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Susceptibilities of 14 Cell Lines to Bluetongue Virus Infection

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The effect of bluetongue virus (BTV) infection was investigated in 14 cell lines. The cell lines included the following vertebrate cells: baby hamster kidney, African green monkey kidney (Vero), rabbit kidney, bovine kidney, canine kidney, bovine turbinate, bovine endothelium (CPAE), bighorn sheep tongue, equine dermis, gekko lung, rainbow trout gonad, and mouse fibroblast (L929); they also included the following invertebrate lines: mosquito and biting midge. Comparisons between the cell lines were made on the basis of time to observed cytopathic effects, titer in 50% tissue culture infectious doses, and titer in plaque-forming units. The CPAE cell line produced the highest BTV 50% tissue culture infectious dose of all cell lines tested. The Vero and L929 cells gave the most discrete plaques in plaque assays. Of the 14 cell lines tested, the CPAE cells were the most susceptible to both cell culture-adapted and animal source BTV. Bovine endothelial cells demonstrate significant potential as a cell culture system for BTV investigations.

Bluetongue virus (BTV), an orbivirus, can induce disease in ruminants (9). Many different cell lines are susceptible to BTV-induced cytopathic effect (CPE); however, they vary in the extent of CPE (2). Two cell lines, baby hamster kidney (BHK-21) and African green monkey kidney (Vero), are frequently used in BTV investigations (1); however, both lines have limitations. BHK-21 cells are quite susceptible to cell culture-adapted BTV yielding high viral titers, but the cells quickly deteriorate when overcrowded and need close monitoring and frequent passaging. Vero cells are more tolerant of overcrowding, so they are better suited for long-term assays, but they are slower to demonstrate BTV-induced CPE (personal observation). Another limitation is that these cell lines are relatively insensitive to animal source BTV; therefore, intravascular inoculation of embryonated chicken eggs (ECE) is the recommended procedure for primary isolation of BTV field isolates (3).

The goal of the current investigation was to compare the susceptibility of various cell lines to BTV with that of the BHK-21 and Vero cell lines currently used in our laboratory. We were looking for a cell line that would demonstrate easily observed CPE to BTV within a reasonably short period of time (2 to 5 days), produce BTV titers similar to or higher than those produced in BHK-21 or Vero cells, be reasonably tolerant to overcrowding, and, if possible, be similar to ECE in sensitivity for detection of animal source BTV.

MATERIALS AND METHODS

Cells. Cells were obtained and maintained as listed in Table 1. Heat-treated fetal bovine serum was used for all cells except BHK-21 cells. The serum and all cell lines were routinely checked for the presence of bovine viral diarrhea virus and mycoplasma.

Virus. BTV serotype 11, passaged 12 times in cell culture, was used for all trials with cell culture-adapted virus. The titer (50% tissue culture infectious dose [$TCID_{50}$] per ml) in BHK-21 cells was 8.0 $\log_{10}$.

For trials with animal source BTV, blood samples from sheep experimentally infected with BTV serotypes 2, 10, 13, and 17 and brain suspensions from mice inoculated with BTV serotype 11 were used. Heparinized blood was taken from sheep that became infected after intradermal or subcutaneous inoculation with cell culture-adapted BTV or after being bitten by BTV-infected *Culicoides variipennis* (gnat or biting midge), the principal BTV insect vector in the United States (8). Brain samples were taken from mice that died following intracranial inoculation with BTV as newborns. The blood and brain samples were stored at 4°C and sonicated prior to assays.

Tissue culture infectivity titration of virus. The $TCID_{50}$ was determined for each cell type that demonstrated BTV-induced CPE. Serial 10-fold dilutions of samples were made in Hanks balanced salt solution. For each cell type, 50 μl of each virus or virus-free diluent was placed in each of 8 replicate wells of a 96-well tissue culture plate to which 100 μl of cell suspension was added. The concentrations at which the cells were seeded are recorded in Table 2. BHK-21 cells were used as standards for each assay. The plates were incubated at 37°C in 6% CO_2 in air. Cells were observed daily. The assays were stopped when control cells sloughed or at 8 to 10 days, whichever occurred first. The $TCID_{50}$ per milliliter was determined by using the method of Reed and Muench (10). Each cell line was tested at least twice.

Virus plaque assay. For 9 cell types that demonstrated BTV-induced CPE, virus titer in plaque-forming units was determined by using a modification of a medium-agarose overlay procedure (D. R. Sundin, personal communication). Serial 10-fold dilutions of BTV were made in Hanks balanced salt solution. A 0.1-ml sample of each dilution was placed into each of duplicate wells of a sterile 8-well tissue culture plate for each cell type tested. Control wells received 0.1 ml of virus-free diluent. Each well then received 1.5 ml of cells at the indicated concentrations (Table 3). The plates were incubated for 3 h at 37°C in 6% CO_2 in humidified air. The supernatant fluid was aspirated, and 2 ml of a 0.6% low-gelling-temperature agarose (FMC Corp., Rockland, Maine)-medium solution was gently applied to each well. Plates were incubated at 37°C in 6% CO_2 in air until plaques were evident or 6 days after seeding. Cells were fixed with Formalin, overlays were removed, and monolayers were stained with 1% crystal violet. The plaques were counted,

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TABLE 1. Cell types, sources, and media used for BTV susceptibility studies

Cell line (designation)[a]	Original source[b]	Passage frequency	Medium[c]
Bighorn sheep fetal tongue explant (BHFTE)	UCD	1×/wk	MEM, 10% FBS, 4 mM glutamine
Baby hamster kidney (BHK-21; clone 13)	ATCC	2×/wk	BME, 10% TPB, 5% FBS
Bovine turbinate (BT)	ATCC	1×/wk	199, 10% FBS
Aedes albopictus (C6/36)	ATCC	1×/wk	MEM, 10% FBS
Pulmonary artery endothelial, bovine (CPAE)	ATCC	1×/wk	MEM, 10% FBS
Culicoides variipennis (CuVa)[d]	ABADRL	1×/wk	Schneider, 18% FBS
Gekko lung (GL)	ATCC	1×/wk	BME, 10% FBS
Mouse fibroblast (L929)	ATCC	2×/wk	MEM, 10% FBS
Rabbit kidney (LLC-RK1)	ATCC	1×/wk	199, 10% FBS
Madin-Darby bovine kidney (MDBK)	ATCC	2×/wk	199, 10% FBS
Madin-Darby canine kidney (MDCK)	ATCC	1×/wk	MEM, 10% FBS
Equine dermis (NBL-6)	ATCC	1×/wk	199, 10% FBS
Rainbow trout gonad (RTG)	ATCC	1×/2wk	MEM, 10% FBS
African green monkey kidney (Vero)	MARU	1×/wk	199, 10% FBS

[a] Unless otherwise indicated, the designation listed is the official ATCC catalog designation.
[b] UCD, A. E. Castro, University of California-Davis, Davis, Calif.; ATCC, American Type Culture Collection, Rockville, Md.; ABADRL, Arthropod-Borne Animal Diseases Research Laboratory; MARU, Gorgas Memorial Institute, Middle America Research Unit, Canal Zone.
[c] Medium indicated is growth medium; for viral assays, the percent of fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, N.Y.) was reduced by 50% and for BHK-21 cells the medium was minimum essential medium with nonessential amino acids and glutamine (MEM) (GIBCO). All media were adjusted to pH 7.2 except the medium for CuVa, which was kept at pH 6.7. BME, Basal Media Eagle with glutamine (GIBCO); TPB, tryptose phosphate broth (GIBCO); 199, medium 199 with Earle salts and glutamine (GIBCO); Schneider, Schneider *Drosophila* medium (GIBCO).
[d] Cell line originated by author (S.J.W.) with official designation ABAD-CuVa1087.

the mean of duplicate wells was determined, and the titer was calculated in plaque-forming units per milliliter.

Egg infectivity titration of virus. The 50% chicken embryo intravascular lethal dose ($CEIVLD_{50}$) of each sheep blood sample was determined by intravascular inoculation of 11-day-old ECE as described by Foster and Luedke (3) and Goldsmit and Barzilai (5). Briefly, 0.1 ml of serial 10-fold dilutions of sonicated sample was injected (6 eggs per dilution). The eggs were incubated at 33°C. The daily number of deaths was recorded, and assays were terminated at 7 days. The $CEIVLD_{50}$/ml was calculated by using the Reed and Muench method (10). Virus from dead eggs was amplified in cell culture prior to testing for BTV by using immunoperoxidase staining as described below.

Detection of BTV antigen by immunoperoxidase staining of samples. Samples were examined for BTV by using a commercial immunoperoxidase-staining procedure (Vector Laboratories, Burlingame, Calif.). A rabbit polyclonal antibody to BTV serotype 11, provided by J. O. Mecham (Arthropod-Borne Animal Diseases Research Laboratory), was used as the primary antibody.

Experimental procedure. Pilot studies were conducted to determine whether virus titers in tissue culture infectivity assays were affected by the time between monolayer development and BTV exposure. BTV was added to BHK-21 cells and to Vero cells either at the time of seeding or at 24 h after seeding. Final BTV titers did not differ between the two methods. For all subsequent assays, cells and virus were added concurrently.

The susceptibility to BTV of the different cell lines except gekko lung (GL) and rainbow trout gonad (RTG) was first determined by using cell culture-adapted BTV in tissue culture infectivity titration assays. The preliminary exposure of GL and RTG cells was performed by exposing a monolayer of cells in a closed 25-cm^2 flask to tissue culture virus

TABLE 2. Results of tissue culture infectivity titration of BTV in 14 cell lines

Cell line	Cell concn[a]	Titer comparison[b]	Days to titer	CPE
BHFTE	2.0×10^5	0 ± 0	4	Cell death
BHK-21	8.0×10^4	0	3	Cell death
BT	2.0×10^5	−1 ± 0	4	Cell death
C6/36	2.0×10^5	−1.9 ± 0.1	4	Vacuolation
CPAE	2.0×10^5	+0.5 ± 0.1	5	Cell death
CuVa	2.0×10^5	−0.7 ± 0.2	6	Vacuolation
GL	ND	N		None
L929	2.0×10^5	−0.2 ± 0.1	5	Cell death
LLC-RK1	3.4×10^5	−2.9 ± 1.1	7	Cell death
MDBK	2.0×10^5	−1.0 ± 0	5	Cell death
MDCK	2.5×10^5	N		None
NBL-6	2.0×10^5	−1.5 ± 0.5	3	Cell death
RTG	ND	N		None
Vero	2.5×10^5	−0.8 ± 0.3	10	Cell death

[a] Concentration of cells per milliliter at time of seeding for tissue culture infectivity titration of cell culture-adapted BTV. ND, Not done.
[b] For each assay, the BTV $\log_{10}$ $TCID_{50}$ per milliliter of BHK-21 cells was considered to be 0. The difference from the BHK-21 titer was calculated for each cell line tested and is indicated with one standard deviation. Each cell type was tested at least twice. N, No CPE.

TABLE 3. Results of plaque assays of BTV with different cell lines

Cell line	Cell concn[a]	Titer comparison[b]	Plaque description
BHFTE	4.0×10^5	−0.2 ± 0.05	Not discrete[c]
BHK-21	4.0×10^5	NC	Not discrete
BT	4.0×10^5	−1.6 ± 0.4	Not discrete
C6/36	4.0×10^5	+0.4 ± 0.2	Discrete
CPAE	4.0×10^5	+0.5 ± 0	Discrete
CuVa	9.0×10^5	NP	No plaques
L929	4.0×10^5	−0.05 ± 0.05	Very discrete
MDBK	4.0×10^5	0 ± 0	Not discrete
NBL-6	4.0×10^5	+0.4 ± 0.1	Discrete
Vero	7.0×10^5	0	Very discrete

[a] Number of cells per milliliter that were seeded for plaque assays testing cell culture-adapted BTV.
[b] For each plaque assay, the BTV titer in plaque-forming units per milliliter for Vero cells was considered to be 0. The mean difference from the Vero titer was calculated for each cell line and is indicated with one standard deviation. Cell lines were tested twice. NC, Plaques were not discrete enough to be countable; NP, no plaques.
[c] Discrete plaques had more regular borders and were easier to enumerate than nondiscrete plaques that had very irregular borders.

at 1 multiplicity of infection. Cells in control flasks were exposed to virus-free diluent. Virus was allowed to adsorb for 1 h. Medium (5 ml) was added to the flasks that were incubated in darkness at 37 and 25°C. Cells were observed daily for 10 days or until the control cells sloughed, whichever occurred first. If no CPE was noted, samples of the cells were scraped off with a rubber policeman and examined for BTV by immunoperoxidase staining.

Cells that exhibited CPE were assayed in a plaque assay using cell culture-adapted virus. For all plaque assays, Vero cells were used as control standards.

Six cell lines were tested by using blood samples from BTV-exposed sheep. These sheep were infected with BTV serotype 2, 10, or 13. The blood samples were also assayed in ECE. Cells that gave results similar to those of ECE for the initial BTV-infected sheep blood were further tested against additional BTV-infected samples that were also tested in ECE. These samples included blood from sheep that were infected with BTV serotype 2 or 17 and brain samples from mice inoculated with mouse-passaged BTV serotype 11.

RESULTS

Of the 14 cell lines that were exposed to BTV in tissue culture infectivity assays, 11 demonstrated BTV-induced CPE. No CPE was observed in three cell lines (Madin-Darby canine kidney, rainbow trout gonad, and gekko lung). Staining with immunoperoxidase detected no viral antigens in these cells. Four cell lines (mosquito [C6/36], biting midge, Madin-Darby bovine kidney [MDBK], and rabbit kidney [LLC-RK1]) demonstrated subtle CPE indicated by cell vacuolation and a low percentage of cell death in response to BTV infection (Table 2). Most cell lines demonstrated BTV-induced CPE by 4 to 5 days (Table 2).

The bovine endothelial cell line (CPAE) was the only cell line that demonstrated a higher $TCID_{50}$ titer than that of BHK-21 cells (Table 2). Five cell types (bovine turbinate [BT], C6/36, LLC-RK1, equine dermis, and Vero) demonstrated $TCID_{50}$ titers lower than those of BHK-21 cells.

Most cell lines demonstrated BTV-induced CPE by 4 to 5 days (Table 2). BHK-21 cells began to slough after 4 days in culture. The other cell lines were much more resistant to overcrowding; their monolayers remained in good condition for at least 8 to 10 days.

In the plaque assays, only BT cells gave a BTV titer markedly lower than that produced in Vero cells (Table 3). All other cell lines that produced plaques gave titers equivalent to those found in Vero cells. Plaques in four cell lines (BHK-21, bighorn sheep fetal tongue [BHFTE], BT, and MDBK) were not discrete and were difficult to enumerate (Table 3).

Six cell lines (BHFTE, BHK-21, BT, CPAE, mouse fibroblasts [L929], and MDBK) were tested to determine whether they could detect BTV in blood from BTV-exposed sheep in a manner similar to that in ECE. Only the CPAE cell line detected animal source BTV with a frequency similar to that observed in ECE (Table 4). The CPAE cell line was further tested by using additional animal source BTV, and the results were compared with those obtained in ECE. The cells detected BTV in 6 of 7 samples that were positive in ECE (Table 5).

DISCUSSION

Fibroblastlike and epitheliumlike cell types from both ectothermic and endothermic animals were tested for susceptibility to BTV infection. Of the 14 different cell lines tested, 80% were susceptible to BTV. No general pattern of susceptibility by cell type was detected. It is not known why some of the cell lines were resistant to BTV infection. This observed variation in susceptibility to BTV infection could possibly be explained by variations in the presence of cellular receptors with which BTV interacts; cells with few or no appropriate receptors probably would not become infected. The demonstrated in vitro susceptibility of the bovine endothelial cell line (CPAE) to BTV is consistent with in vivo studies that have demonstrated localization of BTV to the endothelial lining of blood vessels of infected animals (12).

When different cell lines were tested in virus infectivity assays, all but one, CPAE, gave $TCID_{50}$ titers of cell culture-adapted BTV that were similar to or lower than titers found in BHK-21 cells. Only the CPAE cells gave titers slightly higher than titers detected in BHK-21 cells.

In plaque assays, several cell lines produced monolayers in which BTV-induced plaques were difficult to separate into individual plaques. This kind of result would not be desirable for use in plaque assays. Interestingly, C6/36 cells which grow in very lightly adherent monolayers demonstrated very clear plaques. Both Vero and L929 cells gave very discrete plaques in the plaque assay, as has been previously reported (6, 13; D. R. Sundin, personal communication).

Isolation of BTV from animal source samples can be very difficult and should be conducted in several different isolation systems (7). Several reports cite instances of BTV detected in one system but not in others (4, 11, 13). We found similar results when comparing detection of animal source BTV by using several different cell lines and ECE. These samples included tissues from animals that had been

TABLE 4. BTV titers in 6 cell lines and ECE tested with BTV-exposed sheep blood samples

Blood sample no.	Log_{10} $CEIVLD_{50}$/ml in ECE	Log_{10} $TCID_{50}$/ml in:					
		BHFTE	BHK-21	BT	CPAE	L929	MDBK
1	6.7	0	0	0	6.1	0	0
2	3.6	2.6	0	3.2	2.5	0	0
3	0	0	0	ND[a]	0	ND	0
4	5.3	ND	0	3.2	4.2	0	ND

[a] ND, Not done.

TABLE 5. Comparison of detection of animal source BTV in ECE or in CPAE cells

Sample	Serotype	Titer	
		ECE[a]	CPAE[b]
Sheep blood[c]	2	0	0
Sheep blood[c]	2	6.3	5.4
Sheep blood[d]	10	6.7	6.1
Sheep blood[c]	10	3.7	2.5
Mouse brain[e]	11	5.8	5.2
Sheep blood[c]	13	5.3	4.2
Sheep blood[d]	17	0.9	0
Sheep blood[d]	17	1.2	2.8

[a] Log_{10} $CEIVLD_{50}$/ml.
[b] Log_{10} $TCID_{50}$/ml.
[c] Sheep were experimentally infected by bites of BTV-infected *Culicoides variipennis*.
[d] Sheep were infected by intradermal or subcutaneous inoculation of cell culture-adapted BTV.
[e] Newborn mice were injected intracranially with mouse-passaged BTV.

inoculated with cell culture-adapted BTV as well as from animals that were infected with animal-passaged BTV of 5 different serotypes. Although the actual titers obtained in ECE and in CPAE cells were frequently different, the CPAE cells were more consistent than other cell lines for detection of the presence of BTV in samples without requiring prior adaptation of the sample virus. The CPAE cell line is quite susceptible to both cell culture-adapted and animal source BTV, demonstrates easily observable cell death within 2 to 5 days, and is reasonably tolerant to overcrowding in our assay conditions. The bovine endothelial cell line demonstrates significant potential as a cell culture system for use in BTV investigations.

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