Studies on Bluetongue
IV. Studies of Three Strains in Primary Bovine Foetal Kidney Cell Cultures

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SUMMARY

Three different strains of bluetongue virus were adapted to grow in primary bovine foetal kidney cell cultures. The cytopathic effects observed from the three strains were similar, and were characterized by shrinkage of cells and increased granularity. The specificity of the changes was confirmed by the fluorescent antibody technique. No significant immunological cross-reaction was detected by serum-virus neutralization tests from the strains studied.

SOMMAIRE

Trois souches différentes de la fièvre catharrale ovine ont été adaptés aux cultures de cellules primaires de rein fétal bovin. On a observé des effets cytopathogènes semblables chez les trois souches. Ils étaient caractérisés par un rétrécissement des cellules et une augmentation de la granularité. L'épreuve de la séro-neutralisation n'a pas démontré de relation immunologique réciproque entre les souches étudiées. Les mêmes globulines anti-cyprus (type-3) conjuguées à la fluorescine ont servi à confirmer la présence des trois différentes souches dès les premiers jours après inoculation en cultures cellulaires.

Literature relating to propagation of bluetongue virus (BTV) in tissue culture was rather limited until recently. In the last few years, however, this virus has received increased attention particularly from workers connected with its propagation and detection by means of cytopathic effects in tissue culture cells (1-4). Noteworthy is the report of Pini (5) during the last year on the successful use of the fluorescent antibody technique to demonstrate the presence of the virus in tissue culture.

The present paper describes the adaptation of three strains of BTV to bovine primary foetal kidney cells and confirmation of this by virus-serum neutralization tests and by the immunofluorescence technique.

Materials and Methods

TISSUE CULTURE

Primary bovine foetal kidney (BFK) cells were prepared weekly by trypsinization of kidney cortex tissue from bovine foeti of 5 to 7 months development, obtained from a local abattoir. Excised and minced portions of kidney cortex were trypsinized for approximately 20 minutes in 0.25 percent trypsin solution in phosphate-buffered saline. The suspension of free cells thus obtained was filtered through three thicknesses of sterile gauze to remove fibrous tissue strands and cell debris. The cells, packed by centrifugation for two minutes at 1000 RPM, were resuspended in growth medium to an approximate concentration of 0.5 percent (V/V). The suspended cells were then dispensed in 1.0 ml. amounts into 16 x 100 mm. tubes and/or in 5.0 ml. amounts into 28 x 85 mm. Leighton tubes containing 18 x 75 mm. cover slips. All tubes were incu-

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bated in a stationary position at 37°C. After 5 to 6 days' incubation a sheet of epithelial and fibroblast cells had formed on the surface of the glass. Immediately prior to inoculation the growth medium was removed, the cells were washed with Hank's solution, and maintenance medium was introducted.

The growth medium consisted of Hank's balanced salt solution containing 0.5 per cent lactalbumin hydrolysate, 0.1 per cent proteose peptone No. 3, 0.001 percent cysteine HCl (W/V) and 10 percent (V/V) newborn calf serum[1]. Penicillin (200 I.U./ml.) and Streptomycin (100 mg/ml.) were incorporated in the medium. The lactalbumin was added to the maintenance medium in order to avoid the use of calf serum which may contain substances inhibitory to tissue culture (4, 5).

Cells were maintained in medium 199 (Hank's base) to which 0.5 percent lactalbumin hydrolysate was added. Antibiotics were used at the same concentrations as in the growth medium.

Virus strains

Three different antigenic types of BTV were used. California (type 10) strain was received in 1962 from the Division of Veterinary Services, Onderstepoort, Republic of South Africa, as freeze-dried mouse brain, tenth serial passage. This material had been passaged 22 times in suckling mice, and held at −40°C for stock virus. The Cyprus (type 3) strain, also from Onderstepoort, was obtained in 1960 as a freeze-dried 10 per cent suspension of chick embryo tissues of the third egg passage. This strain had been passaged 13 times in suckling mice before inoculation of tissue culture cells. The Texas Station strain inoculum consisted of fresh whole blood collected from sheep inoculated 3 days previously with infected ovine whole blood in OCG solution. It was obtained in 1965 from Dr. C. W. Livingston, College of Veterinary Medicine, Texas A & M University.

Preparation of inocula

Frozen infected suckling mouse brains were suspended in maintenance medium by grinding, with a glass tissue grinder, approximately 1.0 gm. of tissue in 5.0 ml. of medium. Suspensions were then centrifuged at 2000 RPM for 10 minutes and the supernatant fluid was considered to be the stock suspension. Immediately before inoculation of Leighton tubes, when blood was used as virus source, one part of distilled water was added to two parts of defibrinated sheep blood to produce haemolysis. Fresh whole blood without any alteration was also used as inoculum.

Infection of Monolayers

Immediately prior to inoculation the growth medium was removed, the monolayers washed with Hank's solution and inoculated with 0.5 ml. of the appropriate virus dilution. Tubes were held horizontally in racks for 2 to 3 hours at 33°C to allow absorption of virus to take place. Monolayers were then rinsed with maintenance medium until the washings were clear, 4.0 ml. of the same medium was added, and tubes reincubated at 33°C.

Serum Neutralization Tests

The hyperimmune sera used were obtained from sheep inoculated with various BTV strains (7). Normal lamb serum served as controls in the tests. All sera were inactivated by heating in a waterbath at 56°C for 15 minutes, and were then diluted 1:10 with maintenance medium. Ten-fold dilutions in the same medium were conducted with the BTV strains. Serum-virus neutralization tests were performed separately on each strain by mixing 1.0 ml. of each virus dilution with 1.0 ml. of the 1:10 serum dilution. The mixtures were then held at 4°C in the dark for one hour. Growth medium was drained from tubes of monolayer cultures and a set of 4 tubes was inoculated with 1.0 ml. of each serum-virus mixture employed. The inoculated tubes were then incubated at 33°C in roller drums and examined daily for 4 to 7 days for the presence of cytopathic effects (CPE). Titration end-points were determined by the method of Reed and Muench (8).

Fluorescent Antibody Technique (FAT)

Cover slips were removed from Leighton tubes 3 and 5 days after inoculation and examined by immunofluorescence. With infected cultures, longer incubation often resulted in more detached cells. This was especially true when whole blood was used

[1]Newborn calf serum W_2 (Special), Hyland Laboratories, Los Angeles, California.

TABLE I. Virus Titration Tests of 3 BTV Strains Following Neutralization with Homologous and Heterologous Antisera

VIRUS			VIRUS TITRATION WITH			
Strain	Passage	Titration	anti-Cyprus	anti-Station	anti-Cal.	Negative
Cyprus	14	$10^{-6.2}$	0*	10^{-6}	10^{-6}	$10^{-6.5}$
Texas	6	10^{-5}	$10^{-4.9}$	0*	$10^{-4.5}$	10^{-5}
California	20	$10^{-5.5}$	$10^{-5.5}$	$10^{-4.2}$	0*	$10^{-6.6}$

*Complete inhibition of virus after 5 days of incubation

as inoculum. The technique employed will be described in detail in another publication (6), which will present the results obtained with infected animal tissue. The same fluorescein-conjugated Cyprus (type 3) serum was used in studying the three strains.

Results

Adaptation of Bluetongue Virus to Bovine Foetal Kidney Cells

California strain — The bovine foetal kidney cells infected with mouse brain-adapted California strain did not show visible CPE after 8 days' incubation. A blind passage was necessary before CPE could be demonstrated. The cytoplasm of the epithelial-type cells began to appear granular by the third day after inoculation (d.p.i.). At this time, the cells appeared to contract and become irregular, a process which was observed first at the edge of the monolayer. Later, the fibroblastic type cells lost their typical star-shape appearance and became elongated. Finally, the cells separated from one another, detached from the surface of the tubes and by the 7th day, most were free and suspended in the medium.

At the third passage level, similar cytopathic changes were observed with the exception that nearly all of the cells had become detached by the 5th d.p.i. After approximately twenty serial passages, cytopathic changes in the monolayers were observed as early as the 1st d.p.i. in cultures inoculated with the 10^{-1} virus dilution, and detachment from the glass by the 3rd d.p.i.

That these changes were specific, was clearly demonstrated by the fluorescent antibody technique. Presence of the viral antigen was detected in BFK cells infected with the original mouse brain-adapted California strain, by the FAT within the 3rd d.p.i., even in the absence of detectable CPE. The antigen appeared as fine granules distributed in the cytoplasm of cells. A detailed description will be presented elsewhere (6).

Cyprus strain — This mouse brain-adapted BTV strain, when inoculated into BFK cell cultures produced on first passage foci of degeneration along the margins of the monolayer cultures by the 4th d.p.i. By the 7th d.p.i. these lesions had extended throughout the cell sheets. After 4 or more serial passages, focal lesions were observed as early as 48 hours after incubation, and complete destruction had occurred by the 4th d.p.i.

Cells infected with the Cyprus strain demonstrated specific fluorescence within 48 hours after inoculation.

Texas Station strain — Cytopathic changes were not observed in BFK cells inoculated with the original material but were apparent after five days incubation of subpassages. The presence of this strain of virus in these tissue cultures was confirmed within 3 days by FAT.

Serum Neutralization Tests

Using a constant amount of serum diluted 1:10 with maintenance medium and tenfold dilutions of virus, complete inhibition of virus was obtained with homologous antiserum, as indicated in Table I. No significant cross-reaction was observed when one strain of virus was tested in the presence of heterologous antiserum.

Discussion

The data presented show that bluetongue virus can be isolated and propagated in bovine foetal kidney cell cultures. The cytopathic effects observed with the three different BTV strains used were very similar, and were characterized by shrinkage of cells and increased granularity. These could easily have been confused with normal or toxic degeneration of cells, es-

pecially when tissue material has been used as inoculum. However, the presence of viral antigen was readily confirmed by the fluorescent antibody technique. The FAT would appear therefore to be a most useful adjunct to the tissue culture method for the diagnosis of BTV since virus can be detected within a few days after inoculation of tissue culture cells with infected material.

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An Editor's View of Expo 67

Now that summer is in full swing, the big show in Montreal, Expo 67, has gone through its initial break-in period. From all reports of those veterinarians, both Canadian and foreign, who have taken in the Fair the reactions are impressive and complimentary. One of the most staggering features of the exhibition is its tremendous size. Expo 67 is situated on nearly 1000 acres of land, and those country veterinarians who have chased a steer over a client's back forty will appreciate the amount of walking required to keep up with your teen-agers as they scurry from pavilion to pavilion. A good pair of comfortable walking shoes is the first order of the day. The second most important piece of equipment is an Expo guide-book. A certain amount of planning is necessary unless you have a month to see the sights.

While reservations have long-since been taken at the major hotels, there are still many rooms left in private homes who offer reasonable rates and a personal, friendly touch that you might miss in a motel or hotel. Food and facilities are excellent at Expo, and the rumors that first circulated about excessive food prices are simply not true. If you want a gourmet meal, be prepared to spend up to $40 for two, but a hamburger can be had for 50 cents and a soft drink for 15 cents. Prices for food can be found spread out between the two extremes. The food at the Atlantic Provinces Pavilion is superb and at very reasonable rates, but many other fair-goers have found this out and the waiting lines reach back to Halifax. There are well over 50 other places to eat.

When you first arrive at Expo, a ride on the Mini-Rail will give you a good overall impression of the island sites. Similar rides can be taken by hover-craft, motor launch or gondola. The Expo express costs nothing and transportation from one island to another takes a moment. After the pavilions close at 9:30 in the evening, La Ronde is in full swing for late night fun until 2:30 in the morning. La Ronde, the amusement area, is Coney Island with a wholesome, family approach. Free entertainment is provided all evening at various spots. Teen-agers seem to have the best time of all, and the Youth Pavilion swings with the latest in spine-cracking dances.

Highlights of the exhibition are the Bell Telephone pavilion with its superb and novel movie, the Canadian pavilion and most of the provincial pavilions. The theme pavilions provide excellent educational fare and are fun too. In fact, almost every exhibit contains something of interest to every fair-goer. There is so much to see and it is spread over such a large area that you should give yourself a day's break in the middle of your visit to rest and re-charge your energy. Montrealers have developed an occupational disease from repeated visit to the fair — sore feet and low-back pain. Above all, don't plan to "do" the exhibition in a day or two — it can't be done.