SOME OBSERVATIONS ON THE PROPAGATION OF AVIAN INFECTIONOUS BRONCHITIS VIRUS IN TISSUE CULTURE

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Introduction

A number of workers have described the propagation of infectious bronchitis (IB) virus in tissue culture (see Cunningham 1970). Although different isolates have been adapted to growth in tissue culture, the one most frequently used has been the highly egg adapted Beaudette strain of IB (Coria 1969; Cunningham 1970).

There have been no published reports of the propagation in tissue culture of the Australian isolates of IB virus which induce nephritis. An attempt was made to adapt some of these isolates to tissue culture in order to evaluate this method of growth for potential vaccine production and as a diagnostic tool. During the course of this work a reovirus was isolated from one of the cultures. The identification procedures for this virus and the cytopathic effects that it produced were compared with those for an IB virus propagated in tissue culture.

Materials and Methods

Viruses

The A (titre $10^{7.3}$ EID₅₀/ml) and T (titre $10^{6.4}$ EID₅₀/ml) isolates of IB virus, in the 31st and 11th egg passages respectively, have been described by Cumming (1963, 1967). The B19 (titre $10^{6.8}$ EID₅₀/ml) and B21 (titre $10^{6.6}$ EID₅₀/ml) isolates of IB virus were from field cases in New South Wales and were in their 30th egg passage. The W (titre $10^{7.3}$ EID₅₀/ml) and G (titre $10^{6.6}$ EID₅₀/ml) isolates of IB virus were from two commercial vaccines which were already egg adapted (30-40 egg passages).

Tissue Culture

Chick kidney (CK) cells were prepared by a modification of the method suggested by Churchill (1965). Kidneys, taken from 2 to 3 chicks less than 1 week old, were finely macerated with scissors and washed twice with warm (37°C) phosphate buffered saline. The macerated kidneys were stirred with 20 ml of 0.25% trypsin in 37°C phosphate buffered saline (without Ca or Mg ions) for 5 minutes. The red cells were then removed by decanting the supernatant layer. The kidney debris was then suspended in 50 ml of 0.05% trypsin in phosphate buffered saline and stirred at 37°C for 30 minutes. The cell suspension was centrifuged at 500 rpm for 5 minutes and the cells resuspended in saline for counting. Normally, cells were distributed in 50 ml or 100 ml medical flats at the rate of 4×10^5 cells per ml and 5 ml or 10 ml of medium per vessel respectively.

Growth medium consisted of Hank's balanced salt solution supplemented with 5% foetal calf serum, 0.15% tryptose phosphate broth, 0.25% lactalbumen hydrolysate, penicillin, streptomycin and fungizone. Maintenance medium consisted of Eagle's basal medium made

up in Earle's balanced salt solution with 2% foetal calf serum and otherwise supplemented as for growth medium. Generally, confluent monolayers resulted in 4 to 5 days and could be maintained for a further 7 to 10 days.

Coverslip cultures were produced by including one flying coverslip in a culture vessel. These were normally stained with May-Grunwald Geisma.

Infection of Eggs and Tissue Culture

Tissue Culture—Confluent monolayers of CK cells were infected by removal of the medium and the addition of 0.5 ml of infected allantoic fluid directly to the cell sheet. After incubation at 37°C for 30 minutes, fresh medium was added. Viruses were blind passaged by the transfer of 0.5 ml of supernatant fluid to fresh cultures every 4 days.

Eggs—The inoculation of eggs via the allantoic route and the preparation of kidney specimens for virus isolation has been described by Cumming (1969). Infection of the chorio-allantoic membranes of 9-day-old embryos was by the method described by Cunningham (1963).

Viruses were stored in 0.2 ml or 1.0 ml aliquots in sealed vials at -20°C or in liquid nitrogen.

Virus Titrations

The methods used for virus titrations followed those of Churchill (1965). Viruses were normally diluted in half log. steps. In eggs, 0.1 ml of each dilution was inoculated into each of five 9-day-old embryos. Deaths occurring in the first 24 hours were disregarded. End points were determined on the basis of death, or stunting and curling of the embryos, eight days after infection. In tissue culture, 0.5 ml of each dilution was inoculated into each of three tissue culture vessels. The vessels were observed for cytopathic effect for 6 days.

Titres were calculated by the method of Reed and Muench (1938).

Virus Neutralisation

Neutralisation tests were carried out in eggs by the constant virus method of Fontaine *et al* (1963). A one in four serum dilution was expected to neutralise 100 EID₅₀ of virus to be positive. A similar method was used with tissue culture except that 100 TCID₅₀ were used and three culture vessels per inoculum.

Serums

Two groups of ten 4-week-old cockerels were vaccinated with either the A or T isolate of IB virus. Three weeks after vaccination the birds were bled and their serum pooled for neutralisation tests.

Gel Diffusion

The gel diffusion technique used has been described by Chubb and Cumming (1971).

Fluorescent Microscopy

Fluorescent staining with coriophosphine was by the method described by Keeble and Jay (1962).

Electron Microscopy

Electron microscopy using 3% phosphotungstic acid, pH 6.4, followed the method of Spradbrow and Francis (1969).

Experimental Procedures

The isolation facilities used have been described by Cumming (1967, 1969). The experimental chickens

were White Leghorn x Black Australorp cockerels obtained from a local hatchery at one-day-old and reared in isolation until 4 weeks of age.

Experiment 1—Each of the viruses tested (A, T, B19, B21, W and G) was given by infra-orbital instillation of one drop of infected tissue culture media into each of ten 4-week-old cockerels housed in separate isolation pens for each virus. Three weeks after infection all the birds were challenged with virulent T virus and kept together under cold stress at 16°C. Virus titres were all greater than 10^5 EID₅₀/ml.

Experiment 2—A similar procedure to Experiment 1 was carried out, except that the viruses used were four different tissue culture passage levels of the W virus and 50 birds were infected with each passage level.

Experiment 3—The infectivity of W/17 (the seventeenth tissue culture passage of the W virus) was assessed by infecting 9 four-week-old cockerels by the infra-orbital route and at 1, 2 and 3 days after inoculation taking kidneys and tracheas from three birds for attempts to reisolate the virus in eggs.

All birds that died were autopsied. Only birds with typical macroscopic kidney lesions (Cumming 1963) were classified as dying from the challenge virus infection.

Results

All the samples of IB viruses were blind passaged in CK cell cultures at least five times. The viruses multiplied in the cultures to at least 10^5 EID₅₀/ml. A granular degeneration of the cell sheet developed after one or two blind passages of the A, B19, B21 and T viruses. Although this occurred somewhat earlier than control cultures, it was not sufficiently obvious to be used as a criterion for infectivity. Only the W and G virus samples gave any definite cytopathic effect.

The cytopathic effect of the W virus (Figures 4, 5, 6) was observed from the third passage. It consisted of foci of enlarged, rounded, refractile cells. These appeared throughout the cell sheet

and eventually lifted off, leaving a skein of fibroblastic cells on the glass. Histological examination revealed that the large cells were generally multinucleated, the number of nuclei ranging from 2 to 8. However, there was not a similar growth in cytoplasm, so the nuclei took up most of the

Figure 2. Cytopathic effect of G (reovirus) 24 hours after inoculation. Note large syncytia.

cytoplasm of the cell. Vacuolation was also apparent in the cells (Figures 4, 5). Large doses of virus produced cytopathic effects within 24 hours, while smaller doses took 3 to 5 days to affect the cultures. The cytopathic effect allowed titration of the virus in tissue culture. Comparisons of titrations using tissue culture and eggs are given in Table 1. It can be seen that tissue culture gave titres approximately one tenth of those obtained from growth in eggs. Antiserums

Figure 1. Monolayer of normal chick kidney cells (x10).

Figure 3. Cytopathic effect of G (reovirus) 48 hours after inoculation. Syncytia degenerate and flake off the monolayer.

TABLE 1

The Growth of Infectious Bronchitis Virus (W) in Tissue Culture

Virus	Tissue Culture Passage Level	Log EID ₅₀ /ml	Log TCID ₅₀ /ml (Based on Cytopathic Effect)
W	4	6.4	5.2
	5	—*	5.4
	7	7.5	5.8
	9	—	6.3
	11	6.9	5.8
	15	—	6.3
	17	6.8	5.6

*Not tested.

prepared against the A and T isolates of IB virus neutralised the W virus in both tissue culture and in eggs.

Some difficulty was experienced in storing the W virus at -20°C . A comparison of the TCID₅₀

Figure 4. Cytopathic effect of W (coronavirus) 24 hours after inoculation. Note small darkly stained cells (x10).

of the 16th CK passage of the W virus stored at -20°C and in liquid nitrogen showed a steady drop in the titre of the virus held at -20°C over a period of 21 weeks (Table 2).

TABLE 2

A Comparison of the Effects of Storage of Tissue Culture Grown Virus, W/16, at -20°C and -196°C

Temperature	Weeks of Storage			
	0	1	5	21
-20°C	6.8*	4.8	3.9	3.6
196°C	6.8	6.4	5.8	6.2

*Log TCID₅₀.

Figure 5. Enlargement of arrowed area of figure 4 (x40). Note multinucleated cells.

Fluorescent microscopy of W infected CK cells, using the cytochrome coriophosphine, gave a similar picture to that described by Berry (1967). No intranuclear staining was observed. RNA staining was apparent in the cytoplasm of affected cells either in the form of distinct granules or as large aggregates.

Electron microscopy of lysed W infected CK cells revealed coronavirus-like particles (Figure 7). These particles were of a similar size and morphology to that described for IB virus (Berry *et al* 1964).

The G virus sample produced a cytopathic effect at the fourth CK cell passage (Figures 2, 3). Large syncytia were prominent and formed flakes on the cell sheet. These flakes lifted off the glass surface early in the infection. Eventually, the whole cell sheet degenerated into an amorphous mass. Histological examination revealed large syncytia (Figure 2). The cytoplasm to nuclei ratio of these cells was normal, unlike the multinucleated cells seen with the W virus. Fluorescent staining failed to demonstrate abnormal distribution of RNA or DNA or cytoplasmic aggregates of RNA. Electron microscopy of lysed infected cells revealed many reovirus-like particles (Figure 8).

The original G virus sample had produced typical IB dwarfing and curling of embryos when inoculated into the allantoic cavity of 9-day-old embryonated eggs, as had the original W virus and its subsequent tissue culture passages. Tissue culture propagated G virus did not produce IB virus lesions when inoculated into eggs via the

allantoic route, but produced large irregular plaques on the chorio-allantoic membrane (Figure 9). Neither the original G virus sample or the W virus produced plaques on the chorio-allantoic membrane.

The Effect of Tissue Culture Virus in Birds (Experiments 1, 2 and 3)

Neither neutralising nor precipitation antibodies were detected in any serums taken from birds in Experiment 1 (Table 3) or Experiment 2 (Table 4) at the time of challenge with the T virus.

TABLE 3

The Resistance Induced in Birds by various Tissue Culture Propagated Viruses

Virus	Tissue Culture Passage	Death after Challenge
A31	6	4/10*
W	6	2/10
B21	5	0/10
B19	5	1/10
G	5	5/10
T	5	0/10
Control	—	6/10

Birds were immunised at 3 weeks of age and challenged 3 weeks later under cold stress.
Challenge virus was virulent T virus.

*Numerator: number of birds that died after challenge.
Denominator: number of birds challenged.

TABLE 4

The Resistance Induced in Birds by Various Passage Levels of W Virus in Tissue Culture.

Passage Level	Deaths After Challenge
5	7
7	11
11	10
17	9
Control	11

Number of birds immunised and challenged in each group = 50.
Other procedures as outlined for Table 3.

Although the first experiment suggested that resistance was induced by some of the viruses, the second experiment using different passage levels of W virus showed no evidence of resistance. In Experiment 3, virus was isolated from one trachea only, 3 days after infection with the W virus.

Discussion

The ease with which IB virus can be adapted to tissue culture seems to vary from worker to worker and from isolate to isolate. Cunningham (1970) states that at least some degree of adaptation to growth in eggs seems to be essential before IB virus can be adapted to tissue culture. It has been shown by Lukert (1965) that kidney and lung cells derived from embryos are the most susceptible cells for the growth of IB virus. Kawamura *et al* (1961) adapted 15 of 17 IB viruses isolated in Japan to give a cytopathic

Figure 6. Cytopathic effect of W (coronavirus) 48 hours after inoculation. Many rounded cells have lifted off the monolayer (x10).

effect in kidney cells. Estola (1966) found that a number of IB viruses isolated in Finland were easily adapted to chick embryo kidney cells. Churchill (1965) had little difficulty in adapting the Allen isolate of IB virus to CK cells but could not adapt the Massachusetts strain 41 virus which had undergone bird to bird passage only. Krauss and Peters (1968) found that an IB isolate capable of producing nephritis induced a cytopathic effect in tissue culture from its third egg passage.

We have only been able to adapt one of five Australian nephritis inducing isolates of IB virus to CK cells. The difficulty of infecting tissue

Figure 7. Electron micrograph of lysed cells infected with W virus showing coronavirus-like particles (x100,000).

culture cells with IB viruses may be due to the complexity of the virus receptor sites (Lukert 1972a) or in the method of attachment to the cell. Lukert (1972b) reported that not only was attachment of IB virus to cells inefficient compared with other viruses, but that interference to attachment occurred within a relatively short time period after infection.

The cytopathic effect produced by the W virus resembles the effect described by Chomiak *et al*

Figure 8. Electron micrograph of lysed cells infected with G virus showing reovirus-like particles (x100,000).

(1958) rather than the descriptions given by Churchill (1965), or Akers and Cunningham (1968). Fluorescent microscopy, using coriophosphine, showed similar changes to those described by Berry (1967) and Akers and Cunningham (1968). Electron microscopy confirmed the presence of IB virus-like particles (Berry *et al* 1964; Berry and Almeida 1968). It is unfortunate that because of Australian quarantine restrictions direct comparisons of European and American IB isolates with isolates held in this laboratory were not possible. Apart from the minor difference in description of the cytopathic effect of CK cells, however, the W virus isolate seems to follow the characteristics of other isolates of IB virus (Cunningham 1970).

The results of electron microscopy, cytopathic effect in tissue culture and the effect in embryonated eggs described here suggest that the virus isolated from the IB virus vaccine sample G is a reovirus similar to the viruses described by Rhim *et al* (1962), Deshmukh and Pomeroy (1969) and Mustaffa-Babjee and Spradbrow (1971).

It is not certain whether the reovirus (G) was a contaminant of the original IB vaccine

material or the tissue cultures used for its propagation. Six weeks after this isolation, another reovirus was isolated from normal CK cells. Since then, reovirus contamination has appeared in kidney cells derived from chicks from a commercial breeder and from both University flocks which are held in isolation from outside sources and each other. This contamination persisted for at least 6 months. At the initial isolations of reovirus in normal CK cells, this virus was also detected in fibroblast cultures from the same source, although contamination of fibroblast cultures as judged by electron microscope examination, lasted only 5 weeks. Reoviruses were not seen in cultures of the W virus up to the 17th cell passage.

In certain aspects the cytopathology of the reovirus (G) in tissue culture resembled the descriptions generally given for IB virus. It would seem to be a potential contaminant of tissue cultures derived from chickens in Australia where flocks free from the known poultry viruses are not available at present. In this case, routine screening of normal cultures with the electron microscope (Berry and Stokes 1968; Spradbrow and Francis 1969) proved valuable.

The poor response of birds to infection with the various tissue culture propagated IB viruses is not surprising. The necessity to adapt the viruses to growth in eggs before adaptation to tissue culture can reduce pathogenicity for birds (Delaplane and Stuart 1941; Cunningham 1970). It should be noted, however, that European workers (Hoekstra 1960; Hoekstra and Rispen 1960a, b) have passaged IB virus in eggs for at least 100 times without loss of resistance inducing

Figure 9. Chorio-allantoic membrane showing pocks produced eight days after infection with G (reovirus).

properties in birds. It has been shown by von Bulow (1966a, b) that while passage in tissue culture may have little effect on the pathogenicity of IB virus for embryos, 5 to 10 tissue culture passages reduces its virulence in birds. The low recovery rate, and poor resistance induction experienced here supports this latter contention. Coria and Hofstad (1971), however, have used 20th tissue culture passaged virus to induce resistance to subsequent challenge with virulent IB virus. It was disappointing that the virus isolates used in the present study, despite their ability to produce a response in birds before propagation in tissue culture, showed little effect in birds after growth in tissue culture. Further work is needed to elucidate the changes in virulence of IB virus brought about by its culture in different host systems.

Summary

Samples of six egg adapted Australian infectious bronchitis nephritis viruses were tested in chick kidney cells for growth and production of cytopathic effects. Four of the samples produced no marked cytopathic effect in five blind passages, although some virus multiplication occurred.

Two of the samples produced cytopathic effect. One produced large rounded refractile and multinucleated cells, whereas the other produced syncytial cells which flaked off the cell sheet. Electron microscopy showed the first to have a similar morphology to a coronavirus and the second a similar morphology to a reovirus. Serum neutralisation tests, coriophosphine staining and embryo lesions confirmed the coronavirus as an infectious bronchitis virus.

Tissue culture grown infectious bronchitis viruses were tested for their immunological potential in birds with equivocal results.

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