# MECHANISM OF PRODUCTION OF PULMONARY LESIONS IN MICE BY NEWCASTLE DISEASE VIRUS (NDV)

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That viruses can be found in the lesions of a virus disease is well established, but the mechanism by which such lesions are produced is not clear. Infecting virus particles become associated with susceptible host cells, invade these cells, and multiply within them. Manifest disease has, in the main, been considered a result of the multiplication process itself. There appears to be reason to doubt that multiplying virus is the sole factor involved. The following findings, in this connection, are pertinent: (*a*) Lesions produced by pneumonia virus of mice (PVM) and influenza viruses appear only after a high titer of new virus particles is attained (1–3), and are formed at a rate different from that of the new particles (1, 3). (*b*) Certain strains of influenza virus multiply extensively in the mouse lung without production of pneumonia, whereas a closely related variant may, under the same conditions, produce pneumonic consolidation (4). (*c*) Some strains of Coxsackie virus multiply to an equal degree in a number of organs but do not produce lesions except at certain specific sites (5).

That formation of lesions is associated with an increasing quantity of virus seems irrefutable, but the mechanism responsible for them requires further study. It appeared that an investigation of how the virus acts on susceptible cells might enhance our knowledge of the pathogenesis of virus lesions.

Identifiable toxic action has been attributed to relatively few viruses. In fact, only the influenza (6–10) and psittacosis-lymphogranuloma (11) groups of viruses have been adequately demonstrated to bring about host injury through their toxicity. It has been shown in each instance (8, 10, 11) that the toxic effect is due to the direct action of the infectious virus particle, and is, in fact, inseparable therefrom. Since the injurious effects of these viruses are widespread and take place in organs not conveniently studied, the mechanism of production of local lesions has not been investigated. Burnet had observed that two successive intranasal inoculations of Newcastle disease virus (NDV) result in extensive pulmonary consolidation in the mouse (12). When, in this laboratory, it was demonstrated that a single inoculation was sufficient to produce marked pneumonic consolidation, a detailed study of the mechanism of

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the phenomenon was undertaken. It is the purpose of this paper to present additional evidence that Newcastle disease virus (NDV) produces severe pulmonary lesions, and that furthermore the damage results from a series of reactions between NDV and lung cells which are similar to those thought to occur in the initiation of a virus infection.

## *Materials and Methods*

*Viruses.*—Two strains of Newcastle disease virus, referred to as NDV, were employed in this study: (*a*) the Hickman strain isolated by Dr. F. R. Beaudette, New Jersey Agricultural Experiment Station, New Brunswick; (*b*) the Australian strain, received through the kindness of Dr. B. A. Briody, Yale University Medical School, New Haven. The Hickman strain of NDV was employed in most experiments. The influenza A viruses employed were the PR8 and MA strains. The latter strain, isolated in this laboratory in March, 1950, had been passaged 3 times in the chick embryo, but not inoculated previously into mice. All viruses were maintained by passage in the allantoic sac of 11 to 13 day old chick embryos (13), and between experiments were stored as undiluted infected allantoic fluid in a solid $CO_2$ cabinet at $-65°C.$ (14).

*Mice.*—The Rockefeller Institute strain of Swiss albino mice was employed throughout this investigation. Unless otherwise specified, those 3 to 4 weeks of age were used. Light ether anesthesia was employed to prepare the animals for intranasal or intracerebral inoculations.

*Virus Infectivity and Hemagglutination Titrations.*—Infectivity titrations were carried out with serial tenfold dilutions of infected allantoic fluid in 11 to 13 day old chick embryos as previously described (13). In order to determine the virus infectivity titer of mouse lungs, a 10 per cent suspension was prepared by grinding them with appropriate quantities of 10 per cent normal horse serum broth containing 500 units of penicillin and 0.5 mg. of streptomycin per cc., to insure sterility. Lungs were ground in a modified Waring blendor for 3 minutes following which each suspension was centrifuged at 1500 R.P.M. for 3 minutes. Infectivity titrations were carried out on the supernates in chick embryos, and the titer expressed as the final dilution of mouse lung which infected 50 per cent of the chick embryos (E.I.50) (15). Hemagglutination titrations were carried out in a manner identical with that previously described (13). Serial twofold dilutions of infected allantoic fluid in 0.85 per cent NaCl buffered at pH 7.2 (0.01 M phosphate) were prepared and to them was added an equal volume of a 1 per cent suspension of chicken RBC. The mixtures were placed at 4°C. for 60 minutes after which the red blood cell patterns were observed. The results of all titrations are expressed in terms of final concentration.

*The Scoring of Lung Lesions.*—Lung lesions were scored in terms of the amount of consolidation present, from $\pm$ to 5+ (16), and expressed as the ratio of observed lesions to the maximum score; *i.e.*, 30 per group of 6 mice. In certain experiments titration of the lung lesion-producing factor was carried out on serial twofold dilutions of NDV-infected allantoic fluid, and the titer expressed as the 50 per cent maximum score (M.S.50) (16).

*Immune Sera.*—Immune rabbit sera were prepared in a manner similar to that previously described (17). Rabbits received an intravenous injection of 10 cc. of undiluted, infected allantoic fluid which was followed in 1 week by an intraperitoneal injection of 10 cc. of similar fluid. Serum was collected 2 weeks after the second injection.

*Receptor-Destroying Enzyme* (*RDE*).—A culture filtrate of *V. cholerae* was prepared as described by Schlesinger (18) and will be referred to in all experiments as RDE (19). An 18 hour culture of *V. cholerae* grown in nutrient broth was centrifuged at 7,000 *g* for 10 minutes, and the supernate passed through a Coors No. 3 filter. The potency of the preparation was

tested in a manner similar to that described by Burnet (19). To twofold dilutions of RDE was added an equal volume of chicken RBC, and the mixture incubated at 37°C. for 60 minutes. To each tube was added influenza A virus, PR8 strain, to make a final concentration of 8 hemagglutinating units, and the pattern of agglutination of the treated RBC was observed after 60 minutes at room temperature.

## EXPERIMENTAL

*Neutralization of the Action of NDV Allantoic Fluid by Homologous Immune Serum.*—A first necessity was to identify the agent responsible for the production of the lung lesions observed after the intranasal inoculation of NDV allantoic fluid.

TABLE I

*Effects of Immune Serum on Lesion Production by NDV*

| Inoculum 0.05 cc. intranasal | | Interval | Lung lesions | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mouse No. | | | | | | Score | Pulmonary consolidation, score |
| Virus* | Rabbit serum* | | 1 | 2 | 3 | 4 | 5 | 6 | | |
| | | *days* | | | | | | | *L/M*‡ | *per cent* |
| NDV§ | Normal | 4 | D3‖ | D4 | +++ | +++ | ++ | + | 19/30 | 63 |
| " | NDV-immune | " | 0 | 0 | 0 | 0 | 0 | 0 | 0/30 | 0 |
| " | PR8-immune | " | D3 | D4 | +++ | ++ | + | ± | 16/30 | 53 |
| " | NaCl control | " | D3 | D3 | +++ | ++ | ++ | ± | 17/30 | 57 |

* Mixture of equal volumes of each.

‡ Numerator = lesion score; denominator = maximum score.

§ $10^{8.7}$ E.I.D. (embryo infectious doses).

‖ Dead mice had complete pulmonary consolidation. Number denotes day of death.

Mixtures of equal volumes of bacteria-free NDV allantoic fluid and normal rabbit sera, NDV immune rabbit sera, PR8 immune rabbit sera, and saline were incubated at 37°C. for 15 minutes, following which 0.05 cc. of each mixture was inoculated intranasally into each of a group of 6 mice. The surviving mice were killed in 4 days and the lung lesions scored.

The results of an experiment are presented in detail in Table I. Extensive pneumonia and death in one-third of the mice resulted from the inoculation of NDV allantoic fluid mixed with saline or normal rabbit serum. In contrast, the inoculation of infected allantoic fluid mixed with NDV immune rabbit serum produced no pulmonic lesions, whereas the lesions were undiminished in extent when the heterologous, PR8, immune serum was employed. It is clear from the data presented that the lesions were not produced by a latent pulmonary virus (20, 21) provoked by the intranasal inoculation. On the contrary, it is plain that NDV was the agent responsible for the pulmonary consolidation.

Further experiments have demonstrated that the lesion-producing factor is

not confined to a single strain of NDV, though these differ in effectiveness. When equal concentrations of the Hickman strain and a strain originally isolated in Australia were compared, it was noted that, although the latter strain produced pulmonary consolidation after intranasal inoculation, the extent of the pneumonia was significantly less than that induced by the Hickman strain.

*Failure to Pass NDV in Series in the Mouse Lung.*—The observation that NDV allantoic fluid produced extensive pulmonary consolidation in mice raised the possibility that the virus readily multiplies in the mouse lung. It has been reported by other workers (22) that NDV is incapable of sustained multiplication in mice, yet it seemed possible that the virus employed in the present experiments was of a strain which might be less fastidious in its requirements. The following experiment was carried out to test this hypothesis.

NDV allantoic fluid was inoculated intranasally into a group of 6 mice. The lungs of those that died were removed aseptically and stored at $-28°C.$ until the completion of the experimental period. The mice surviving 4 days after inoculation were killed, the lungs examined, and lesions scored. A 10 per cent suspension of the pooled lungs of all animals of each group was prepared as described under Methods, and inoculated into another group of mice. The infectivity titer of the mouse lung suspension was determined in chick embryos. Three such passages were carried out, and the chick embryo infectivity titer determined at the end of each.

The results of a typical experiment are presented in detail in Table II; two such experiments were carried out, and the results were similar in each. It is clearly evident that, although the infected allantoic fluid containing $10^9$ embryo infectious doses (E.I.D.) of NDV produced extensive pneumonia and death in the majority of mice, the virus did not multiply sufficiently on serial passage to maintain itself. In the second passage no lung lesions were produced, and only $10^{1.5}$ E.I.D. of virus was present; no NDV could be demonstrated in the lungs of the third mouse passage. The presence of virus in the lungs of the second mouse passage may be regarded as due to survival of NDV from the original inoculum. These findings indicate that the lung lesions observed were not attributable to extensive multiplication of the virus.

*Correlation of Production of Pulmonary Lesions by NDV and Concentration of Infectious Virus in the Mouse Lung.*—Although multiplication of NDV does not occur to a sufficient extent to sustain serial passage in the mouse lung, some of the virus can survive for a short time in this organ. In order to determine its period of survival, and to learn whether there was a correlation between the titer of infectious NDV and the production of pulmonary lesions, the following experiments were carried out.

A number of mice were inoculated intranasally with NDV allantoic fluid containing $10^{9.5}$ E.I.D. Commencing 5 minutes after inoculation and thereafter at 1 or 2 day intervals, groups of 6 mice were selected at random, their lungs removed under aseptic conditions at autopsy, and the lung lesions scored. Mice which died at any time were autopsied as soon as possible,

lungs stored at −28°C. and pooled with the lungs of mice killed at the next specified time. All lungs were stored at −28°C. until infectivity titrations were carried out in the chick embryo as described above.

The results of three experiments are summarized graphically in Fig. 1. It seems clear that, under the experimental conditions outlined, there was no multiplication of infectious NDV particles in the mouse lung; indeed, throughout the experimental period there was a continuous decrease of virus of the order of 0.93 log unit per day. It is of interest to compare the quantity of infectious virus with the presence of pneumonic consolidation in the same lungs. Despite the continuous decrease in virus titer, pulmonary lesions continued to develop. Two days after inoculation the amount of NDV was 99 per cent less

TABLE II

*Attempt at Serial Passage of NDV in Mouse Lungs*

| Inoculum 0.05 cc. intranasal | Interval | Lung lesions | | | | | | | | Chick embryo infectivity titer mouse lungs |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mouse No. | | | | | | Score | Pulmonary consolidation, score | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | | | |
| | *days* | | | | | | | *L/M* | *per cent* | *E.I.50** |
| NDV $10^9$ E.I.D.‡ | 4 | D3 | D3 | D4 | ++++ | ++ | ± | 21/30 | 70 | $10^{-5.2}$ |
| Passage 1 mouse lung | " | 0 | 0 | 0 | 0 | 0 | 0 | 0/30 | 0 | $10^{-1.5}$ |
| Passage 2 mouse lung | " | 0 | 0 | 0 | 0 | 0 | 0 | 0/30 | 0 | $<10^{-1.0}$ |

* E.I.50 = 50 per cent embryo infectivity end point.

‡ E.I.D. = embryo infectious doses.

than 5 minutes after inoculation, yet 40 per cent of the lung volume was consolidated. On the third day the maximum of pneumonia was observed; one-third of all the mice inoculated were dead and showed complete pulmonary consolidation. At this time, when the lesions were most extensive, there remained less than 0.1 per cent of the original virus. A marked decrease in the amount of lung involved and resolution of the lesions occurred thereafter. At the completion of the experimental period, an average of 20 per cent of the lung was still affected, but less than 0.00001 per cent of the NDV was demonstrable. No specific immune bodies could be demonstrated in the serum of mice during the experimental period.

The report of Schlesinger (18) that influenza A virus which was not adapted to the mouse brain could produce in that organ an increase in non-infectious virus particles which hemagglutinate chicken erythrocytes, suggested a similar possibility for NDV in the mouse lung. Extensive experiments, however, have failed to reveal, either by hemagglutination or complement-fixation techniques,

the presence of such "immature" non-infectious virus particles. Newcastle disease virus particles, however, cannot be easily dissociated from lung tissues, even after long periods of incubation in the presence of large quantities of the receptor-destroying enzyme (RDE) (19). Moreover, theoretical yields of virus were never obtained with artificial mixtures of NDV and normal mouse lung suspension by means of hemagglutination techniques. It should be noted that PR8 similarly could not be quantitatively recovered from artificial mixtures. Therefore, the possibility that such partial multiplication of non-infectious virus may occur cannot be definitely eliminated.

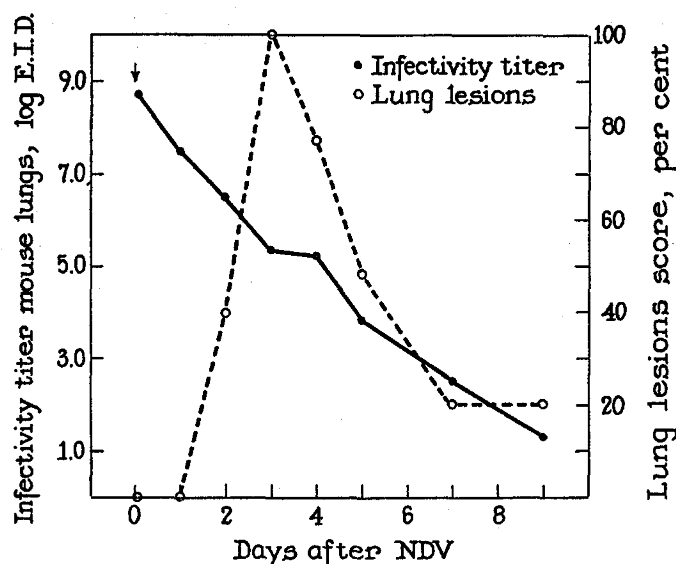


FIG. 1. Correlation of titer of infectious NDV and extent of pulmonary consolidation in lungs of mice inoculated intranasally with $10^{9.5}$ E.I.D. of virus. Groups of 6 mice were employed; each end-point represents the geometric mean of results from three experiments.

*Titration of the Lesion-Producing Capacity of NDV Allantoic Fluid.*—To determine the amount of NDV allantoic fluid necessary to produce pulmonary consolidation and whether prolongation of the experimental period would give a greater maximum score of lesions the following experiments were done.

Twofold dilutions of NDV were made in broth, and each dilution was inoculated intranasally into 2 groups of 6 mice each, one of which was killed 4 days and the other 7 days after inoculation. Lung lesions were scored, and the 50 per cent maximum score (M.S.50) titer was determined (15).

The results of a typical experiment, presented in Table III, clearly indicate that extensive pulmonary consolidation was produced only by a high concentration of infected allantoic fluid, and that the amount of pneumonia induced

was not proportional until, on dilution, the M.S.50 end-point was reached. Upon further dilution a roughly linear relationship between virus dilution and lung lesion score was noted. Only in rare experiments did inoculation of undiluted material regularly result in death. Even when virus was concentrated tenfold by centrifugation, complete consolidation of the lungs and death of all mice did not follow. This finding may be explained in terms of the mechanical factors involved in intranasal inoculation which are responsible for the fact

TABLE III

*Titrations of the NDV Factor Which Produces Pulmonary Lesions*

| Dilution virus inoculated | Interval | Lung lesions | | | | | | | | NDV factor titer |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mouse No. | | | | | | Score | Pulmonary consolidation, score | |
| 0.05 cc. intranasal | | 1 | 2 | 3 | 4 | 5 | 6 | | | |
| | *days* | | | | | | | *L/M* | *per cent* | *M.S.50* |
| NDV undiluted* | 4 | D3‡ | D3 | D4 | ++++ | +++ | + | 23/70 | 77 | |
| " 1:2 | " | D3 | D4 | ++++ | +++ | +++ | +++ | 23/70 | 77 | |
| " 1:4 | " | D3 | D3 | D4 | D4 | +++ | ++ | 25/30 | 83 | |
| " 1:8 | " | D3 | D3 | D3 | D4 | ++ | + | 22/30 | 73 | 1:10 |
| " 1:16 | " | D3 | D4 | + | ± | 0 | 0 | 11/30 | 37 | |
| " 1:32 | " | +++ | ± | ± | ± | 0 | 0 | 4/30 | 13 | |
| " 1:64 | " | ++ | ± | 0 | 0 | 0 | 0 | 2/30 | 07 | |
| NDV undiluted | 7 | D3 | D3 | D5 | D6 | ++ | 0 | 22/30 | 73 | |
| " 1:2 | " | D3 | D3 | D4 | ++ | ++ | ++ | 21/30 | 70 | |
| " 1:4 | " | D3 | D3 | D3 | D3 | 0 | 0 | 20/30 | 67 | |
| " 1:8 | " | D3 | D3 | D4 | D5 | 0 | 0 | 20/30 | 67 | 1:4 |
| " 1:16 | " | D4 | + | 0 | 0 | 0 | 0 | 6/30 | 20 | |
| " 1:32 | " | ± | ± | 0 | 0 | 0 | 0 | 1/30 | 03 | |
| " 1:64 | " | 0 | 0 | 0 | 0 | 0 | 0 | 0/30 | 00 | |

* $10^{9.3}$ E.I.D.

‡ All mice which died had complete consolidation of the lungs.

that 10 per cent or less of the original inoculum can be detected in the lungs. The results of these experiments confirm the data presented in Fig. 1, and further demonstrate that resolution of the pneumonia commences after the 3rd to 4th day following NDV inoculation. In fact, examination of the lungs 4 days after virus inoculation yielded an M.S.50 titer 2.5 times greater than when lesions were scored at 7 days.

*Dependence of Lesions upon NDV Particles.*—Neutralization experiments with potent homologous and heterologous immune sera indicated that NDV is the agent responsible for the extensive lesions produced by infected allantoic fluid. However, these experiments did not permit conclusions to be drawn as

to whether the virus particle itself, or a soluble substance in the infected allantoic fluid, is the actual etiologic factor. This problem was investigated by two approaches: (*a*) high speed centrifugation to attempt separation of the properties of infectivity, hemagglutination, and lesion production; and (*b*) adsorption experiments with chicken RBC to attempt similar separation.

NDV allantoic fluid was centrifuged in a Spinco centrifuge at 114,500 *g* for 45 minutes. The upper half of the supernate was carefully removed and saved; the remainder was discarded and the sediment was resuspended in sterile saline to the original volume. The uncentrifuged infected allantoic fluid, the upper half of the supernate, and the resuspended sediment were then each employed to determine: (*a*) ability to produce lung lesions in the mouse; (*b*) hemagglutination titer; and (*c*) chick embryo infectivity titer.

TABLE IV

*Sedimentation of Hemagglutinating, Infective, and Lesion-Producing Factors of NDV*

| NDV-pooled allantoic fluid | Hemagglutination | | Chick embryo infectivity | | Mouse lung lesions* | | |
|---|---|---|---|---|---|---|---|
| | Titer‡ | Reduction | Titer | Reduction | Score | Pulmonary consolidation, score | Reduction |
| | | *log* | *E.I.50* | *log* | *L/M* | *per cent* | *log* |
| Before centrifugation | 1024 | | $10^{-9.0}$ | | 20/30 | 67 | |
| Upper 1/2 supernate after centrifugation at $5.1 \times 10^6$ *g* min. | 16 | −1.83 | $10^{-7.2}$ | −1.8 | 0/30 | 0 | −1.83 |
| Sediment resuspended in original volume | 1024 | 0 | $10^{-8.7}$ | −0.3 | 17/30 | 57 | −0.07 |

* Inoculum = 0.05 cc. NDV intranasally per mouse. Lesions scored 4 days after virus inoculation.

‡ Expressed as the reciprocal.

The results of this experiment are summarized in Table IV. The production of pulmonary lesions by the supernate and its infectivity and hemagglutination titers were each correspondingly less than in the uncentrifuged material, but the amounts of the lesion-producing factor and virus in the resuspended sediment were closely similar to the quantities in the uncentrifuged pool.

These results indicate that within the limits of the experimental techniques employed, high gravitational fields have similar if not identical effects upon the properties of lesion production, hemagglutination, and infectivity. Because sedimentation of the virus was accompanied by proportional sedimentation of the component responsible for the production of pulmonary lesions, it would appear that the two were not separable under the conditions of the experiment.

Newcastle disease virus particles under proper conditions react with chicken erythrocytes, bringing about their agglutination (12). During this process the virus particles are adsorbed by and then eluted from the red blood cells (23).

These reactions between NDV and chicken RBC were utilized in an effort to separate the lesion-producing factor from the virus particle.

To obtain a high degree of adsorption of NDV upon and subsequent elution from chicken RBC, the conditions required are different from those for influenza A and B (24) and mumps (25) viruses. It is difficult to demonstrate adsorption of this virus to RBC except at 4°C., at which temperature 90 to 95 per cent is adsorbed (23). Since elution from the RBC is slow, and approximates completion more effectively when the reaction mixture is held at room

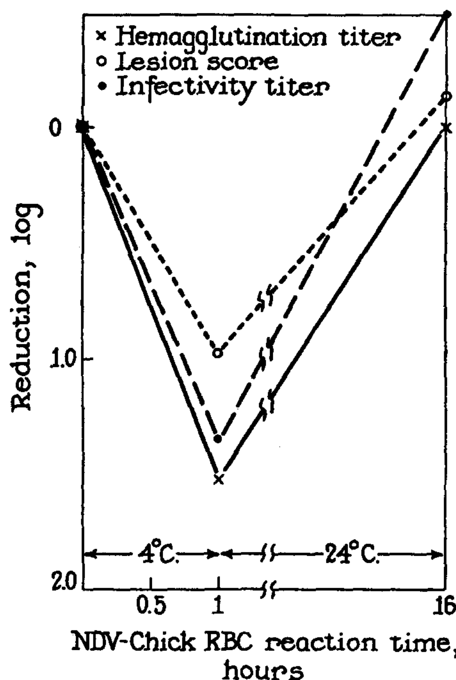


FIG. 2. Adsorption to chicken RBC and later elution of the NDV factors of infectivity, hemagglutination, and lung lesion production. Each point is the geometric mean of the results of two experiments.

temperature (22 to 24°C.) than at 37°C. (26), these temperatures were maintained in the present experiments.

Equal volumes of NDV allantoic fluid, having an infectivity titer of $10^{-9}$, and a 10 per cent suspension of chicken RBC were mixed at 4°C. and held at this temperature for 60 minutes, after which the mixture was centrifuged at 2,000 R.P.M. for 2 minutes at 4°C., and the supernate removed and saved. The RBC were then mixed with sterile saline to restore the original volume, the mixture let stand at room temperature for 15 hours, and then centrifuged at 2,000 R.P.M. for 2 minutes. The supernate, termed eluate, was removed and saved. Finally, an aliquot of the original NDV allantoic fluid, the supernate obtained after keeping the virus-RBC mixture for 60 minutes at 4°C., and the eluate were tested to determine infectivity titer, hemagglutination titer, and capacity to produce lung lesions.

The results of two experiments are summarized graphically in Fig. 2 in which the logarithm of the reduction in lung lesion score, infectivity titer and hem-

agglutination titer are plotted against time of reaction of the NDV-chicken RBC mixture. As virus, determined by infectivity titer, was adsorbed to the erythrocytes, there was a corresponding adsorption of the factor which produced lung lesions and hemagglutination of RBC. Not only were these properties of the NDV fluid adsorbed to a similar degree, but within the limits of the experimental procedures employed they also eluted to a similar extent.

The results of the experiments indicate that not only does the lesion-producing factor sediment along with the virus particle, but also that it reacts with RBC as does the virus. These facts strongly suggest that the injury is a function of the virus particle of Newcastle disease, *per se*.

*Relation of Lesion-Producing Factor to Infectivity.*—Table IV and Fig. 2 indicate that the virus particle itself causes the pulmonary lesions, though virus multiplication could not be demonstrated with the experimental techniques employed. To learn whether lesions can be produced only by the infectious particle, or whether inactive NDV can cause similar pulmonary consolidation, the following techniques were utilized: (*a*) inactivation of NDV by ultraviolet irradiation; (*b*) inactivation of NDV by heat; and (*c*) storage of virus at 4°C. The rate and amount of inactivation of infectivity and of the factor producing lesions were determined by each procedure.

*A. Ultraviolet Inactivation.*—NDV allantoic fluid was dialyzed for 16 to 20 hours at 4°C. against two changes of saline; the volume of each was more than 50 times greater than the volume of allantoic fluid. Ten to 12 cc. of the dialyzed fluid was then put in a Petri dish, attached to a vibrator to provide constant mixing throughout the period of irradiation, and vibrator and Petri dish were placed 7 inches from a General Electric germicidal lamp. Aliquots were removed at intervals during irradiation, and the titer of infectivity and the capacity to produce lung lesions were determined.

In Fig. 3, which summarizes graphically the results of four experiments, the per cent reduction of the infectivity titer and of pulmonary consolidation are plotted against the period of irradiation of virus. Irradiation for 45 seconds inactivated 99.6 per cent of infectious virus and lung lesions produced by this sample were 53 per cent less extensive than those resulting from inoculation of non-irradiated NDV; irradiation for 60 seconds led to a reduction of 99.99 per cent and 77 per cent, respectively. Within the limits of quantitation, loss of infectivity was closely correlated in both rate and degree to a decrease in the capacity to produce lesions.

*B. Heat Inactivation.*—NDV allantoic fluid which had been stored at −65°C. was heated at 48, 50, and 52°C. in a water bath for varying intervals of time. At each interval an aliquot was removed, and infectivity titer and lung lesion-producing capacity were determined.

A summary of the findings is presented in Table V. Incubation at 48°C. for as long as 60 minutes produced a decrease in neither infectivity titer nor lesion-producing factor. Indeed, there was no significant reduction in either infectiv-

ity titer or lesion score, following incubation for 30 minutes at 50°C. When, however, virus was incubated at 52°C. for 60 minutes, the infectivity titer was reduced by 92 per cent and the lung lesions by 95 per cent. Further incubation lessened the infectivity of the virus preparation by over 99 per cent and no lung lesions whatever were caused. These results suggest that within the limits of the procedures employed a concurrent elimination of these properties took place.

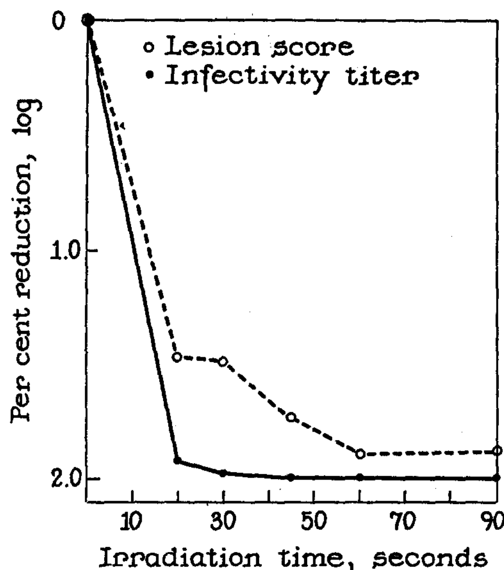


FIG. 3. Rate of inactivation by ultraviolet irradiation of the factors responsible for the infectivity of NDV and lung lesion production. Infectivity was determined by titrations in chick embryos and the extent of the lung lesions was estimated directly. Each point is the geometric mean of the data from four experiments.

*C. Storage at 4°C.*—A pool of NDV allantoic fluid was divided, and one portion stored at 4°C., the other at −65°C. At various intervals the infectivity titer and the lung lesion-producing factor of each was determined.

The results presented in Table VI show that NDV, under the conditions employed, is a hardy agent; even when the virus was stored at 4°C. for as long as 5 weeks, no significant inactivation of infectivity and little, if any, loss of capacity to cause lung damage could be demonstrated. Indeed, after 7 weeks of storage, a reduction in infectivity titer of only 0.8 of a log unit occurred. A corresponding reduction of the capacity to produce lesions was observed.

The results of experiments in which NDV was inactivated by heat and ultraviolet irradiation indicate a similar rate and degree of reduction of infectivity and of the capacity to produce lung lesions. Conversely, during prolonged

storage at 4°C., both infectivity and lesion-producing factor were preserved. These findings strongly suggest that the capacity of NDV to produce lesions is a property of the fully infectious virus particle, *per se*, and that in proportion as the virus becomes non-infectious it becomes non-injurious.

TABLE V

*The Abolition by Heat of the Infectivity and the Lesion-Producing Properties of NDV*

| Treatment NDV | | Chick embryo infectivity titer | Mouse lung lesions* | |
|---|---|---|---|---|
| Temperature | Time | | Score | Pulmonary consolidation, score |
| *°C.* | *min.* | *log* | *L/M* | *per cent* |
| — | — | −8.8 | 17/30 | 57 |
| 48 | 30 | — | 22/30 | 73 |
| 48 | 60 | −8.7 | 21/30 | 70 |
| 50 | 30 | −8.5 | 18/30 | 60 |
| 52 | 60 | −7.7 | 1/30 | 3 |
| 52 | 90 | −6.7 | 0/30 | 0 |

* Inoculum = 0.05 cc. NDV intranasally per mouse. Lesions scored 4 days after virus inoculation.

TABLE VI

*Correlation of Virus Infectivity and Lesion-Producing Properties after Storage of NDV at 4°C.*

| Period NDV stored at 4°C. | Chick embryo infectivity titer | Mouse lung lesions* | |
|---|---|---|---|
| | | Score | Pulmonary consolidation, score |
| *weeks* | *E.I.50* | *L/M* | *per cent* |
| 0 | $10^{-9.1}$ | 50/60 | 83 |
| 5 | $10^{-8.8}$ | 20/30 | 67 |
| 7 | $10^{-8.3}$ | 8/30 | 27 |

* Inoculum = 0.05 cc. NDV intranasally per mouse. Lesions scored 4 days after virus inoculation.

*Susceptibility of Adult Mice of Various Ages to the Lesion-Producing Factor.*—The experiments described above were carried out with 3 week old Swiss albino mice. That susceptibility of animals to certain virus infections is often dependent upon the host age is well known, and it is notably marked in mice (27–30). The following experiment was carried out to determine whether mice of various ages differed in susceptibility to the lesion-producing factor.

Mice ranging from 3 to 32 weeks old were inoculated intranasally with $10^{9.5}$ E.I.D. of NDV; 6 mice per age group were used. Those that survived for 4 days were killed then and the lungs examined and scored.

The results of this experiment (Table VII) indicate that mice 20 weeks old are as susceptible to the effects of NDV on the lungs as are newly weaned mice

3 weeks of age. In fact, even the mice nearly 8 months old exhibited a marked response to NDV, in that more than 50 per cent of the lung volume was consolidated. It is of interest to note the good reproducibility of the lung lesion score in a single experiment with mice varying between 3 and 20 weeks of age.

*Effect of NDV Inoculated by the Intravenous, Intracerebral, and Intraperitoneal Routes*.—Toxicity of influenza viruses is manifest in the mouse when inoculated by the intravenous, intracerebral, or intraperitoneal routes (7, 8). However, when undiluted NDV allantoic fluid ($10^9$ to $10^{9.5}$ E.I.D.) was so inoculated, no significant degree of damage could be demonstrated. Following intracerebral inoculation the mice had ruffled fur for 24 to 48 hours, but no paralysis or convulsions occurred, and only one of 18 died. Even when the virus was concentrated 10 times by centrifugation, no toxicity was exhibited except after

TABLE VII

*Age as Influencing Susceptibility of Mice to the Lesion-Producing Factor of NDV*

| Inoculum 0.05 cc. intranasal | Age mice inoculated | Mouse lung lesions | |
|---|---|---|---|
| | | Score | Pulmonary consolidation, score |
| | *weeks* | *L/M* | *per cent* |
| NDV* | 3 | 23/30 | 77 |
| " | 6 | 23/30 | 77 |
| " | 8 | 22/30 | 73 |
| " | 12 | 23/30 | 77 |
| " | 20 | 21/30 | 70 |
| " | 32 | 17/30 | 57 |

* $10^{9.3}$ E.I.D.

intranasal inoculation. Some very rapid deaths followed intravenous administration of concentrated virus; these may well have been the result of vascular phenomena such as follow upon the intravenous injection of tissue extracts (31, 32). It would appear that the effect of NDV is local and confined to the lungs.

*Pathology of Lung Lesions Produced by NDV*.—The pulmonary lesions produced by NDV appeared indistinguishable from those due to influenza viruses (33), or to pneumonia virus of mice (PVM) (20). In order to determine if any basic difference existed in the pathology of lesions produced by influenza virus and NDV, a series of mice was inoculated intranasally with each agent.

A $10^{-2}$ dilution of PR8 and undiluted NDV allantoic fluid, Hickman strain, were employed. Groups of 3 mice were each killed 2, 3, and 4 days after inoculation, the lungs removed, and the lesions examined. The lungs were then fixed in Zenker's fluid and microscopic sections prepared.

Gross and microscopic examinations of these lungs indicated that the lesions produced by NDV were indistinguishable from those due to influenza A infec-

tion. Epithelial degeneration and eventual sloughing, interstitial infiltration of mononuclear cells and red blood cells, atelectasis, and marked terminal pulmonary edema were observed. The pathology of experimental influenza A virus infection of mice has been described in detail by Straub (33, 34) and Loosli (35).

*Effect of V. cholerae Filtrate (RDE) on Production of Mouse Lung Lesions by NDV.*—The experimental evidence thus far presented indicated that the fully infectious NDV particle causes pulmonary consolidation. This brought up the possibility that the mechanism by which such lesions are produced might be similar to a true virus infection.

Following the demonstration by Fazekas de St. Groth that receptor-destroying enzyme (RDE) can destroy mouse lung receptors for influenza virus and prevent cell-virus combination in the excised lung (36), Stone pointed out that RDE can limit influenza virus infection in the mouse (37). It has also been shown that RDE destroys red blood cell receptors for NDV as it does for influenza and mumps viruses (19). During the present investigation it was demonstrated that incubation of mouse lung suspensions with RDE or injection of RDE into living mouse lung prevented the union of each with NDV. These facts suggested that, if NDV reacts with the living lung cell to produce an injurious effect in a manner similar to that of influenza virus on infection of a host cell, RDE should prevent the production of pulmonary consolidation by NDV.

Intranasal injection of RDE, 0.05 cc. per mouse, was followed in 6 hours by intranasal inoculation of an undiluted, 1:2 or 1:4 dilution of NDV. Control mice received broth or RDE heated at 100°C. for 5 minutes. The mice were killed 4 days after virus inoculation, the lungs examined, and the degree of pulmonary consolidation scored. The RDE employed had a RBC-receptor–destroying titer of 1:1024 (19).

Results of three experiments are summarized in Table VIII. It is clear that RDE, injected 6 hours before large quantities of virus, completely inhibited the production of pulmonary consolidation, whereas heated RDE and broth had no such effect. The fact that heated RDE did not prevent production of lung lesions suggests that it was the enzyme which was responsible for this prophylactic action. Other experiments in this laboratory have shown that RDE, injected intranasally 6 hours before mice are killed reduces adsorption of NDV in the excised lungs of these animals. It seems reasonable to infer, therefore, that prevention of NDV lung lesions by RDE is due to restriction of the association of virus with the susceptible lung cells.

*Effect of Intranasal Injection of NDV Immune Serum after Virus Inoculation.*—The evidence presented indicates that the infectious particle of NDV is directly concerned in the production of pulmonary lesions and, furthermore, that the commencement of this process is not unlike that of a virus infection. Infection is itself initiated by rapid fixation of virus to susceptible host cells

and, in so far as determinable, the fixation is irreversible (38, 39). Once infection has taken place, the injection of specific immune serum fails to modify the course of a virus disease. Utilizing this fact, the following experiments were carried out to obtain further evidence concerning the mechanism of production of lung lesions by NDV.

A 1:4 dilution of NDV allantoic fluid was inoculated intranasally into groups of 6 mice and at intervals thereafter intranasal injection of heat-inactivated NDV immune or normal rabbit serum was given. In addition, groups of mice were injected with normal or immune

TABLE VIII

*Prevention of the Injurious Action of NDV by the Receptor-Destroying Enzyme of V. cholerae*

| 1st injection 0.05 cc. intranasal | Interval | 2nd injection 0.05 cc. intranasal | Interval | Mouse lung lesions | |
|---|---|---|---|---|---|
| | | | | Score | Pulmonary consolidation, score |
| | *hours* | | *days* | *L/M* | *per cent* |
| Broth | 6 | NDV undiluted* | 4 | 40/60 | 67 |
| " | " | " 1:2 | " | 20/30 | 67 |
| " | " | " 1:4 | " | 90/150 | 60 |
| RDE | " | " undiluted | " | 2/60 | 3 |
| " | " | " 1:2 | " | 0/30 | 0 |
| " | " | " 1:4 | " | 0/90 | 0 |
| Heated RDE‡ | " | " undiluted | " | 32/60 | 53 |
| " " | " | " 1:2 | " | 16/30 | 53 |
| " " | " | " 1:4 | " | 25/60 | 42 |

* $NDV^{9.0}$ E.I.D.

‡ RDE heated in 100°C. water bath for 5 minutes.

rabbit serum either 15 minutes before or accompanying NDV. Surviving mice were killed 4 days after virus inoculation, the lungs examined, and the lesions scored.

The results of four experiments are summarized in Table IX. Injection of NDV immune serum before virus inoculation or at the same time completely prevented pulmonary consolidation, but lesions still occurred when immune serum was injected 5 minutes after NDV although the pneumonia was significantly limited in its extent. The amount of pulmonary consolidation was also lessened when antiserum was injected 15 minutes after virus, but when it was given after 30 minutes or more there was no effect on pulmonary lesions; consolidation became as extensive as in control animals.

It will be noted in Table IX that intranasal injection of normal rabbit serum after NDV had no effect on the production of lung lesions, and other experiments have shown that injection of normal horse serum, broth, RDE, or

saline at various periods after virus inoculation did not influence the extent of lesions. In contrast, the injection of similar materials during the course of influenza virus infection does increase the extent of the virus pneumonia (2).

These experiments indicate that NDV is rapidly fixed to the lung cells, and, once cell-virus association has been accomplished, it cannot be reversed by highly potent, specific immune serum.

*Interference with NDV Lung Lesions by Influenza A Virus.*—Interference with multiplication of one virus by another, both related and unrelated, has been demonstrated for numerous combinations (40). Present evidence suggests that interference results because the initial virus depletes the host cell of some

TABLE IX

*Effect of Time of Injection of Immune Serum upon the Production of Lung Lesions by NDV*

| 1st injection 0.05 cc. intranasal | Interval | 2nd injection 0.05 cc. intranasal | Interval | Mouse lung lesions | |
|---|---|---|---|---|---|
| | | | | Score | Pulmonary consolidation, score |
| | *min.* | | *days* | *L/M* | *per cent* |
| NDV 1:4* | — | None | 4 | 141/210 | 67 |
| " " | 15 | NRS‡ | " | 50/120 | 67 |
| " " | 5 | IRS§ | " | 15/80 | 19 |
| " " | 15 | " | " | 21/90 | 23 |
| " " | 30 | " | " | 76/120 | 63 |
| IRS | 15 | NDV 1:4 | " | 0/120 | 0 |
| NDV 1:2 + IRS | — | — | " | 0/60 | 0 |

* $10^{8.7}$ E.I.D.

‡ Normal rabbit serum.

§ Newcastle disease virus immune rabbit serum.

substance or metabolic system which is required by the second virus for multiplication (17, 41, 42). That influenza A virus can block the multiplication of NDV has been reported (43). If the production of pulmonary lesions by NDV follows a course like that of a virus infection, it seemed possible that multiplication of influenza A virus (IAV) in the mouse lung would interfere with the production of these lesions. In order to test this possibility, the following experiments were carried out.

A recently isolated IAV (MA strain), which had been passed only in chick embryos, was inoculated intranasally into mice. Three days after inoculation, twofold dilutions of NDV were inoculated intranasally in the same mice. Control groups received intranasal injection of normal allantoic fluid in place of either IAV or NDV. Four days after NDV the mice were killed, the lungs removed aseptically, the lesions scored, and lungs from certain groups were employed to determine the quantity of IAV and NDV by titrations in chick embryos. The lungs from mice which died during the experiments were stored at −28°C. and pooled with those of the animals surviving at the termination of the experimental period. Infectivity

titrations were carried out as follows: equal volumes of dilutions of lung suspensions and either immune NDV or IAV rabbit serum were mixed, incubated at 37°C. for 15 minutes, and inoculated in 0.2 cc. volume into the allantoic sac. Four embryos were employed per dilution of mouse lung suspension. The embryos were incubated for 2 days at 35°C., and infection determined by the hemagglutination technique.

Results of a typical experiment are presented in Table X. Influenza A virus, even though unadapted to the mouse lung, multiplied extensively, and markedly interfered with the production of pulmonary consolidation and death by NDV. Even when as much as $10^9$ E.I.D. of NDV was inoculated, previous infection with IAV reduced the lung lesions by about four-fifths as compared with those present in mice which received only NDV. When one-half or one-quarter the quantity of NDV was inoculated 3 days after infection with IAV, no lesions resulted which could be attributed to NDV. It is to be noted, however, that the initial multiplication of IAV did not significantly alter the rate of disappearance of NDV from the mouse lung: the quantity of infectious NDV in mice previously infected with IAV was only 0.9 log unit less than that in control mice whose mean survival time was about ½ a day less (*cf.* Fig. 1). That earlier multiplication of an unrelated virus does not significantly influence

TABLE X

*Effect of Infection with Influenza A Virus (IAV) upon the Production of Lung Lesions by NDV*

| 1st injection 0.05 cc. Intranasal | Interval | 2nd injection 0.05 cc. intranasal | Interval | Lung lesions | | | | | | | Infectivity titer of mouse lung suspensions in the presence of immune rabbit serum | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Mouse No. | | | | | | Pulmonary consolidation, score | | |
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | | NDV* | IAV (MA)‡ |
| | *days* | | *days* | | | | | | | *per cent* | *E.I.50* | *E.I.50* |
| IAV (MA) undiluted§ | 3 | — | — | ± | ± | ± | 0 | 0 | 0 | 3 | | $10^{-6.7}$ |
| " " | " | Normal allantoic fluid | 4 | ± | ± | 0 | 0 | 0 | 0 | 3 | | |
| " " | " | NDV undiluted‖ | " | ++ | + | ± | ± | 0 | 0 | 13 | $10^{-3.3}$ | $10^{-4.0}$ |
| " " | " | " 1:2 | " | ± | ± | ± | 0 | 0 | 0 | 3 | | |
| " " | " | " 1:4 | " | ± | ± | 0 | 0 | 0 | 0 | 3 | | |
| Normal allantoic fluid | " | " undiluted | " | D3 | D3 | D4 | +++ | ++ | + | 70 | $10^{-4.2}$ | |
| " " | " | " 1:2 | " | D3 | D3 | D4 | +++ | +++ | ++ | 76 | | |
| " " | " | " 1:4 | " | D3 | D4 | +++ | +++ | ++ | ± | 60 | | |

* Carried out in the presence of anti-IAV (MA) immune rabbit serum.

‡ Carried out in the presence of anti-NDV immune rabbit serum.

§ $10^{7.3}$E.I.D.

‖ $10^{9.2}$E.I.D.

the quantity of infectious NDV, despite interference with its damaging action, is additional evidence suggesting that multiplication of NDV does not occur in the mouse lung.

Previous studies in the chick embryo (17, 42) have demonstrated that interference with multiplication between viruses does not result because the initial virus prevents association of the second agent with the host cells. In order to interpret the experiments described above, it was necessary to determine whether influenza A infection in the mouse blocked association of NDV with lung cells.

Twenty mice were infected with IAV (MA strain) and 20 mice were injected with normal allantoic fluid as described above, and all were killed 3 days later. The lungs with trachea still attached were removed and washed in saline. The lungs from each infected and control

TABLE XI

*Fixation of NDV in Excised Lungs Previously Infected with Influenza A Virus (IAV)*

| Inoculation 0.05 cc. intranasal | Interval before lungs and trachea removed | Intratracheal instillation 0.1 cc. | Interval at 4°C. | Hemagglutination titer* fluid expressed from trachea | NDV fixed |
|---|---|---|---|---|---|
| | *days* | | *min.* | | *per cent* |
| Normal allantoic fluid | 3 | NDV‡ | 0 | 128 | — |
| " " " | " | " | 60 | 16 | 87 |
| IAV $10^{7.0}$ E.I.D. | " | " | 0 | 64 | — |
| " " " | " | " | 60 | 8 | 87 |

* Expressed as the reciprocal.

‡ $10^{9.0}$ E.I.D.; hemagglutination titer of 1:1024.

mice were divided into two groups of 10. The lungs of each mouse were treated as follows: The trachea was cannulated with a 23-gauge needle from which the point had been ground off. A ligature held the needle securely in place; 0.1 cc. of undiluted NDV was injected through it and the trachea closed with a second ligature. The ligatures on the trachea of 10 infected lungs and 10 control lungs were cut immediately, and the fluid within the tracheobronchial tree was expressed by centrifuging the lungs at 12,700 *g* for 20 minutes. This procedure was repeated on the remaining 10 lungs of each group after they had been held at 4°C. for 60 minutes. The fluid expressed from each of the groups of lungs was mixed with an equal volume of RDE, incubated at room temperature 18 hours, and then at 37°C. for 2 hours. The hemagglutination titer of each sample was determined.

The results of a typical experiment are presented in Table XI. NDV became fixed to the same extent on the lung tissue of mice infected 3 days previously with IAV as on that of control mice. The lungs of mice infected with IAV at the same time as those demonstrating this fact had an infectivity titer of $10^{-7}$ in the chick embryo. These data indicate that IAV produces its inhibitory effect at some intracellular site, and not by interfering with cell-NDV association.

## DISCUSSION

The evidence here presented indicates that infectious Newcastle disease virus as such, acting in the absence of any formation of new virus particles of demonstrable activity, causes the extensive pneumonia observed in the mouse. In all other respects, the production of pulmonary lesions by NDV parallels the reactions between virus and host cell which occur in a true virus infection. The first step in lesion production appears to be association of NDV and lung cells. The combination is rapid and cannot be reversed by highly potent, specific immune serum. Not only is combination with pulmonary tissue required, but it is probable that at least one intracellular reaction occurs. The fact that unadapted influenza A virus did not induce cellular damage but did interfere with the production of lung lesions by NDV suggests that influenza virus and NDV have some intracellular reaction in common. This reaction, however, appears not to be that responsible for the production of lesions by NDV.

Sugg has reported that certain strains of influenza A virus, unadapted to the mouse lung, produce extensive pneumonia on primary inoculation, but that despite active multiplication the lesions are not produced on passage (44). He has correlated the production of lesions with the titer of virus present (45). A similar phenomenon was noted in this laboratory when NDV was inoculated intranasally into hamsters, and serial passages with the hamster lungs carried out. Both of these findings can be interpreted as due to the injurious action of a large amount of virus. As the virus titer diminishes on passage, lesions fail to be produced despite the fact that active multiplication occurs. It would appear that pulmonary consolidation, after the inoculation of unadapted influenza virus in the mouse (and NDV in the hamster), may be the result of the injurious action of a large amount of virus like the pneumonia induced by NDV in the mouse.

Extensive studies correlating the rate of virus multiplication and rate of formation of lesions by pneumonia virus of mice (1) and by influenza A virus (3) have demonstrated that: (*a*) gross lesions appear after extensive virus multiplication has occurred, and (*b*) the rate of production of pulmonic lesions is slower than the rate of production of new virus particles. The fact that lesions are formed at a rate different from that of virus suggests that factors other than the multiplication process as such are involved in the production of lesions. This appears to hold true as well with NDV.

## SUMMARY

Infectious NDV particles produce extensive pulmonary consolidation in the mouse in the absence of demonstrable virus multiplication. The lesions are indistinguishable from those of influenza A virus infection. This effect of NDV was blocked by intranasal injection of RDE or immune serum before virus

inoculation, but not by immune serum injected 5 minutes or more after NDV. Influenza A virus infection did not diminish fixation of NDV in excised lungs but did interfere with the injurious action of this agent in the living mouse. The analogy between these reactions and those which take place in a progressive virus infection is pointed out, and the mechanism of production of lesions in virus pneumonias discussed.

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