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INFECTIOUS BRONCHITIS VIRUS INTERFERENCE WITH GROWTH OF NEWCASTLE DISEASE VIRUS.

I. STUDY OF INTERFERENCE IN CHICKEN EMBRYOS (1)

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INTRODUCTION

The introduction of two live viruses into a susceptible host presupposes knowledge of their relative actions and the consequent respsonse of the host to the two agents. Under such circumstances there are three possibilities: 1) independent action of each virus, 2) interference of one virus with the other, and 3) synergism.

There is disagreement among the investigators who studied the effects of IBV-NDV combination in birds. Some investigators 16 believe that dual infection and subsequent immune response are possible, whereas others sustain the thesis of interference of one virus over the other, with consequent impaired immune response. A desire to elucidate the contrasting reports and common use of combined infectious bronchitis (IB) and Newcastle disease (ND) vaccines suggested a systematic investigation of the factors involved in dual administration of the above viruses. Also considered were the interval between inoculation of the viruses and their relative amounts since these two factors are known to be responsible for either interference or dual infection.

It was deemed necessary to confine the initial studies to a relatively simple system, such as the embryonating chicken egg (ECE), in which the

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effects of the two viruses may be related directly to their invasion and growth 15 without detectable antibody and immune responses as observed in birds. Furthermore, differences in tropism between NDV and IBV may account for failure of demonstrating interference 11 . In ECE, the advantage of the multiple tropsim of NDV over IBV is curtailed because the strains of IBV and NDV used were highly pathogenic for embryos.

Other reasons for choosing the chicken embroys were : simplicity of demonstrating NDV in the allantoic fluid (AF) by the hemagglutination (HA) test, the availability of an egg-adapted strain of IBV, and an adequate supply of fertile eggs.

Data are presented herein to show that the determining factor in the interference of ECE is an excess of IBV over NDV.

MATERIALS AND METHODS

The eggs used were from dams previously exposed to IB and ND vaccinations. Fabricant 7 reported, and experience confirmed, no significant differences in susceptibility to either ND or IB viruses between ECE from dams susceptible to ND and IB and ECE from dams immune to these two diseases. All ECE were incubated in a Jamesway incubator at 37° C until 10 days of age, and only apparently ones were selected (after candling). The shell over the air cell was swabbed with 70% ethyl alcohol, drilled, and reswabbed with 10% tincture of iodine. Subsequently 0.2 ml of the virus was inoculated into the chorioallantoic cavity and the ECE were incubated at 35.5° C. Cold sterile tryptose broth (Difco) was used as diluent throughout the experiments.

The egg-adapted DA strain of IBV used (originally from the Lederle Laboratories, through the courtesy of Dr. F.S. Markham) has an ELD 50^{17} per ml of at least $10^{6.3}$ .It is similar to the Beaudette egg-adapted strain.

The GB (Texas) strain of NDV, with an HA titer of at least 1 :640 and an ELD_{50} of at least $10^{6.8}$,was used. The tube HA test was performed according to the method described by Beach, 4 and the plate HA test used was that described by Brandly *et al.* 5

To avoid introduction of unnecessary variants, antibiotics were not used. Freedom from aerobic bacteria was ascertained by streaking each dilution onto 5% bovine blood agar plates, and by inoculating a tube of tryptose broth. At the beginning of the experiment, the IB and ND virus suspensions were found to be free of *Mycoplasma gallisepticum* by seeding them into Mycoplasma broth. 1

Table 1. Design of preliminary experiments.

Trial A[a]	Trial B[a]
1. IBV and NDV, approximately equal amounts	1. IBV and NDV, approximately equal amounts
2. IBV alone	2. Excess of IBV over NDV
3. NDV alone	3. Excess of NDV over IBV
	4. IBV alone
	5. NDV alone
	6. Diluent alone

[a]The plate hemagglutination test was used for NDV.

EXPERIMENTAL PROCEDURE AND RESULTS

Two preliminary experiments developed into four major series of experiments. Three corollary experiments were performed to substantiate the data obtained.

The design of the two preliminary experiments, in which NDV was inoculated immediately after IBV, was as shown in Table 1.

Trial A-1. Fifteen ECE were inoculated. The amount of inoculum was 0.2 ml of each virus. Allantoic fluid (AF) was sampled from each dead ECE for the plate HA test, and the results were tabulated (Table 2).

Trial A-2. A titration of IBV to serve as IBV control for Trial A-1 was performed. Five ECE per dilution were used. An ELD^{50} per ml of $10^{7.1}$ was obtained.

Table 2. Results of inoculation of IBV immediately followed by NDV. Slight excess of NDV over IBV.

Dilutions of		Plate HA Test		Number of EOE	
IBV[a]	NDV[b]	Positive	Negative	Alive	Total
10^{-6}	10^{-6}	0	5	0	5
10^{-7}	10^{-7}	4	1	0	5
10^{-8}	10^{-8}	2	1	2	5

[a]$10^{7.1}$ ELD_{50} per ml.
[b]$10^{7.7}$ ELD_{50} per ml.

Trial A-3. A similar titration was carried out for NDV. The ELD^{50} per ml was $10^{7.7}$, and AF from all dead ECE were positive on the plate HA test. As controls to handling and incubation, two dozen uninoculated ECE were incubated, candled, and kept for the same duration as those in Trial A-1. One died within 60 hours of incubation. Attempts failed to isolate a lethal agent.

Table 3. Inoculation of IBV immediately followed by NDV. Excess of NDV over IBV.

Dilutions of		Plate HA test		Number of ECE	
IBV[a]	NDV[b]	Positive	Negative	Alive	Total
10^{-3}	10^{-3}	1	3	0	4
10^{-4}	10^{-4}	3	1	0	4
10^{-5}	10^{-5}	2	2	0	4
10^{-6}	10^{-6}	1	3	0	4
10^{-7}	10^{-7}	3	1	0	4
10^{-8}	10^{-8}	1	1	2	4
10^{-9}	10^{-9}	1	0	3	4

[a]$ELD_{50} = 10^{6.3}$ per ml. [b]$ELD_{50} = 10^{7.8}$ per ml.

Trial B-1. Twenty-eight ECE were inoculated with IBV immediately followed by NDV. Four ECE per dilution, and seven dilutions of each virus, were used. An approximately equal amount of each virus was anticipated, but titrations of IBV and NDV in Trials B-4 and B-5, conducted almost concurrently, showed that IBV had an ELD^{50} of $10^{6.3}$ per ml whereas NDV had an ELD^{50} of $10^{7.8}$ per ml. Nevertheless, the data obtained (Table 3) showed an irregularity of HA activity in the 10^{-3}, 10^{-3}, and 10^{-6} dilutions.

Table 4. Inoculation of IBV immediately followed by NDV. Excess of IBV over NDV.

Dilutions of		Plate HA test		Number of ECE	
IBV[a]	NDV[b]	Positive	Negative	Alive	Total
10^{-1}	10^{-4}	1	3	0	4
10^{-2}	10^{-5}	0	4	0	4
10^{-3}	10^{-6}	0	4	0	4
10^{-4}	10^{-7}	0	4	0	4
10^{-5}	10^{-8}	1	2	0	3[c]
10^{-6}	10^{-9}	0	4	0	4
10^{-7}	10^{-10}	0	2	2	4

[a]$ELD_{50} = 10^{6.3}$ per ml. [b]$ELD_{50} = 10^{7.8}$ per ml.
[c]One egg died in less than 20 hours and was discarded.

Trial B-2. A procedure similar to Trial B-1 was used except that IBV was in excess of NDV. The results (Table 4) clearly indicate marked interference of NDV by IBV in the plate HA test.

Trial B-3 was similar to Trial B-1, the only difference being that NDV exceeded IBV by $2\frac{1}{2}$ logs. Table 5 presents data showing an irregularity in HA activity similar to that in Trial B-1.

Trial B-4 was a titration of IBV, and the results are reported under Trial B-1.

Trial B-5. Samples of AF from all dead ECE in the NDV titration were HA-positive on the plate test.

Table 5. Inoculation of IBV immediately followed by NDV. Excess of NDV over IBV.

Dilutions of		Plate HA test		Number of ECE	
IBV[a]	NDV[b]	Positive	Negative	Alive	Total
10^{-2}	10^{-1}	4	0	0	4
10^{-3}	10^{-2}	3	1	0	4
10^{-4}	10^{-3}	1	3	0	4
10^{-5}	10^{-4}	1	3	0	4
10^{-6}	10^{-5}	0	4	0	4
10^{-7}	10^{-6}	2	1	0	3[c]
10^{-8}	10^{-7}	3	0	0	3[d]

[a]$ELD_{50} = 10^{6.3}$ per ml. [b]$ELD_{50} = 10^{7.5}$ per ml.
[c]One egg broke during harvest.
[d]One egg died in less than 20 hours and was discarded.

Trial B-6. Twenty-eight ECE were inoculated only with tryptose broth to serve as diluent controls. All survived the 7-day incubation period except one, which died within 30 hours of incubation. Its AF was negative for HA activity.

Since most of the deaths of the ECE inoculated with the various combinations of NDV and IBV occurred between the 33rd and 68th hours after inoculation it was necessary to establish that within the above times the GB (Texas) strain of NDV alone was capable of multiplying sufficiently to hemagglutinate at significant titers. The absence of HA activity within that period was considered an indication of interference whenever both IBV and NDV were inoculated. Accordingly, a corollary experiment (Experiment C-1) was designed as shown in Table 6.

Table 6. Design of experiment C-1

Dilutions	No. eggs to be inoculated	Hours of incubation prior to sampling for HA activity, and no. of eggs to be sampled.					
		5	17	30	41	50	65
10^{-1}	25	5	5	5	5	5	0
10^{-2}	20	0	0	5	5	5	5
10^{-3}	20	0	0	5	5	5	5
10^{-4}	20	0	0	5	5	5	5
10^{-5}	20	0	0	5	5	5	5
10^{-6}	20	0	0	5	5	5	5
10^{-7}	15	0	0	0	5	5	5
10^{-8}	15	0	0	0	5	5	5

The results (Fig. 1) showed HA activity as early as 17 hours after inoculation of NDV (10^{-1} dilution). HA titers were significant from the 30th hour on.

Since the major criterion used to judge interference was HA activity, it was necessary to ascertain *in vitro* that IBV would not prevent hemagglutination by NDV; to this end, corollary experiment C-2 was executed. Data

from Table 7 show that an excess of IBV did not interfere with the expected HA titer, when titers of 1 :640 were obtained with NDV irrespective of the presence or absence of IBV in the same test tubes.

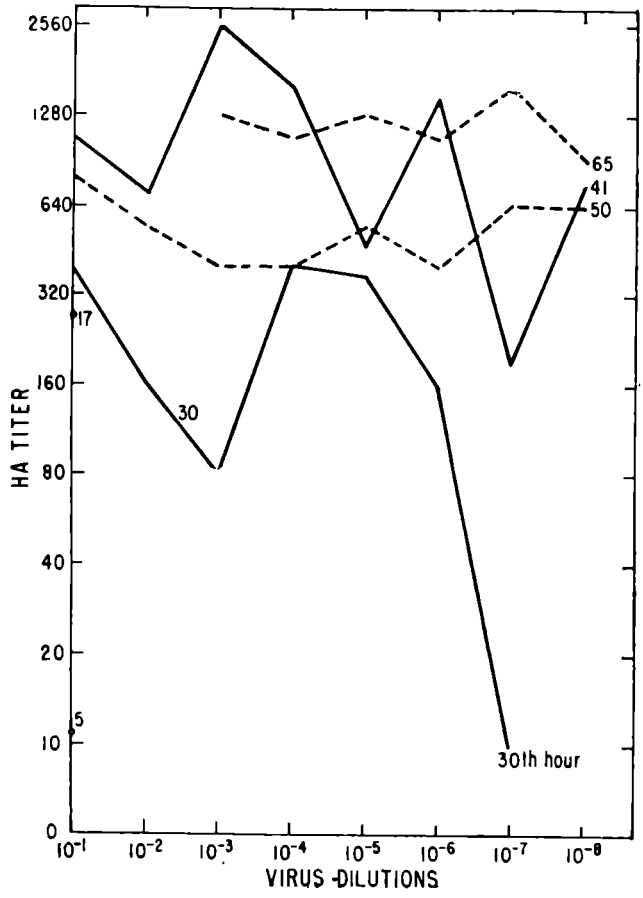

Fig 1. Geometric mean hemagglutination titers of GB (Texas) strain Newcastle disease virus at various times and dilutions.

Since interference occurred when the two viruses were inoculated almost simultaneously in the preliminary experiments, it was deemed necessary to explore the phenomenon by inoculating IBV and NDY at different time intervals. Hence, a subsequent series of experiments were performed as shown

in Table 8. The tube HA test for NDV was used throughout these experiments.

Series 1 and 2 were conducted simultaneously. Each series was allotted 200 ECE, each group comprised 40 ECE, and AF from all dead ECE were

Table 7. Hemagglutination titers of Newcastle disease virus (NDV) in the presence of excessive amounts of infectious bronchitis virus (IBV).

IBV dilutions of 1 to:	NDV dilutions of 1 to:		
	10 to 640 incl.	1280	2560
10	4	3	2
20	4	3	—
10	4	2	—
20	4	3	—
GB-NDV	4	3	—
IB alone	—	—	—
RBC alone	—	—	—

sampled for HA activity. The results are tabulated in Tables 9-18. Attempts to re-isolate IBV were made in the E groups to ascertain that IBV was responsible for the death of the embryos. A fair degree of success was achieved (Tables 17, 18).

Table 8. Design of experiments series 1-4.

Series 1: IBV 4 hr Before NDV.	Series 2: NDV 4 hr Before IBV.
A. Approximately equal amounts of each virus B. Excess of IBV over NDV C. Slight excess of NDV over IBV D. Increasing amounts of IBV to decreasing amounts of NDV E. Excess of IBV over NDV with attempts to re-isolate IBV F. NDV alone G. IBV alone	A. Approximately equal amounts of each virus B. Excess of IBV over NDV C. Slight excess of NDV over IBV D. Decreasing amounts of NDV to increasing amounts of IBV E. Excess of IBV over NDV with attempts to re-isolate IBV
Series 3: IBV 4 hr before NDV and further testing of randomly selected samples	**Series 4: inactivated IBV 4 hr before NDV**
A. Approximately equal amounts of each virus B. Excess of IBV over NDV C. Excess of NDV over IBV	A. Inactivated IBV alone B. Excess of inactivated IBV over NDV C. Excess of NDV over inactivated IBV D. Increasing amounts of inactivated IBV to decreasing amounts of NDV

Table 9. Inoculation of infectious bronchitis virus (IBV) 4 hours before Newcastle disease virus (NDV). Approximately equal amounts of each virus.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:										No. of ECE	
IBV[a]	NDV[b]	0	10	20	40	80	160	320	640	1280	2560	Alive	Total
10^{-2}	10^{-2}	3			1	1						0	5
10^{-3}	10^{-3}	4					1					0	5
10^{-4}	10^{-4}	3			1	1						0	5
10^{-5}	10^{-5}	4			1							0	5
10^{-6}	10^{-6}	1						)	1		2	0	5
10^{-7}	10^{-7}								1	2		2	5
10^{-8}	10^{-8}							1				4	5
10^{-9}	10^{-9}											5	5

[a] $10^{6.1}$ ELD_{50} per ml. [b] $10^{6.8}$ ELD_{50} per ml

Trial 1-A. HA titers considerably lower than expected were found in the 10^{-2} through 10^{-6} dilutions, indicating interference.

Table 10. Inoculation of NDV 4 hours before IBV—approximately equal amounts of each virus.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:										No. of EQE	
NDV[a]	IBV[b]	0	10	20	40	80	160	320	640	1280	2560	Alive	Total
10^{-2}	10^{-2}							1	3	1		0	5
10^{-3}	10^{-3}				1			2	2			0	5
10^{-4}	10^{-4}					3	1	1				0	5
10^{-5}	10^{-5}						1	3	1			0	5
10^{-6}	10^{-6}							1		2	2	0	5
10^{-7}	10^{-7}											5	5
10^{-8}	10^{-8}											5	5
10^{-9}	10^{-9}											5	5

[a] $10^{6.8}$ ELD_{50} per ml. [b] $10^{6.4}$ ELD_{50} per ml.

Trial 2-A. Interference in HA titers is still present, but not as marked as in Trial 1-A because NDV had an advantage of 4 hours over IBV.

Table 11. Inoculation of IBV 4 hours before NDV—excess of IBV over NDV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:					No. of ECE	
IBV[a]	NDV[b]	0	10	20	40	80	Alive	Total
10^{0}	10^{-3}	5					0	5
10^{-1}	10^{-4}	5					0	5
10^{-2}	10^{-5}	5					0	5
10^{-3}	10^{-6}	5					0	5
10^{-4}	10^{-7}	5					0	5
10^{-5}	10^{-8}	5					0	5
10^{-6}	10^{-9}	1					4	5
10^{-7}	10^{-10}	2					3	5

[a] $10^{6.4}$ ELD_{50} per ml. [b] $10^{6.8}$ ELD_{50} per ml.

Trial 1-B. Absence of HA titers is evident throughout the trial. The deaths observed in the last 3 dilutions are probably due to IBV.

Interference by IBV over NDV was observed to varying extents in both series. HA titers were 1 :640 or greater in the AF of all dead ECE in group F of series 1 receiving NDV alone. No HA titer was found in the AF of all dead ECE of group G, series 1, receiving IBV alone.

Table 12. Inoculation of NDV 4 hours before IBV...excess of IBV over NDV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:				No. of ECE	
NDV[a]	IBV[b]	0	10	20	40	80	Alive	Total
10^{-3}	10^{0}	4		1			0	5
10^{-4}	10^{-1}	5					0	5
10^{-5}	10^{-2}	5					0	5
10^{-6}	10^{-3}	4				1	0	5
10^{-7}	10^{-4}	5					0	5
10^{-9}	10^{-5}	3				1	1	5
10^{-9}	10^{-6}						5	5
10^{-10}	10^{-7}	1					4	5

[a] $10^{6.5}$ ELD_{50} per ml [b] $10^{5.1}$ ELD_{50} per ml.

Trial 2-B. Interference with the HA titers is marked, notwithstanding the 4 hours during which NDV had an opportunity to invade undisturbed the susceptible cells of the chorio-allantois.

Table 13. Inoculation of IBV 4 hours before NDV—slight excess of NDV over IBV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:										No. of ECE	
IBV[a]	NDV[b]	0	10	20	40	80	160	320	640	1280	2560	Alive	Total
10^{-2}	10^{-1}	2				3						0	5
10^{-3}	10^{-2}	1					1	3				0	5
10^{-4}	10^{-3}					5						0	5
10^{-5}	10^{-4}				1	1	1	1	1			0	5
10^{-6}	10^{-5}	1			1				1	2		0	5
10^{-7}	10^{-6}								1	2	2	0	5
10^{-8}	10^{-7}							1				4	5
10^{-9}	10^{-8}	1										4	5

[a] $10^{6.4}$ ELD_{50}/ml. [b] $10^{6.8}$ ELD_{50}/ml.

Trial 1-C. Interference with the HA titers is much less evident than in Trial 1-B, because IBV was less than NDV. Interference is less pronounced as the amount of IBV decreases.

Experiments of series 3 were undertaken to determine the cause of death for randomly selected ECE, thereby indicating the presence or absence of interference pheonomena on an individual basis. This was accomplished by neutralizing IBV present in the AF of ECE that died after inoculation of varying combinations of IB and ND viruses. A portion of the neutralized material was then inoculated into other ECE and incubated for 6 days. NDV was considered absent if the embryos survived this period. If death

Table 14. Inoculation of NDV 4 hours before IBV—slight excess of NDV over IBV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:										No. of ECE	
NDV[a]	IBV[b]	0	10	20	40	80	160	320	640	1280	2560	Alive	Total
10^{-1}	10^{-2}							2	1	1	1	0	5
10^{-2}	10^{-3}							3		2		0	5
10^{-3}	10^{-4}							[illegible]			1	0	5
10^{-4}	10^{-5}					2	1		1		1	0	5
10^{-5}	10^{-6}								1	1	3	0	5
10^{-6}	10^{-7}									1	4	0	5
10^{-7}	10^{-8}											5	5
10^{-8}	10^{-9}	1										4	5

[a]$10^{6.5}$ ELD_{50}/ml. [b]$10^{6.4}$ ELD_{50}/ml.

Trial 2-C. Interference is barely detectable, since NDV has the double advantage of time and dosage over IBV.

Table 15. Inoculation of IBV 4 hours before NDV—increasing amounts of IBV to decreasing amounts of NDV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:										No. of ECE	
IBV[a]	NDV[b]	0	10	20	40	80	160	320	640	1280	2560	Alive	Total
10^{-7}	10^{-1}	1[c]							2	2		0	5
10^{-6}	10^{-2}							2	1	2		0	5
10^{-5}	10^{-3}					1	2	2				0	5
10^{-4}	10^{-4}	3		1		1						0	5
10^{-3}	10^{-5}	5										0	5
10^{-2}	10^{-6}	5										0	5
10^{-1}	10^{-7}	5										0	5
10^{0}	10^{-8}	5										0	5

[a]$10^{6.4}$ ELD_{50}/ml. [b]$10^{6.8}$ ELD_{50}/ml. [c]Death of embryo due to trauma.

Trial 1-D. Interference with the HA titers becomes evident as the concentrations of IBV increase.

Table 16. Inoculation of NDV 4 hours before IBV—decreasing amount of NDV to increasing amount of IBV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:										No. of ECE	
NDV[a]	IBV[b]	0	10	20	40	80	160	320	640	1280	2560	Alive	Total
10^{-1}	10^{-7}									2	1	0	5[c]
10^{-2}	10^{-6}								1	3	1	0	5
10^{-3}	10^{-5}						2	2		1		0	5
10^{-4}	10^{-4}			1	1	3						0	5
10^{-5}	10^{-3}	1		1	1	2						0	5
10^{-6}	10^{-2}	5										0	5
10^{-7}	10^{-1}	5										0	5
10^{-8}	10^{0}	5										0	5

[a]$10^{6.8}$ ELD_{50}/ml. [b]$10^{6.4}$ ELD_{50}/ml.
[c]2 embryos died in less than 20 hours and were discarded.

Trial 2-D. The results are very similar to Trial 1-D, irrespective of the 4-hour advantage of NDV over IBV.

occurred within 6 days, the remaining neutralized material was thawed and titrated for NDV. AF from dead ECE of the 10^{-1} dilution of these titrations was sampled for HA activity. The results are presented in Tables 19-21, and Tables 19A-21A are evaluations of those results after further work was performed.

Table 17. Inoculation of IBV 4 hours before NDV—excess of IBV over NDV with attempts to re-isolate IBV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:								No. of ECE		IBV[c]
IBV[a]	NDV[b]	0	10	20	40	80	160	320	640	Alive	Total	re-isolated
10^{-1}	10^{-4}	5								0	5	Yes
10^{-2}	10^{-5}	5								0	5	Yes
10^{-3}	10^{-6}	5								0	5	No
10^{-4}	10^{-7}	5								0	5	No
10^{-5}	10^{-8}	5								0	5	No
10^{-6}	10^{-9}	5								0	5	No
10^{-7}	10^{-10}	5								0	5	No
10^{-8}	10^{-11}	5								0	5	Yes

[a] $10^{8.1}$ ELD_{50}/ml. [b] 10^{-4} ELD_{50}/ml

[c] Procedure for re-isolation:

1) Randomly select 1 ECE from each dilution. Harvest the allantoic fluid (AF) and store frozen at -15°C.
2) Neutralize the AF with ND antiserum (1:1) for 30 minutes at room temperature prior to inoculation into other ECE. If death occurred, IBV was considered responsible.
3) To confirm that the deaths of step 2 were actually due to IBV, the AF of dead ECE from step 2 was neutralized with IB antiserum (1:1) for 30 minutes at room temperature and again inoculated into other ECE to observe absence of death.

Trial 1-E. The results are similar to those obtained in Trial 1-B. Evidence is presented that the ECE died of IBV, since this virus could be isolated in three instances. The failure to re-isolate IBV in the other 5 dilutions may be due to the prolonged storage of the original AF.

Table 18. Inoculation of NDV 4 hours before IBV—excess of IBV over NDV with attempts to re-isolate IBV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:								No. of ECE		IBV[c]
NDV[a]	IBV[b]	0	10	20	40	80	160	320	640	Alive	Total	re-isolated
10^{-4}	10^{-1}	2			1	1				0	5[d]	Yes
10^{-5}	10^{-2}	3	2			1				0	5	Yes
10^{-6}	10^{-3}	3	1			1				0	5	Yes
10^{-7}	10^{-4}	5								0	5	Yes
10^{-8}	10^{-5}	3		1		1				0	5	Yes
10^{-9}	10^{-6}	5								0	5	No
10^{-10}	10^{-7}	5								0	5	Yes
10^{-11}	10^{-8}	5								0	5	Yes

[a] $10^{8.3}$ ELD_{50}/ml [b] 10^{-1} ELD_{50}/ml.

[c] Procedure for re-isolation same as that used in Trial 1-E.

[d] Yolk ruptured during process of harvesting, no HA test performed on one ECE.

Trial 2-E. The results are comparable to those of Trial 2-B. The death of ECE was due of IBV.

Tables 19-21 and 19A-21A show that IBV interfered not only with the development of HA but also with the growth of NDV as judged by embryo lethality. It is known that the lethality test for ECE is a much more sensitive

criterion for the presence of NDV than the HA test, since only 10 NDV particles suffice to kill a chicken embryo 3 whereas approximately 10^7 NDV particles are required for hemagglutination. 10

Table 19. Inoculation of IBV 4 hours before NDV—approximately equal amounts of each virus.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:							No. of ECE	
IBV[a]	NDV[b]	0	10	20	40	80	160	320 or more	Alive	Total
10^{-3}	10^{-3}	4			1				0	5
10^{-4}	10^{-4}	2	1		1			1	0	5
10^{-5}	10^{-5}	5							0	5
10^{-6}	10^{-6}	4							0	5[c]
10^{-7}	10^{-7}	2				1		1	0	5[c]
10^{-8}	10^{-8}	1						2	1	5[d]
10^{-9}	10^{-9}							1	3	5[c]

[a] $10^{9.1}$ ELD_{50}/ml. [b] $10^{8.3}$ ELD_{50}/ml.
[c] Yolk ruptured during process of harvesting, no HA test performed on one ECE.
[d] 5th ECE died within 24 hours and was discarded.

Trial 3-A. Interference is marked. The time factor appears to be important when the respective amounts of each virus are similar.

Table 19A. Evaluation of HA results in Table 19 after further testing of randomly selected samples from each dilution.

Dilutions of		Egg	HA	Approx.	Growth of NDV after	Death	Interference of NDV by
IBV[a]	NDV[b]	no.	titer	ELD_{50} (I)	IBV neutr. (II)	due to NDV (III)	IBV (IV)
10^{-3}	10^{-3}	1	0	0	—[c]	—	+
10^{-3}	10^{-3}	2[d]	1:40	10^4 or more	+	+	+
10^{-4}	10^{-4}	7	0	10^9	+	—	+
10^{-4}	10^{-4}	8	1:10	10^8	+	?	+
10^{-5}	10^{-5}	11	0	0	—	—	+
10^{-5}	10^{-5}	12	0	10^1or less	+	—	+
10^{-6}	10^{-6}	17	0	10^1 or less	+	—	+
10^{-6}	10^{-6}	19	0	10^1 or less	+	—	+
10^{-7}	10^{-7}	22[d]	1:40	10^8	+	+	+
10^{-7}	10^{-7}	23	0	10^1 or less	+	—	+
10^{-8}	10^{-8}	28	1:320	10^6 or more	+	+	—
10^{-8}	10^{-8}	30	1:320	10^5 or more	+	+	—
10^{-9}	10^{-9}	32	1:320	10^6 or more	+	+	—

[a] $10^{9.1}$ ELD_{50}/ml. [b] $10^{8.3}$ ELD_{50}/ml.
[c] + yes; — no. [d] IBV re-isolated.

I. Titration of original allantoic fluid (AF) after 30 minutes neutralization with IB antiserum at room temperature.
II. The original AF was neutralized with IB antiserum for 30 minutes at room temperature. The above mixture was inoculated into other ECE, and if no embryos died within 6 days after inoculation NDV was considered absent
III. Conclusions based on the HA titers, the expected ELD_{50}, and the presence of NDV after neutralization with IB antiserum
IV. On the basis of either absence of NDV or presence of NDV in much smaller amounts than expected if IBV had not been present

The soundness of the reisolation procedure for NDV was substantiated. The presence of NDV was determined by the embryo lethality of each dilution of the original AF after neutralization with IB immune serum.

To validate the above procedure, it was necessary to determine that after dilution of the neutralized IBV serum mixture, 6 no residual IBV[2] could have been responsible for the death of the embryos. To this purpose Expt. C-3 was

Table 20. Inoculation of IBV 4 hours before NDV—excess of IBV over NDV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:							No. of ECE	
IBV[a]	NDV[b]	0	10	20	40	80	160	320 or more	Alive	Total
10^{-1}	10^{-4}	3						1	0	5[c]
10^{-2}	10^{-5}	3	1						0	5[d]
10^{-3}	10^{-6}	4							0	5[c]
10^{-4}	10^{-7}	4							0	5[c]
10^{-5}	10^{-8}	5							0	5
10^{-6}	10^{-9}	5							0	5
10^{-7}	10^{-10}	5							0	5

[a] $10^{8.1}$ ELD_{50}/ml [b] 10^{-3} ELD_{50}/ml
[c] Yolk ruptured during process of harvesting, no HA test performed on one ECE.
[d] 5th ECE died within 24 hours and was discarded.

Trial 3-B. Interference is more noticeable than in Trial 3-A as evident in all dilutions used. This marked effect is due to an excess of IBV over NDV

Table 20A. Evaluation of HA results in Table 20 after further testing of randomly selected samples from each dilution.

Dilutions of		Egg	HA	Approx.	Growth of NDV after	Death due to	Interference of NDV by
IBV[a]	NDV[b]	no.	titer	ELD_{50} (I)	IBV neutr. (II)	NDV (III)	IBV (IV)
10^{-1}	10^{-4}	2	0	0	—[c]	—	+
10^{-1}	10^{-4}	4[d]	1:320	10^{8}	+	+	—
10^{-2}	10^{-5}	6	1:10	10^{4} or more	+	?	+
10^{-2}	10^{-5}	7	0	0	—	—	+
10^{-3}	10^{-6}	11	0	0	—	—	+
10^{-3}	10^{-6}	12	0	0	—	—	+
10^{-4}	10^{-7}	17	0	0	—	—	+
10^{-4}	10^{-7}	18	0	10^{4}	+	?	+
10^{-5}	10^{-8}	21	0	0	—	—	+
10^{-5}	10^{-8}	25	0	0	—	—	+
10^{-6}	10^{-9}	27	0	0	—	—	+
10^{-6}	10^{-9}	28	0	0	—	—	+
10^{-7}	10^{-10}	31	0	0	—	—	
10^{-7}	10^{-10}	32	0	0	—	—	

[a] $10^{8.1}$ ELD_{50}/ml. [b] 10^{-3} ELD_{50}/ml.
[c] + yes; — no. [d] IBV re-isolated.

I. **Titration of original allantoic fluid (AF) after 30 minute neutralization with IB** antiserum at room temperature.
II. The original AF was neutralized with IB antiserum for 30 minutes at room **temperature. The above mixture was inoculated into other ECE, and if no embryos** died within 6 days after inoculation NDV was considered absent.
III. **Conclusions based on the HA titers, the expected ELD_{50}, and the presence of NDV after neutralization with IB antiserum.**
IV. On the basis of either absence of NDV or presence of NDV in much smaller amounts than expected if IBV had not been present.

dilution was netralized for 30 minutes at room temperature (25° C) with an equal amount of undiluted IB immune serum having a neutralizing titer of

at least 10^6 . Each mixture was in turn diluted to 10^7 with tryptose broth and then inoculated into five ECE. The results showed that the IB immune serum completely neutralized the IBV and no detectable virus was liberated after serial dilutions of the serum-virus mixture.

Table 21. Inoculation of IBV 4 hours before NDV—excess of NDV over IBV.

Dilutions of		Embryonating chicken eggs with allantoic fluid having hemagglutination titer of 1 to:							No. of ECE	
IBV[a]	NDV[b]	0	10	20	40	80	160	320	Alive	Total
10^{-5}	10^{-3}	3				2			0	5
10^{-6}	10^{-4}	3		1			1		0	5
10^{-7}	10^{-5}	1						2	0	5[c]
10^{-8}	10^{-6}					1		3	0	5[d]
10^{-9}	10^{-7}							4	0	5[c]

[a] $10^{8.1}$ ELD_{50}/ml. [b] $10^{8.3}$ ELD_{50}/ml.
[c] 5th ECE died within 24 hours and was discarded.
[d] Yolk ruptured during process of harvesting, no HA test performed on one ECE.

Trial 3-C. The results in this trial are in contrast to those obtained in Trial 3-B. The difference is attributable to the excess of NDV over IBV.

Table 21A. Evaluation of HA results in Table 21 after further testing of randomly selected samples from each dilution.

Dilutions of		Egg	HA	Approx.	Growth of NDV after	Death	Interference of NDV by
IBV[a]	NDV[b]	no.	titer	ELD_{50} (I)	IBV neutr. (II)	due to NDV (III)	IBV (IV)
10^{-5}	10^{-3}	1[d]	1:80	10^8	+[c]	+	+
10^{-6}	10^{-3}	3	0	10^8	+	—	+
10^{-6}	10^{-4}	6[d]	1:80	10^8	+	+	+
10^{-6}	10^{-4}	9	1:20	10^6 or more	+	+	+
10^{-7}	10^{-5}	13	1:10	10^6 or more	+	?	+
10^{-7}	10^{-5}	14	1:320	10^6	+	+	—
10^{-8}	10^{-6}	16	1:320	10^6	+	+	—
10^{-8}	10^{-6}	17	1:320	10^8	+	+	—
10^{-9}	10^{-7}	22	1:320	10^8	+	+	—
10^{-9}	10^{-7}	23	1:320	10^6 or more	+	+	—

[a] $10^{8.1}$ ELD_{50}/ml. [b] $10^{8.3}$ ELD_{50}/ml
[c] + yes; — no. [d] IBV re-isolated.

I. Titration of original allantoic fluid (AF) after 30-minute neutralization with IB antiserum at room temperature.
II. The original AF was neutralized with IB antiserum for 30 minutes at room temperature. The above mixture was inoculated into other ECE, and if no embryos died within 6 days after inoculation NDV was considered absent.
III. Conclusions based on the HA titers, the expected ELD_{50}, and the presence of NDV after neutralization with IB antiserum.
IV. On the basis of either absence of NDV or presence of NDV in much smaller amounts than expected if IBV had not been present.

Changes also in the pH of the diluent may result in releasing active virus, but those changes were negligible since tryptose broth, having a considerable buffering effect, was used throughout all the experiments.

Series 4 was initiated to establish that interference required living IBV

and was not detected when IBV was inactivated. IBV was therefore heat-inactivated at 56° C for 30 minutes in a water bath and inoculated 4 hours prior to active NDV. The relative amounts of each virus varies according to the procedure followed in the other series.

The results of this series of experiments show that inactivated IBV did not affect the HA titers (1 :640 or higher) expected with the NDV present in the various combinations, and that all embryos died. Inactivated IBV alone did not cause death of embryos.

DISCUSSION

The results of the different combinations (Tables 9-21) clearly show that an excess of IBV consistently interfered with the growth of NDV in ECE. The time interval between administration of each virus was less important than the relative amounts of each agent (Table (12). These findings in general agree with those reported by Hanson *et al.*, 8 who studied IBV-NDV interference in birds.

Interference could have taken place either on or within the available cells. In was theorized that IBV interfered with the multiplication of NDV. Since both the DA strain of IBV and GB (Texas) strain of NDV are very pathogenic for ECE, their invasiveness for the same tissue should therefore be pronounced. Table 12, however, shows that NDV failed almost completely to grow even when it was inoculated 4 hours prior to IBV. It is very difficult to conceive that in 4 hours NDV did not have time and opportunity to become adsorbed to the available epithelial cells of the chorio-allantoic membrane, estimated by Hoyle 13 to be approximately 10^8 in 12-day-old ECE. Saturation of available receptor sites, 18 therefore, cannot be invoked as a theoretical explanation of interference in these studies. It is then probable that interference occured intracellularly.

The possible role of interferon was considered. Isaacs and Lindenmann 14 isolated from tissues infected with a virus an aspecific substance, named interferon, capable of preventing multiplication of several other unrelated viruses. One of the characteristics of interferon is its heat resistance. The results of Series 4 clearly show no interference of IBV with NDV when IBV was heat-killed at 56° C in water bath for 30 minutes. It is known that an egg-adapted strain of IBV is killed within 10 minutes when exposed to 56° C in a water bath. It is not known if interferon was present in the IBV suspension. If present, however, it did not appear to play a significant role. Nothing is definitely known about either the chemical nature of the egg-adapted strain of IBV or its metabolism within a cell and conclusions on

the mechanism of interference must therefore await further evidence.

Generally speaking, there was a good direct correlation between the HA titers and those determined by the embryo lethality test.

SUMMARY

The egg-adapted (DA) strain of infectious bronchitis virus (IBV) and the GB (Texas) strain of Newcastle disease virus (NDV) were inoculated into embryonating chicken eggs in varied amounts and at different time intervals. The results of the different combinations clearly showed that an excess of IBV over NDV consistently interfered with the growth of NDV in chicken embryonating eggs, even when NDV was inoculated 4 hours prior to IBV.

Hemagglutinating activity in embryonating chicken eggs inoculated solely with the GB strain of NDV was detected as early as 17 hours after inoculation.

The allantoic fluid harvested from eggs that died but that did not develop hemagglutinating titers after inoculation of the IBV-NDV mixtures was found to contain IBV.

Heat-inactivated IBV did not interfere with NDV.

Interferon did not appear to play a detectable role in the interference of IBV over NDV.

Active IBV, when mixed *in vitro* with NDV, did not interfere with the expected hemagglutinating titer.

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