Replication of Avian Infectious Bronchitis Virus in African Green Monkey Kidney Cell Line VERO

(Accepted 12 June 1972)

Avian infectious bronchitis virus (IBV), a coronavirus, requires initial isolation in, and adaptation to, chicken embryos (CE) before transfer to primary avian cell and chicken tracheal organ cultures. These are the only presently known cell cultures in which IBV replicates and produces cytopathic effects (c.p.e.) in serial passage (Estola, 1966; Cunningham, 1970). Monkey kidney cells have been reported (Fahey & Crawley, 1956; Steele & Luginbuhl, 1964) to support replication of IBV without c.p.e. when first inoculated with virus propagated in CE. Attempts apparently were not made to passage the virus in these cells.

Direct haemagglutination (HA) is not a normal property of IBV (Biswal, Nazerian & Cunningham, 1966) or of the human coronaviruses (Kapikian, 1969). However, human coronaviruses OC 38 and OC 43 adapted to suckling mouse brain (Mcintosh et al. 1969) cause direct HA (Kaye & Dowdle, 1969) and produce syncytia and plaques in African green monkey kidney and BSC-1 cells (Bruckova, cited by Bradburne & Tyrrell, 1971).

The studies reported here were prompted by the possibility that IBV adapted to suckling mouse brain (Simpson & Group6, 1959; Estola, 1966, 1967; McIntosh et al. 1969; Kaye & Dowdle, 1969; Bradburne, 1970) might also cause direct HA and replicate in African green monkey cell line VERO (Yasumura & Kawakita, 1963). This cell line was selected because it was not one of those previously tested by the authors without success by direct inoculation for replication of IBV, and similar lesions are produced by IBV in chicken embryo kidney cells (CEKC) (Cunningham & Spring, 1965; Lukert, 1965; Akers & Cunningham, 1968) and by mouse-brain-adapted OC 38 and OC 43 in African green monkey kidney cell cultures (Bruckova, cited by Bradburne & Tyrrell, 1971).

Stock viruses were IBV-41 (Massachusetts), 5th CE passage; IBV-42 (BEAUDETTE), hundreds of CE passages; IBV-46 (CONNECTICUT), 7th CE passage; and IBV-42C, 135th CEKC passage of IBV-42.

Litters of 10 to 14, 5- or 6-day-old, suckling Swiss albino mice were used for each of three serial intracerebral passages of the respective viruses. Encephalitic signs (Estola, 1966, 1967) were produced by IBV-42 and IBV-42C and virus was recovered in CEKC from the brains of the mice on the day when signs were present (Table 1). Neither IBV-41 nor IBV-46 produced signs through three 'blind' passages in mice and virus was not recovered in CEKC.

The VERO cells were supplied by Dr D. L. Croghan, Veterinary Biologics Division, United States Department of Agriculture, Ames, Iowa, 50010, as passage 129. The growth medium was of Eagle's basal medium with non-essential amino acids supplemented with 5% foetal calf serum (FCS), L-glutamine (1 ml./l. of 200 mM) (Grand Island Biological Company (GIBCO), 3175 Staley Road, P.O. Box 68, Grand Island, New York, 14072) and antibiotics (100 mg./ml. of penicillin and streptomycin, 6000 mg./ml. of tylosin tartrate), buffered to pH 7.4 with 7.5% filtered sodium bicarbonate. Maintenance medium was the same except that the FCS was reduced to 2%.

Confluent monolayers of cells (Fig. 1) in closed tubes, Leighton tubes with coverslips, and 60 x 15 mm. Petri dishes were used. Incubation was at 37°C. Petri-dish cultures were in an atmosphere of 85% relative humidity and 8% CO₂. The growth medium was decanted and
Table 1. Intracerebral serial passage* of IBV in 5- to 6-day-old suckling mice. Virus recovered on the day of encephalitic signs† assayed in CEKC as p.f.u./g. brain

<table>
<thead>
<tr>
<th>Inoculum for 1st passage</th>
<th>Mouse passage</th>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBV-42 6×10^5 EID&lt;sub&gt;50&lt;/sub&gt;/mouse</td>
<td></td>
<td>1</td>
<td>1·8×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1·9×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2·7×10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>3·5×10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2·7×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>IBV-42C&lt;sub&gt;5&lt;/sub&gt; 1×10&lt;sup&gt;5&lt;/sup&gt; p.f.u./mouse</td>
<td></td>
<td>1</td>
<td>6·6×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5·0×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1·4×10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>5·4×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3·5×10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>—</td>
</tr>
</tbody>
</table>

* Inoculum 0·02 ml./mouse.
† Brains pooled for respective viruses and stored at −90°. At the time of use as inoculum for the next passage in mice or for cell cultures, the brain pool was made into a 10% suspension (w/v) with PBS without Ca<sup>2+</sup> or Mg<sup>2+</sup>, pH 7, + 2% FCS + antibiotics.

the cells were washed thoroughly with phosphate-buffered saline (PBS) without Ca<sup>2+</sup> or Mg<sup>2+</sup>, pH 7, + 2% FCS + antibiotics. Tube cultures received 0·2 ml. inoculum. After incubation for 60 min., 2 ml. maintenance medium was added. Petri-dish cultures received 0·5 ml. inoculum. After 90 min. the inoculum was poured off and 4 ml. maintenance medium was added if these cultures were to be used for propagation of virus. All cultures were examined daily for c.p.e. Coverslip cultures were stained with May–Grunwald–Giemsa solution. Maintenance medium was replaced with fresh medium on the 2nd or 3rd day.

Petri-dish cultures for plaque assay were overlaid with 4 ml. agar medium (equal parts of 2% agar gel (GIBCO) and growth medium + 3% pancreatin) in place of maintenance medium after the inoculum was poured off. A 2nd agar overlay was added at the 5th day. Neutral red, 0·5 ml. of a 0·1% solution, was added 5 days later. The cultures were then incubated for 45 to 60 min. at 37° and 60 min. at 4° before the plaques were counted.

VERO cells were first inoculated with IBV-42 and IBV-42C in the 3rd mouse brain passages. Syncytia (Fig. 2) were present throughout the monolayer on the 6th day and all cells were necrotic by the 7th day. Medium from the cells was the inoculum for the next passage. On the 2nd and subsequent passages of the virus, syncytia detectable at 24 hr in stained cultures and at 48 hr in unstained cultures increased in size and number and all cells were necrotic by the 5th day. Medium from cell cultures 5 days after infection was the inoculum used for successive passages of the virus and for plaque assays. Virus was in the cytoplasm, but not the nuclei, of the syncytia and other infected areas of the monolayers (Fig. 3) as observed by the indirect immunofluorescence method (Rodriguez & Deinhardt, 1960) using anti-IBV-41 chicken serum and fluorescein conjugated anti-chicken horse γ-globulin (Roboz Surgical Instrument Co., Inc., 810 18th St., N.W., Washington, D.C. 20006).

Plaques (Fig. 4) were slightly opaque and 2 to 3 mm. in diameter. Assays of IBV-42 from the 4th, 7th, 13th and 14th passages in cells were 1·2×, 1·8×, 3·0×, and 4·6×10<sup>4</sup>p.f.u./ml., respectively. Assay of IBV-42C from the 5th passage was 4·2×10<sup>5</sup>p.f.u./ml. and 5·0×10<sup>4</sup>p.f.u./ml. from the 13th passage.

Anti-IBV-41 chicken serum neutralized mouse-brain-passaged IBV-42 and IBV-42C by plaque reduction in CEKC and VERO cell-passaged virus by plaque and c.p.e. reduction in VERO cells.

There was no direct haemagglutination by either the mouse-brain- or cell-passaged viruses.
Fig. 1. Uninoculated VERO cells. May–Grunwald–Giemsa stain.
Fig. 2. Syncytia produced by IBV-42 in VERO cells 2 days after infection. May–Grunwald–Giemsa stain.
Fig. 3. Immunofluorescence produced by IBV-42C in VERO cells.
Fig. 4. Plaques produced by IBV-42 in VERO cells 10 days after infection. Neutral red stain.
Intracytoplasmic and extracellular virus (Fig. 5) had the typical morphologic features of IBV (Becker et al. 1967; Nazerian & Cunningham, 1968; Uppal & Chu, 1970). Budding was from the walls of the cytoplasmic vacuoles but not at the plasma membrane. Virus was released extracellularly as the result of rupture of the vacuoles.

So far as the authors are aware, this is the first report of serial passage of IBV in a mammalian continuous cell line.

This is Journal Article No. 5888 of the Michigan Agricultural Experiment Station.

C. H. Cunningham
Martha P. Spring

K. Nazerian

Department of Microbiology and Public Health
Michigan State University
East Lansing, Michigan 48823, U.S.A.

U.S. Department of Agriculture
Agricultural Research Service
Regional Poultry Research Laboratory
East Lansing, Michigan 48823, U.S.A.
REFERENCES


LUKERT, P. D. (1965). Comparative sensitivities of embryonated chicken’s eggs and primary chicken embryo kidney and liver cell cultures to infectious bronchitis virus. Avian Diseases 9, 308.


(Received 14 April 1972)