

EFFECT OF DIFFERENT PASSAGE HISTORIES OF INFECTIOUS BRONCHITIS VIRUS ON THE SENSITIVITY TO INHIBITORS IN CHICK SERUM AND THEIR REMOVAL BY TRYPSIN

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Summary. — Sensitivity of the Beaudette strain of infectious bronchitis virus (IBV) to non-antibody inhibitors in neutralization tests depended on the passage history of the virus. The chick embryo kidney cell-adapted (E₇₁ CEK₁₁) virus was the most sensitive, but after one chick embryo (CE) passage (E₇₁ CEK₁₁ E₁), this virus showed reversion to the sensitivity of the parent virus (E₇₁). IBV inhibitors in chicken serum could be removed by treatment with trypsin but not with phospholipase C.

Key words: infectious bronchitis virus; coronavirus; inhibitors

Introduction

For diagnosis of infectious bronchitis by neutralization test, constant serum-varying IBV procedures (Page and Cunningham, 1962; Cunningham, 1973) have been standardized in our laboratory. The antibody titre in field chickens was measured in embryonated CE using the embryo-adapted Beaudette strain as reference virus (Sawaguchi *et al.*, 1983). The antibody titer when assayed in CE as indicator system is considered to be affected by the adaptability of IBV to the CE and by non-antibody inhibitors that are heat-stable and present in most chick sera. These inhibitors probably affected the results of the test which seemed to show high antibody titer (Yachida *et al.*, 1979) and broad cross-reactivity of serologically different types of immune sera (S. Yachida, unpublished data). Influenza virus-neutralizing inhibitor(s) in animal and human sera have been removed with trypsin (Sampaio and Isaacs, 1953) and human coronavirus neutralizing inhibitor(s) in human serum with phospholipase C (Hovi, 1978; Gerna *et al.*, 1981). The present paper deals with the sensitivities to different inhibitors of IBV strains of different passage histories and with the successful removal of the inhibitors with trypsin.

Materials and Methods

Virus. The Beaudette strain of IBV with five different passage histories was used (Yachida *et al.*, 1978, 1979). The 71st embryo (E₇₁)-adapted IBV was passaged in CE kidney cell cultures (Yachida *et al.*, 1979; E₇₁CEK₁₁). The E₇₁CEK₁₁ virus was further passaged either in CE (E₇₁

CEK₁₁E₁₀) or in CE tracheal organ cultures (Yachida *et al.*, 1978) and thereafter in CE (E₇₁CEK₁₁OC₃₀E₁₀). The E₇₁CEK₁₁OC₃₀E₁₀ virus was again passaged in CE kidney cell cultures (E₇₁CEK₁₀OC₃₀E₁₀CEK₅). All viruses were filtered through a 0.22 μ m filter membrane (Millipore) to avoid formation of large aggregates of virus particles, and then stored at -80 °C. Mean death time (day) of embryos inoculated with 10² EID₅₀ of each virus was calculated as described previously (Yachida *et al.*, 1978).

Diluent. The 0.01 mol/l HEPES-buffered Medium 199 (Flow Laboratories, Inc., Rockville, Maryland) containing 10% tryptose phosphate broth (Difco Laboratories, Detroit, Michigan) and 0.5% bovine serum albumin (Fraction V; Miles Laboratories Inc., Elkhart, Indiana) was used as M-10HA, modified as previously described with M-20 diluent (Yachida *et al.*, 1978).

Sera. Sera were collected from White Leghorn chicks obtained from our laboratory's specific pathogen-free flock. Individual normal sera were obtained from nine 11-week-old chickens and four serum pools (11, Y3, P1, G) were collected from chicks of various ages. Two (S1, F) immune serum pools with relatively low neutralizing activity were prepared from 30-day-old chickens as described previously (Yachida *et al.*, 1979).

Neutralization test. The constant serum-varying virus method has been described previously (Yachida *et al.*, 1978; Yachida *et al.*, 1979). Serum was inactivated at 56 °C for 30 min. Portions of each diluted virus were mixed with equal part of the M-10HA or serum and then incubated at 37 °C for 1 hr. The mixture was inoculated into the chorioallantoic sac of five 9- to 11-day-old CE per dilution. All eggs were incubated at 37 °C for 7 days. Eggs with dead embryos before 18 hr post-inoculation (p.i.) were discarded. The endpoint of the median embryo lethal dose (EID₅₀) was calculated by the method of Behrens-Kärber.

The neutralizing index (NI) was determined by subtracting the log₁₀ titre of the virus-immune or -normal serum mixture from the log₁₀ titre of the virus-diluent mixture.

The effect of trypsin at a final concentration of 6.6 mg/ml and of phospholipase C at a final concentration of 8.75 mg/ml for removal of the inhibitor were preliminarily examined by plaque reduction in chicken kidney monolayers. The plaque techniques were performed as described previously (Yachida *et al.*, 1980).

Treatment of sera with trypsin or phospholipase C. Immune serum F and normal serum G were used. For trypsin treatment, the sera were dialyzed at 4 °C for 18 hr against 400 volumes of phosphate-buffered saline (PBS) to remove the trypsin inhibitor and then subjected to trypsin digestion; a solution of 5 or 15 mg/ml of trypsin (Type I; Sigma Chemical Co., St. Louis) in 0.2 mol/l PBS, pH 7.3 was mixed with an equal vol of dialyzed undiluted serum. This mixture was incubated at 37 °C for 2 hr in a water bath. On completion of trypsin digestion, 1/7 vol of the final amount of 25 mg/ml soybean trypsin inhibitor (Sigma; Type II-S) was added to this mixture to block the enzyme activity. For phospholipase C treatment, the solution of 20 mg/ml of the reagent (Type I, Sigma) in 0.2 mol/l PBS, pH 7.4, was mixed with an equal vol of undiluted serum. This mixture was incubated at 37 °C for 1 hr in a water bath. Next, 1/7 vol of the final amount of 12 mg/ml phenanthroline was added to this mixture to block the enzyme activity. The treated sera were dialyzed at 4 °C for 18 hr against 800 vol of PBS.

Results

Effect of passage histories on sensitivity to inhibitors

Thirteen undiluted normal and immune sera were preliminarily tested for neutralization inhibiting activity with Beaudette strain of five different passage histories. Table 1 shows that the highest NI was found either in normal (SPF) chicken sera or in immune sera, respectively, when the neutralization test was performed using the E₇₁CEK₁₁ virus, but this virus after only one embryo passage (E₇₁CEK₁₁E₁) showed reversion to the sensitivity of the parent virus (E₇₁). The NI of normal sera and of immune sera were approximately the same against other viruses including E₇₁CEK₁₁CET₃₀CEK₅.

The effect of the strain passage history on NI in selected normal and immune sera was subsequently investigated using E₇₁, E₇₁CEK₁₁ and E₇₁CEK₁₀E₁

Table 1. Effect of passage histories of the Beaudette strain on NI of 13 normal and 2 immune chicken sera

Chicken serum No.		Passage history of the Beaudette strain*				
		E ₇₁	E ₇₁ CEK ₁₁	E ₇₁ CEK ₁₁ E ₁	E ₇₁ CEK ₁₀ OC ₃₀ E ₁₀	E ₇₁ CEK ₁₀ OC ₃₀ E ₁₀ CEK ₅
Normal	1	1.0**	1.5	Not done	0.2	-0.2
	2	0.6	1.4	-0.1	0.5	0.6
	3	0.3	1.3	0.5	0.5	0.4
	4	0.5	1.3	0.1	0.4	0.2
	5	0.5	1.3	0.7	0.6	0.6
	6	0.3	1.4	0.1	0.7	0.6
	7	0.1	1.7	0.1	-0.5	1.2
	8	0.1	2.5	0.5	0.7	0.2
	10	1.2	0.8	0.7	0.7	0.6
	11 (Pooled)	0.1	1.5	0.1	0.6	0.6
	Y3 (Pooled)	1.3	1.5	1.0	0.6	1.0
	P1 (Pooled)	0.5	1.7	0.5	0.5	0
	G (Pooled)	0.1	1.5	0.7	0.6	0.2
Mean ± standard error		0.51 ±0.12	1.49 ±0.10	0.41 ±0.10	0.47 ±0.09	0.46 ±0.11
Immune	S1 (Pooled)	2.3	2.7	2.7	1.8	2.0
	F (Pooled)	2.0	3.3	2.5	1.7	1.6

* E = embryo passage CEK = chick kidney monolayer passage OC = organ culture passage (chick embryo trachea). Values indicate the number of passages in each host.

** Neutralizing index (NI).

Table 2. Effect of passage histories of Beaudette strain on NI of selected normal and immune chicken sera

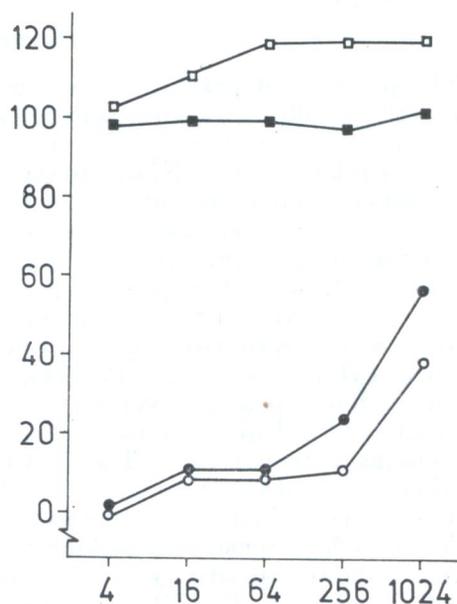
Reference virus	Serum			
	Exp. 1		Exp. 2	
	Normal I1	Immune F	Normal G	Immune F
E ₇₁	0.53 ± 0.07 (3)*	2.80 ± 0.20 (3)	0.47 ± 0.19 (7)	2.86 ± 0.18 (7)
E ₇₁ CEK ₁₁	2.07 ± 0.24 (3)	4.20 ± 0.12 (3)	1.74 ± 0.16 (7)	3.83 ± 0.13 (7)
E ₇₁ CEK ₁₀ E ₁	0.53 ± 0.13 (3)	3.06 ± 0.13 (3)	0.66 ± 0.14 (7)	2.86 ± 0.18 (7)

* Mean neutralizing index (NI) ± standard error (No. tested)

which had mean death times (days) of 2.0, 2.6 and 2.0, respectively. High NI in normal sera were obtained using E₇₁ CEK₁₁ virus, although the NI of the sera against E₇₁ and E₇₁ CEK₁₁ E₁ viruses were relatively low (Table 2).

Removal of inhibitor by treatment with trypsin or phospholipase C in the plaque reduction test

According to the plaque reduction test, serum inhibitors were completely removed with 6.6 mg/ml trypsin but not affected by treatment with 8.75 mg/ml phospholipase C (Table 3). The effects of trypsin treatment on removing the inhibitor from chicken serum diluted for plaque reduction are given in Fig. 1. Immune (F) and normal (G) sera treated with trypsin demonstrated less IBV inhibition (more plaques) than untreated sera.

**Fig. 1.**

Effect of trypsin treatment on removal of non-antibody inhibitors from chicken serum

● —● Trypsin-treated antiserum (F)
 ○ —○ Non-treated antiserum (F)
 □ —□ Trypsin-treated normal serum (G)
 ■ —■ Non-treated normal serum (G)
 Abscissa: serum dilution; ordinate: number of plaques

Table 3. Effect of trypsin or phospholipase C treatments on removal of IBV non-antibody inhibitors measured by the plaque technique in normal and immune chick sera*

Chick serum	Treatment with (final conc.)	Plaque reduction (%)**	
		Exp. 1	Exp. 2
Normal (G)	None	40.1	52.7
	Trypsin (6.6 mg/ml)	0	Not done
	Phospholipase C (8.75 mg/ml)	Not done	44.9
Immune (F)	None	93.2	92.2
	Trypsin (6.6 mg/ml)	87.5	Not done
	Phospholipase C (8.75 mg/ml)	Not done	98.3

* Equal volumes of undiluted sera treated with M10HA, trypsin or phospholipase C were mixed with the E₇₁CEK₁₁ virus (about 130 PFU) and incubated at 37 °C for 1 hr. Next, 0.2 ml samples of these mixtures were inoculated into three Petri dishes. The dishes inoculated with M-10HA- or serum-virus mixtures were left standing at 37 °C for 1 hr in 5% CO₂ atmosphere and then overlaid with agar medium.

** Mean reduction in serum-free mixture.

Effect of trypsin treatment on NI of normal and immune sera measured by the constant serum-variable virus neutralization test

The effects of trypsin treatment on NI of normal and immune sera were investigated with a reference virus of the E₇₁CEK₁₁ virus which had the highest sensitivity to inhibitors as shown in Table 4. Some inhibitors were partially or completely removed by treatment with 2.2 and 6.6 mg/ml trypsin, respectively.

Discussion

The presence of naturally occurring viral inhibitors in the serum posed a practical problem in viral serological tests. In IBV neutralization tests, non-antibody inhibitors of IBV present in many chicken sera probably cause the relatively high NI due to the antibody combined with non-antibody inhibitors in IB immune chickens. Our results of NI for normal sera obtained with E₇₁ virus were similar to those of Cunningham (1951) who reported that normal sera had NI values of up to 1.4 when assayed with the embryo-adapted Beaudette strain. Lukert (1973) reported that NI of normal serum was lower in cell culture systems than in embryo systems.

We showed previously that NI of normal serum depended on the passage history of IBV (Takahashi *et al.*, 1983). We examined the inhibitor sensitivity of five different passage levels of the Beaudette strain of IBV. Interestingly, E₇₁CEK₁₁ virus had a high reactivity to inhibitors, although the mechanisms or reasons were not clear. The E₇₁CEK₁₁ virus was cloned from E₇₁ in chicken embryo kidney monolayers and by plaque purification it was possible to select a clone highly sensitive to the inhibitors. The cell culture-passaged IBV had a high sensitivity to inhibitors, but embryo passages caused it to revert to lower sensitivity. Interestingly, the one embryo-passaged virus (E₇₁CEK₁₁E₁) of E₇₁CEK₁₁ showed the same sensitivity as that of the

Table 4. Effect of trypsin or phospholipase C treatments to remove IBV non-antibody inhibitors measured by constant serum-variable virus neutralization test in normal and immune chick sera*

Chick serum	Treatment with (final conc.)	Mean neutralizing index (NI) ± standard error (number tested)
Normal G	None	1.07 ± 0.06 (7)
	Trypsin (2.2 mg/ml)	0.61 ± 0.10 (7)
	Trypsin (6.6 mg/ml)	0.03 ± 0.06 (7)
Immune F	None	2.70 ± 0.11 (7)
	Trypsin (2.2 mg/ml)	2.21 ± 0.11 (7)
	Trypsin (6.6 mg/ml)	2.09 ± 0.10 (7)

* E₇₁CEK₁₁ was used as the reference virus.

E₇₁ virus. The neutralizing inhibitor-sensitive and -resistant strains have been found with the Newcastle disease virus (Kaleta *et al.*, 1973), poliovirus (Takemori *et al.*, 1958) and influenza virus (Chu, 1951).

Treatment of normal serum G with 6.6 mg/ml trypsin completely removed non-antibody inhibitor(s) (Table 4). In the immune serum F treated with trypsin, the mean NI was relatively low compared with those not treated with the reagent, suggesting that the differences in NI with and without treatment were due non-antibody inhibitors in the immune serum.

Our results suggest that the active substance in IBV inhibitors may be protein or protein-containing complex not associated with lipids (Table 3). Human coronavirus-neutralizing and haemagglutinin inhibitors have been suggested to be lipoproteins (Gerna *et al.*, 1980, 1981). A heat-resistant (gamma) myxovirus inhibitor of glycoprotein nature revealing virus-neutralizing activity has been described (Křižanová and Rathová, 1969). We are trying to elucidate further the IBV inhibitors using IB haemagglutination tests.

The trypsin treatment described in this paper did not destroy the immunoglobulins in immune chick serum, because the titer of anti-Newcastle disease haemagglutination-inhibition antibody in chicken serum treated with trypsin did not decrease with trypsin treatment of final concentration up to 25 mg/ml (S. Yachida; unpublished data). The effect of an IBV inhibitor is reversed in chicken serum by the presence of a 1.5% (w/v) concentration of several sugars (Lukert, 1973), but our study shows that the inhibitor is not completely removed by these substances (S. Yachida, unpublished data).

In summary, the sensitivity of IBV to the neutralizing activity of non-antibody inhibitor depends on the cell culture-passage level of the virus; the inhibitor(s) can be removed by treatment of the serum with trypsin but not with phospholipase C.

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