

Plaque Formation by Avian Infectious Bronchitis Virus in Primary Chick Embryo Fibroblast Cells in the Presence of Trypsin

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With 2 Figures

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Summary

Ten strains of avian infectious bronchitis virus (IBV) were titrated as plaque-forming units in primary chick embryo fibroblast cells. In the absence of trypsin, plaques were only formed by Beaudette-42 and Iowa-609 strains. When trypsin was incorporated in the overlay medium of cell monolayers, all the IBV strains tested produced plaques within 4 days after inoculation. Incorporation of 20—40 μg of trypsin per ml of the overlay medium seemed to be suitable for plaque formation of IBV. A preliminary investigation was made of the mode of action of trypsin.

Introduction

Avian infectious bronchitis virus (IBV) replicates in various cultivated cells, but distinct cytopathic effect (CPE) or plaque formation occurs only in chick embryo kidney cells and chick kidney (CK) cells. A few strains of IBV grow in VERO cells (4, 5), BHK-21 cells (10) and chick embryo fibroblast (CE) cells (9, 10). CE cells are easier to prepare than CK cells and it would be advantageous if, by incorporating some agent into the medium, plaque formation could be made to occur. Trypsin markedly enhances the *in vitro* infectivity of various viruses, i. e. rotavirus (11, 12), influenza viruses (1, 2, 8, 15), Sendai virus (7), reovirus (13) and vaccinia virus (6). Quite recently, STORZ *et al.* (14) reported that trypsin enhanced plaque formation of an enteropathogenic bovine coronavirus in bovine fetal thyroid and bovine fetal brain cells. In the present report, we describe plaque formation by IBV in CE cells when trypsin is incorporated into the overlay medium.

Materials and Methods

Viruses

The ten strains of IBV studied were: Beaudette-42 (Be-42), Massachusetts 41 (IB-41), Connecticut A-5968 (A-5968), Connaught, Holte, Iowa-609, KH, Nerima, Ishida and Shiga. None of these strains would produce CPE or plaques in CE cells (10), but after 10 further passages in CK cells Be-42 and Iowa-609 strains both produced CPE in CE cells. We examined all 10 strains after passage in CK cells for their ability to form plaques.

Cells

Monolayers of primary CK and CE cells were prepared as described previously (10) in Eagle's MEM containing 2 per cent heat inactivated calf serum with penicillin and streptomycin at a final concentration of 200 units and 200 μ g per ml respectively.

Trypsin and Trypsin Inhibitor

A 0.1 per cent stock solution of trypsin (1:250) (GIBCO) and 0.1 per cent soybean trypsin inhibitor (MILES) in phosphate buffered saline (pH 7.2) (PBS) were passed through a Seitz filter (Toyo Roshi) and stored in 1.0-ml aliquots at -20° C.

Solidifying Agent and Additives for Overlay Medium

The overlay was prepared using an 0.95 per cent Bacto-agar (DIFCO) and 0.1 per cent tryptose phosphate broth (DIFCO) in Eagle's MEM; the medium was sterilized by autoclaving prior to use.

Plaque Formation

After removal of the growth medium, cell monolayers were washed twice with Eagle's MEM. Glass culture bottles ($30 \times 30 \times 60$ mm) containing cell monolayers were inoculated with 0.2-ml of virus and incubated at 37° C for 60 minutes. Excess inoculum was then removed and 3-ml portions of the overlay was added to each bottle. Cultivation was continued for 2 days at 37° C. Then, 2-ml of overlay containing 0.004 per cent neutral red was added, and cultivation was recontinued for a further 5 days. The number of plaques on each monolayers were then counted.

Results

Requirement for Trypsin in the Overlay Medium

Ten strains of IBV, all passaged in CK cells, were tested for their ability to produce plaques in CE cells with and without trypsin (20 μ g per ml) in the overlay. Be-42 and Iowa-609 strains both produced plaques in the absence of trypsin, but their diameter was small when compared with those produced in the presence of trypsin. None of the other strains produced plaques after 7 days incubation in the absence of trypsin (Table 1). However, the incorporation of trypsin into the medium in the remaining 8 strains of IBV produced plaques with diameters ranging from 1 to 4 mm after 4 days incubation. The number of plaques in CE cells approximated those produced in CK cells.

Plaque Formed by IBV A-5968 Strain in CE Cells in the Presence of Trypsin

To study the effect of trypsin concentration on the number and size of plaques, IBV A-5968 strain was grown in CE cells in the presence of 10 to 60 μ g of trypsin

per ml of overlay. Plaques first appeared 3 days after inoculation using concentrations of 40 and 60 μg per ml; their number was maximal by 4 days using 40 μg per ml, and a concentration of 60 μg per ml appeared toxic to the cells (Fig. 1).

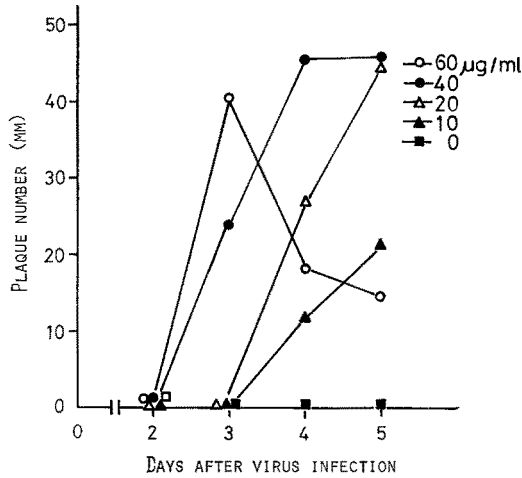


Fig. 1. Relationship between trypsin concentration and plaque number of A-5968 strain

Table 1. Enhancement of plaque formation in CE cells in the presence of trypsin

Virus	Trypsin in overlay (20 $\mu\text{g}/\text{ml}$)	Number of plaques (PFU/ml)	Diameter of plaque (mm)	Days after virus inoculation
Be-42	—	8.3×10^4	1—2	5
	+	2.0×10^5	4—5	3
Iowa-609	—	4.0×10^4	2—3	4
	+	1.5×10^5	4—6	4
A-5968	—	0	— ^a	7
	+	3.3×10^5	1—2	4
IB-41	—	0	—	7
	+	1.9×10^5	2—4	4
Connaught	—	0	—	7
	+	1.0×10^5	2	4
Holte	—	0	—	7
	+	2.4×10^5	1—2	4
KH	—	0	—	7
	+	2.5×10^5	1—2	4
Ishida	—	0	—	7
	+	1.3×10^5	2—3	4
Shiga	—	0	—	7
	+	1.6×10^5	1—2	4
Nerima	—	0	—	7
	+	1.0×10^5	1—2	4

^a No plaques were detected

The virus titer was an average of two titrations performed under the same conditions

By 5 days after inoculation, the numbers of plaques were similar using concentrations of 20 and 40 μg of trypsin. However, trypsin at a concentration of 40 μg per ml produced larger plaques (Fig. 2).

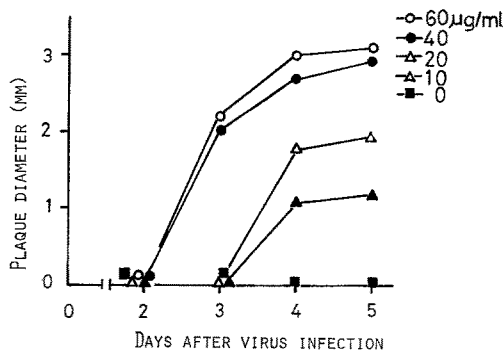


Fig. 2. Relationship between trypsin concentration and plaque diameter of A-5968 strain

Studies on the Mode of Action of Trypsin

IBV A-5968 strain which forms small plaques not only in CK cells but also in CE cells was used in the following studies:

Effect of Trypsin Inhibitor

To exclude the possibility that plaque enhancement was caused by an impurity in the trypsin preparation, a solution of trypsin containing 500 μg per ml was mixed with 1,000 μg per ml of soybean trypsin inhibitor and then used in the plaque assay at a concentration of 20 μg of trypsin per ml. The enhancing action of trypsin was abolished by trypsin inhibitor. Hence, trypsin itself was responsible for the effect on plaque formation.

Pre-Incubation of Medium with Trypsin

To test whether or not trypsin might act by destroying an inhibitor in the medium, agar overlay medium was incubated with 50 μg per ml of trypsin for 24 hours at 37° C. The medium was then heated at 100° C and used as overlay. This medium did not enhance plaque formation. When 20 μg per ml of trypsin was incorporated into this medium it enhanced plaque formation. This result suggested that trypsin did not act by removing a pre-existing virus inhibitor from the medium.

Pre-incubation of CE Cells with Trypsin

To test whether or not trypsin might act by modifying the surface of CE cells, 5-ml volumes of maintenance medium containing trypsin (20 μg per ml) were poured into bottles containing cell monolayers and these were incubated for 5 hours at 37° C. IBV A-5968 strain was then inoculated onto the cells, and after 60 minutes adsorption, the inoculum was replaced with overlay without trypsin. No plaques were formed. Therefore, trypsin did not act by modifying CE cells at the time of virus entry.

Discussion

CHOMIAK *et al.* (3) reported that the Beaudette strain of IBV produced CPE in CE cells after many passages in cell culture. In the present investigation, we showed that the Be-42 and Iowa-609 strains both produced plaques in CE cells in the absence of trypsin. We further showed that the addition of trypsin to the overlay medium enables other strains of IBV to produce plaques in CE cells. In many other investigators (1, 6, 7, 8, 11, 12, 13, 14, 15), trypsin has been incorporated into the overlay medium at a concentration of 10 μg per ml. The results of the present investigation, however, show that the incorporation of 20–40 μg of trypsin per ml of overlay is optimal for plaque formation by IBV.

The mechanism by which trypsin enhances plaque formation is uncertain, but it is possible that it enhances the replication or release of IBV. STORZ *et al.* (14) reported that coronaviral particles produced in trypsin-treated bovine cells had uniformly shorter surface projection than did the viral forms generated by trypsin-free cell cultures. The results reported here show that trypsin was required during the whole period of plaque development and we therefore assume that it acts on successive cycles of virus replication. CAME *et al.* (2) suggested that pancreatin produced its effect by an action on the host cells. In the present investigation, however, IBV A-5968 strain did not form plaques in CE cells which had been pre-incubated with trypsin. Possibly trypsin facilitates the development of IBV plaques by acting on the released virus particles, as has been described for reovirus (13), influenza virus (8) and rotavirus (12). Further studies are necessary to establish the mechanism(s) by which trypsin enhances plaque formation of IBV.

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