine embryonic kidney cells infected with BEK-22 virus. Hematoxylin and eosin stain; $\times 380$.

ence of high concentrations of stock fetal bovine serum. Examination by electron microscopy of negative-stained preparations revealed pleomorphic parainfluenza-like particles measuring 121 to 346 nm, with an average diameter at 192 nm. Disrupted particles released long coils of ribonucleoprotein.

CF tests with the BEK-22 isolate with reference antisera to common respiratory viruses revealed that the virus was parainfluenza type 2 (Table 1). HI tests with reference antisera to the 13 myxoviruses clearly showed the isolate to be SV-5 (Table 1). The identity of SV-5 was confirmed by reciprocal HI tests with known SV-5 reference reagents and the isolate and immune

FIG. 3. Intranuclear inclusion bodies in second subculture of bovine embryonic kidney cells infected with IBR virus. Hematoxylin and eosin stain; $\times 760$.

guinea pig serum prepared against BEK-22 (Table 2).

Attempts to experimentally infect calves with the SV-5 isolate were unsuccessful. The BEK-22 virus was not recovered from the nasal secretions obtained from the exposed calves, and acute- and convalescent-phase calf sera were negative for HAD inhibition and HI antibodies.

A total of 35 bovine serum samples randomly selected from nine herds were tested by HI. HI occurred in one serum sample at a final serum dilution of 1:8.

DISCUSSION

We have shown that later serial passages of uninoculated bovine embryonic cell cultures were infected with two different viruses—one a bovine herpesvirus and the other a simian paramyxovirus. We assume that the IBR isolate was probably of endogenous origin because it can naturally occur in fetal calf kidneys (1, 6) and the virus could not be demonstrated in the fetal bovine serum used. The absence of cytopathic effects in the PBEK cells was not considered unusual because primary viral isolates do not always elicit typical cytopathic effects (8). The virus content of the lot of contaminated PBEK cells was probably low because virus was not detected in all flasks of cells serially passed.

The simian virus was chloroform sensitive, possessed structural and cultural characteristics of the paramyxoviruses, and was serologically identified as SV-5. The other virus was chloroform sensitive, produced herpetic-type cyto-

pathic changes in cell cultures, and was neutralized by specific IBR immune serum.

The origin of the two viruses is of special interest. Although parainfluenza type 3-like viruses have been recovered from fetal bovine serum (8) and bovine parainfluenza virus (PI-3) has been isolated from bovine fetal tissue (12, 14), parainfluenza type 2 SV-5 to our knowledge has not been reported as being associated with bovine infections. No significant levels of HI antibody to the isolate were demonstrated in a limited number of bovine serum samples. The fetal bovine serum used in the cell culture was

TABLE 1. CF and HI titers of reference antisera tested with the two unknown myxovirus antigens

Test	Antiserum	Serum titer with optimal dilution of antigen ^a	
		BEK-22 ^b	BEK-22A ^c
CF	Influenza		
	A	<8	<8
	B	<8	<8
	Parainfluenza		
	1	8	8
	2	64	64
	3	<8	<8
	Mumps	16	8
	NDV	<10	10
HI	Influenza		
	A	<10	<10
	B	<10	<10
	Parainfluenza		
	1 C-35	<10	10
	1 Sendai	<10	10
	2 Greer	10	40
	2 SV-5	160	640
	2 SV-41	<10	<10
	3 C-243	<10	10
	3 SF-4	<10	10
	4A	<10	10
	4B	<10	<10
	Mumps	<10	10
	NDV	<10	10

^a CF antibody titer is defined as the dilution factor of the highest dilution of serum fixing 5 units of complement overnight in the standard CF test. HI antibody titer is defined as the dilution factor of the highest dilution of serum completely inhibiting 4 HA units of virus per 0.025 ml in 1 h at 23°C.

^b Isolate passed seven times in PBEK cells and twice in MK cells.

^c Isolate passed seven times in PBEK cells, five times in HEK cells, and once in MK cells.

TABLE 2. Reciprocal HI titers with parainfluenza type 2 and BEK-22 immune sera against parainfluenza type 2 and BEK-22 viruses

Serum	Reciprocal HI titer ^a		
	Parainfluenza 2 (Greer)	Parainfluenza 2 SV-5 (Hull)	BEK-22
Parainfluenza 2 (Greer)	160	10	10
Parainfluenza 2 SV-5 (Hull)	<10	160	80
BEK-22 A ^b	<10	20	10
BEK-22 B ^c	<10	160	40
BEK-22 C ^d	10	640	160

^a HI titer is defined as the dilution factor of the highest dilution of serum completely inhibiting 4 HA units of virus per 0.025 ml in 1 h at 23°C.

^b A, Guinea pig preinoculation serum.

^c B, Guinea pig serum 21 days after intranasal exposure.

^d C, Guinea pig serum 21 days after intraperitoneal inoculation.

negative for antibodies to BEK-22, and attempts to isolate a virus by serially passing cells with 20% fetal bovine serum were unsuccessful. Furthermore, the failure to infect or produce a seroconversion in two calves with the isolate suggests that the bovine species is not readily susceptible to SV-5 virus. Thus, the SV-5 contaminant almost certainly was not endogenous in the PBEK cells.

An inquiry into the commercial source of the PBEK cells revealed that monkey cell cultures were prepared in the same room as the bovine cells. Because SV-5 virus infection is common in monkeys, we postulated that cross-contamination occurred between the monkey and bovine kidneys during preparation of the cell cultures. This possibility is supported by our observation that SV-5 readily cross-contaminates other culture systems in laboratories where SV-5-infected cells are handled in proximity to other cells (J. C. Hierholzer, unpublished data). Such cross-contamination is a frequent occurrence and is probably due to a combination of the properties of SV-5: rapid replication to high infectivity titers, relative stability of the virus in aerosols, and growth in many different culture systems.

Six months after the BEK-22 virus was isolated, syncytial formations occurred in stock cultures of PK-15 cells. HAD was demonstrated, and the agent referred to as PK-1463 was further passed in cell culture and studied. The PK-1463 virus was chloroform sensitive, and its ultrastructural features were similar to those of the BEK-22 virus. The virus was serologically identified as SV-5 by CF and HI. The CF and HI

titers of reference sera were identical to those reported in Table 1 with the BEK-22 antigen (1:64 and 1:160, respectively). HI titers of SV-5 and BEK-22 immune sera against the PK-1463 antigen were 1:80 and 1:320, respectively, and were similar to the results shown in Table 2 with the SV-5 and BEK-22 antigens.

It is interesting that the SV-5 contamination of the PK-15 cells was not discovered until 6 months after its isolation from the bovine cells. Although all contaminated PBEK cultures were removed from the cell culture laboratory when the virus was recognized, we believe that the source of contamination of the PK-15 cells was the bovine cells. Also, MK cell cultures were never used in the cell culture laboratory. No further problem has been encountered during the 18-month period after the PK-15 contamination.

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