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Studies on Avian Infectious Bronchitis Virus (IBV)

II. Propagation of IBV in Several Cultured Cells

By

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With 6 Figures

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Summary

The growth of ten strains of avian infectious bronchitis virus (IBV) in several cultured cells was examined. The cultured cells used were chick kidney (CK), chick embryo (CE), HeLa and BHK-21 cells. The results obtained can be summarized as follows. 1. All the strains showed similar growth curves in CK cells. Progeny viruses appeared in the culture medium 4 to 6 hours after inoculation and peak virus titers of $10^{6.5}$—$10^{8.5}$ $TCID_{50}$ per 0.1 ml were obtained after 36 hours. A cytopathogenic effect (CPE) was detected within 24 hours. No distinct CPE and low multiplicities were observed on culturing at 30° C. 2. All strains replicated in CE cells, although only small amounts of virus were produced. No CPE was observed. 3. Only Beaudette-42 and Holte strains grew in BHK-21 cells. 4. No IBV strains grew in HeLa cells.

Introduction

Avian infectious bronchitis virus (IBV) replicates in various cultured cells (3, 4, 5, 8, 10, 14), although distinct cytopathic effect (CPE) and plaques are only detected in chick embryo kidney and chicken kidney (CK) cells. No growth of IBV has been reported in BHK-21 and HeLa cells (1, 7, 20). However, certain differences have been reported in the multiplication of IBV in different cell lines. For example, the time from virus inoculation to the peak virus titer in the cultured medium varied from 16 (8, 9, 16) to 60 hours (17), and the virus titer varied from $10^{4.2}$ (2) to $10^{6.8}$ $TCID_{50}$ (14). Furthermore, the multiplicity of IBV in chick embryo (CE) cells differed from strain to strain; some strains produced a CPE (2, 13), while other strains did not grow so well (13, 15). This investigation was undertaken to see whether the multiplication of IBV in CK and CE cells varies according to the strain used, and to determine if IBV can propagate in BHK-21 and HeLa cells.

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Materials and Methods

Virus Strains

The ten strains of IBV examined were Beaudette-42 (Be-42), Massachusetts-41 (IB-41), Connecticut A-5968 (A-5968); Connaught, Holte, Iowa-609, KH, Nerima, Ishida and Shiga strains (18).

Cell Cultures

CK cells were cultured as described previously (18). CE cells were cultured according to the method of Chomiak *et al.* (2). Five milliliter of Eagle's minimum essential medium (Eagle's MEM) with 5 per cent calf serum containing 10^6 cells per ml was poured into each small bottle. HeLa and BHK-21 cells were kindly supplied by Dr. M. Inoue, Hygenic Laboratory of Okayama Prefecture. The growth medium was Eagle's MEM with 0.1 per cent tryptose phosphate broth (TPB) and 10 per cent calf serum, and the maintenance medium was Eagle's MEM with 2 per cent calf serum.

Virus Titration

Virus was titrated as described previously (18).

Growth Curves

Small bottles of monolayers of cells were prepared for each IBV strain. Each virus at a titer of $10^{4.0}$ to $10^{8.0}$ $TCID_{50}$ was inoculated into each bottle and the virus was adsorbed to the cells for 90 minutes at 37° C. Samples of cultured medium were taken at selected times and after microscopical examination for CPE virus growth was determined by titration in CK cells. IBV multiplicity at 30° C was examined by transforming the cells to 30° C and incubating them for 24 hours. After adsorption at 37° C for 90 minutes, the cells were washed with Eagle's MEM, cooled to 25° C, resuspended in maintenance medium at 25° C, and incubated at 30° C.

Virus Adsorption

Virus adsorption tests were based on the method of Coria and Ritchie (4).

Immunofluorescent Antibody (FA) Test

The indirect FA test method described by Jones (11) was used.

Immune Sera

Immune sera were obtained from rabbits and SPF chickens. The rabbits were immunized as described by Estola (9). Two rabbits were each inoculated intravenously 10 times over a period of 3 months with a total of 100 ml of undiluted virus culture medium. The sera had virus-neutralizing antibody titers higher than 1:640. Chicken immune sera were prepared by inoculating each virus strain into five 8-week-old SPF White Leghorn chickens kindly supplied by Dr. Y. Iritani, Aburahi Laboratories, Shionogi Co., Koka-cho, Shiga Prefecture. The chickens were infected both intravenously and intranasally with 1 ml of cultured medium containing 10^6—10^7 $TCID_{50}$ and all birds were bled after 5 weeks. All sera obtained were inactivated by heating for 30 minutes at 56° C. Then, the sera were absorbed with chicken kidney powder, to remove any nonspecific components.

Two commercial conjugates, fluorescein isothiocyanate (FITC)-labelled sheep anti-rabbit globulin (MILES) and FITC-labelled rabbit anti-chicken globulin (MILES), were used. The optimal dilution of each conjugate was determined by a "chessboard" titration using IBV-infected CK cells on a coverslip. Both conjugates were used at a 64-fold dilution.

Fluorescence Microscopy

One drop of each serum dilution was spread over the IBV-infected CK cells grown on a coverslip (approximately 3×32 mm), which was left in a moist chamber for 60 minutes at 37° C. Excess serum was removed by agitating the coverslip in phosphate

buffered saline (PBS), pH 7.1, for 15 minutes. The buffer was drained off, one drop of the diluted conjugate was applied, and the coverslip was left for 60 minutes. After a final rinse in PBS for 15 minutes, the preparations were mounted in phosphate buffered glycerol (pH 7.1) and examined in a Nikon fluorescence microscope.

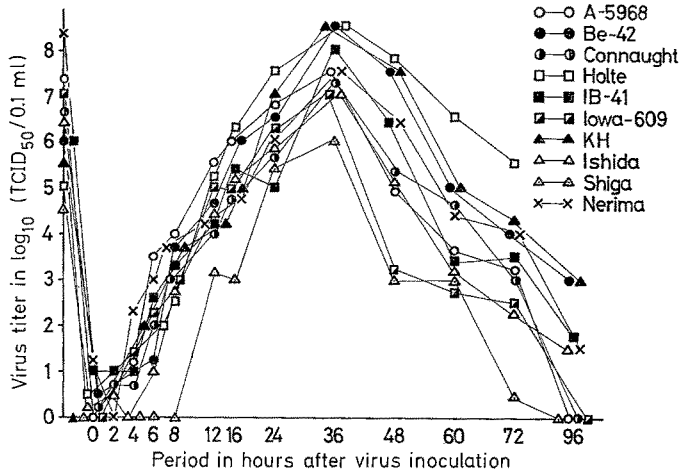

Fig. 1. Growth curves of IBV in CK cells at 37° C. The virus titer was an average of two titrations done under the same conditions

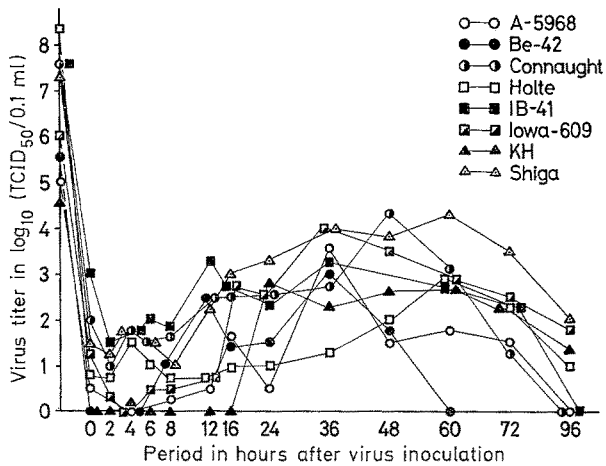

Fig. 2. Growth curves of IBV in CK cells at 30° C. The virus titer was an average of two titrations done under the same conditions

Results

Multiplicity in CK Cells

The growth curves of all the IBV strains at 37° C were similar (Fig. 1). Progeny viruses appeared in the medium 4 to 6 hours after the inoculation of all strains except Ishida and Shiga. Virus yields increased logarithmically in the first 24 hours and the maximum yields were detected at 36 hours for all strains. The virus titer

at 36 hours ranged from $10^{6.0}$ (Shiga strain) to $10^{8.5}$ $TCID_{50}$ (Holte strain) per 0.1 ml. The growth curves were not affected by the inoculum size. After 36 hours, the infectious titre in the medium decreased sharply with all strains. The Shiga strain did not grow as well as the others. CPE was detected at 24 hours in all the strains except Be-42 which appeared at 16 hours.

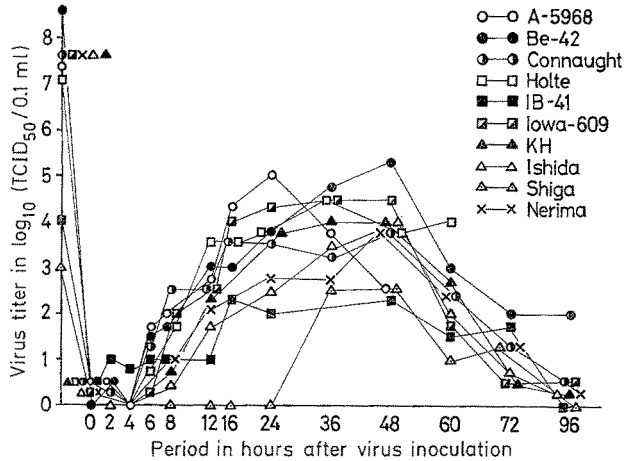

Fig. 3. Growth curves of IBV in CE cells at 37° C. The virus titer was an average of two titrations done under the same conditions

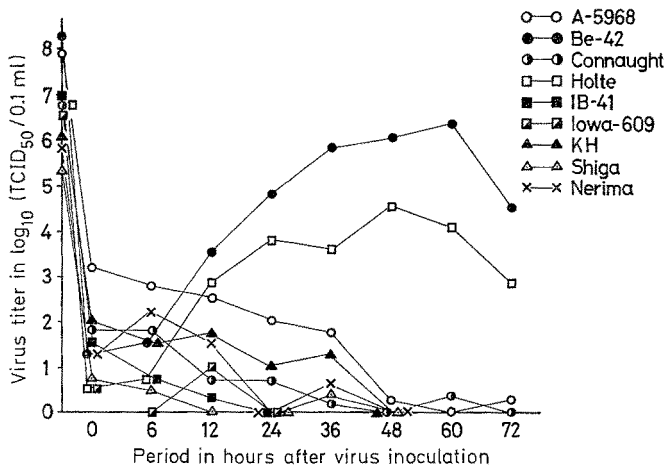

Fig. 4. Growth curves of IBV in BHK-21 cells at 37° C. The virus titer was an average of two titrations under the same conditions

Growth curves of IBV incubated at 30° C differed from those obtained at 37° C. Progeny virus increased slowly up to 36 to 60 hours (Fig. 2) and only a small amount of virus was produced. The CPE for all strains was slight and was not observed before 48 hours.

Multiplicity in CE Cells

All strains grew in CE cells (Fig. 3). The growth curves were similar to those in CK cells at 37° C, although the amount of virus produced was small. The titers of most strains increased to $10^{3.5}$ to $10^{5.3}$ $TCID_{50}$ per 0.1 ml, although the peak titers of IB-41 and Shiga strains were only $10^{2.5}$. As CPE was not observed, growth of IBV was confirmed by the FA test.

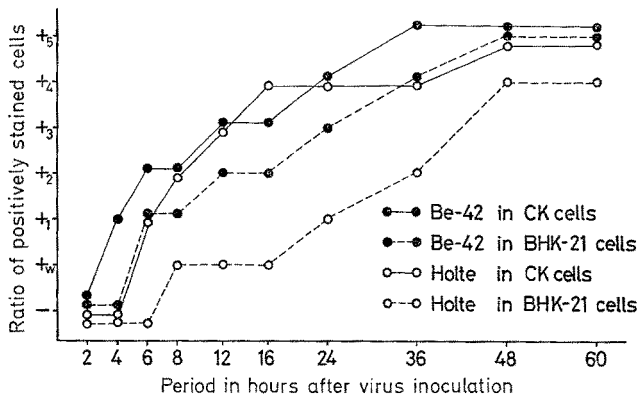

Fig. 5. Growth curves of Be-42 and Holte strains in BHK-21 and CK cells determined by FA tests

Multiplicity in BHK-21 Cells

Figure 4 shows that Be-42 and Holte strains grew in BHK-21 cells. In these strains, the eclipse phase of 6 to 12 hours was followed by an increasing production of virus. The virus titers reached $10^{6.3}$ $TCID_{50}$ per 0.1 ml in 60 hours for the Be-42 strain and $10^{4.8}$ in 48 hours for the Holte strain. Positively immunofluorescent-stained cells were observed only with the Be-42 and Holte strains, showing that only these strains replicated in BHK-21 cells. The growth cycles of these strains in BHK-21 cells were examined with the FA test using CK cells as controls. Growth was graded from $+_w$ to $+_5$ according to the number of positively stained cells and the results shown in Figure 5, confirm the pattern of growth in BHK-21 cells shown in Figure 4. CPE was observed after 60 to 72 hours in BHK-21 cells infected with either the Be-42 or Holte strain. CPE was represented mostly by a rounding up of cells (Fig. 6a), although occasionally syncytium formation was observed with the Be-42 strain (Fig. 6b). Similar CPE was observed in infected CK cells. Plaques were formed by Be-42 and Holte strains in BHK-21 cells after about 120 hours (Fig. 6c). The plaques were 1.0—1.5 mm in diameter, although those formed by Be-42 tended to be larger and more distinct than those formed by the Holte strain. There was essentially a linear relationship between virus dilution and the number of plaques produced. There was no difference in the adsorption of Be-42 to BHK 21 or CK cells. The number of plaques produced in BHK-21 cells was compared with those in CK cells by inoculating them both with the same titer of the Be-42 strain. The efficiency of plating of the Be-42 strain in BHK-21 cells was 50 per cent that in CK cells.

Multiplicity in HeLa Cells

None of the strains tested grew in HeLa cells. Only $10^{1.0}$ (A-5968) and $10^{0.3}$ (Be-42) titers of infective viruses were detected in the culture medium after 36 hours; whilst after 60 hours, no viable virus could be obtained. No immunofluorescence-stained HeLa cells were observed with any IBV strain using the FA test.

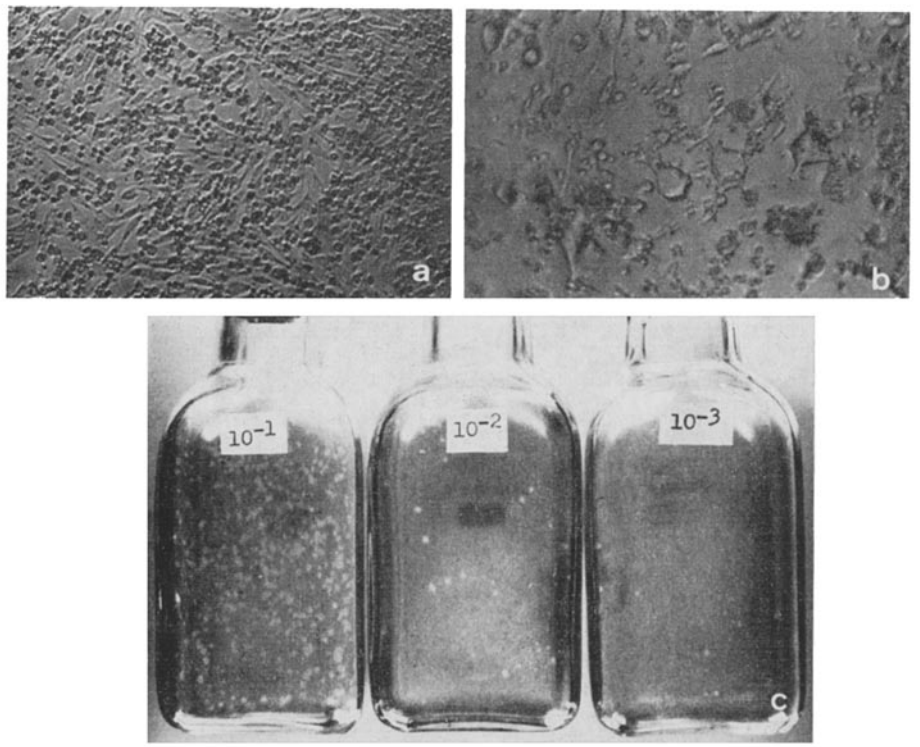

Fig. 6. CPE and plaque produced by Be-42 strain in BHK-21 cells. *a* Round type CPE at 130 hours after infection. ×100. *b* Syncytia at 80 hours. ×100. *c* Plaques produced at 120 hours. Neutral red stain

Discussion

The present investigation was undertaken to clarify the *in vitro* growth characteristics of IBV in a number of cell lines. Previous work has produced conflicting reports on the infectivity of some IBV strains in different cells and in the time of peak virus production. The IBV strains tested in the present investigation were not related to one another in either the time or place of their isolation or in their passage history.

The growth curves of ten IBV strains in CK cells were similar to one another at 37° C. Previous reports have indicated that the growth curves of different IBV strains in CK cells were variable (6, 12). We suggest that the growth curves of most IBV strains adapted to CK cells do not differ much from one to another if their

growth is measured under the same conditions. All the IBV strains propagated in CE cells although showing no CPE. Similar results have been reported previously (10, 20) and furthermore LUKERT (15) has shown that the majority of CE cells are not infected by IBV. We have also shown that IBV is unable to grow in HeLa cells. This confirms similar results from a previous report (7). Finally, this report is the first to show that some IBV strains propagate in BHK-21 cells and produce a CPE. Previous reports had shown that IBV-42 (1), and KH and Nerima strains (20) do not propagate in BHK-21 cells.

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