

THE PATHOGENESIS OF INFECTION WITH A VIRULENT (CG 179)
AND AN AVIRULENT (B) STRAIN OF NEWCASTLE
DISEASE VIRUS IN THE CHICKEN

II. DEVELOPMENT OF ANTIBODY*

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(Received for publication, November 3, 1950)

The pattern of virus multiplication of a virulent (CG 179) and an avirulent (B) strain of Newcastle disease virus (NDV) has been analyzed in the accompanying paper (1). It was found that as birds recovered from an NDV infection virus titers fell first in the circulating blood, then in the viscera, and persisted longest in the central nervous system.

In the present paper the development of serum antibody has been studied in relation to the curve of virus growth and decline. The early appearance of antibody during the course of an acute infection coincided with the disappearance of virus from the blood and tissues. The presence or absence of antibody in the brain of convalescent chickens was found to be poorly correlated with serum levels but rather related to the severity of the virus infection in the central nervous system. The antigenic properties of the CG 179 and B strains are shown to be comparable.

Materials and Methods

The materials and methods for this study were identical with those described in the accompanying paper (1).

Virus Strains.—The CG 179 (virulent) and the B (avirulent) strains of NDV were used.

Hemagglutination Inhibition Titrations.—Titrations were performed using serial twofold dilutions of serum in 0.85 per cent NaCl solution buffered at pH 7.2 with 0.01 M phosphate. Ten hemagglutinating units of B strain virus was added to each serum dilution. An equal volume of washed 1 per cent chicken RBC was added and the test read by the pattern technique after 30 to 60 minutes at room temperature. The hemagglutination inhibition titer was read as the highest serum dilution which completely inhibited hemagglutination.

Neutralization Procedure.—All materials to be tested for neutralization were inactivated

* Supported in part by a grant-in-aid from the National Institutes of Health, United States Public Health Service.

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in a 56° C. water bath for 30 minutes. The logarithmic curve relating the dilution of chicken immune serum to the quantity of virus neutralized has been demonstrated to be a straight line with a slope of somewhat greater than 1.3, *i.e.*, dilution of the serum was accompanied by a slightly more than equivalent loss of virus-neutralizing capacity (2). Therefore, holding either the serum or virus constant is adequate, from a practical point of view, in the performance of a neutralization procedure. In the present study a constant serum dilution of $10^{-0.5}$ (1/3.2) was combined with an equal volume of varying logarithmic dilutions of virus and the mixture held at 4° C. for 30 minutes. Preliminary titrations showed no enhancing effect of a 1 to 2 hour incubation of the virus-serum mixture at 35° C. (2). The mixture was inoculated in 0.1 ml. quantities on the chorio-allantoic membrane of four or five 11 day chick embryos. Normal heated chicken serum was used as a control. The neutralization index was calculated as the difference between the virus titer in the control and the virus titer in the presence of immune serum and expressed as the number of log LD₅₀ of virus neutralized.

Chicken brains, prepared by a thorough saline perfusion and washing as described previously (1), were ground in a final dilution of $10^{-0.5}$. The whole brain suspension was then used in a neutralization procedure identical with that detailed for serum. The use of the supernatant fluid after centrifugation of the brain suspension in a horizontal centrifuge at 2000 R.P.M. for 20 minutes did not modify the outcome of the test.

Sensitized Cell Agglutination.—Chicken RBC were sensitized by incubating a 2 per cent suspension of washed RBC in 100 ml. of a 1/5 dilution of fresh B strain at 35° C. for 1 hour (3). Serial twofold dilutions of serum were made in saline and an equal volume of sensitized RBC was added. The titer of sensitized cell agglutinin was recorded as the highest serum dilution causing a hemagglutination pattern after 30 to 60 minutes at room temperature.

Hyperimmune Serum.—A constant source of pooled immune serum was prepared from three chickens inoculated with the B strain intramuscularly three times over a period of 7 weeks. The serum, stored at -20° C., retained its neutralizing capacity for at least 7 months.

Preparation of Vaccines.—Approximately 100 ml. quantities of freshly harvested allantoic fluid infected with NDV were concentrated and washed three times using a Johnson Foundation angle ultracentrifuge at 12,000 times gravity (tube center) for 1 hour. The final preparation which was concentrated 10 times had infectivity titers of $10^{8.5}$ LD₅₀ for the CG 179 strain and $10^{9.5}$ LD₅₀ for the B strain. Nitrogen determinations by the micro Kjeldahl technique were 0.10 mg./ml. and 0.11 mg./ml. respectively.¹ The opalescent solutions were mixed with equal volumes of 0.4 per cent formalin and stored at 4° C. for 21 days. Embryo inoculation showed this vaccine to be entirely non-infectious.

EXPERIMENTAL

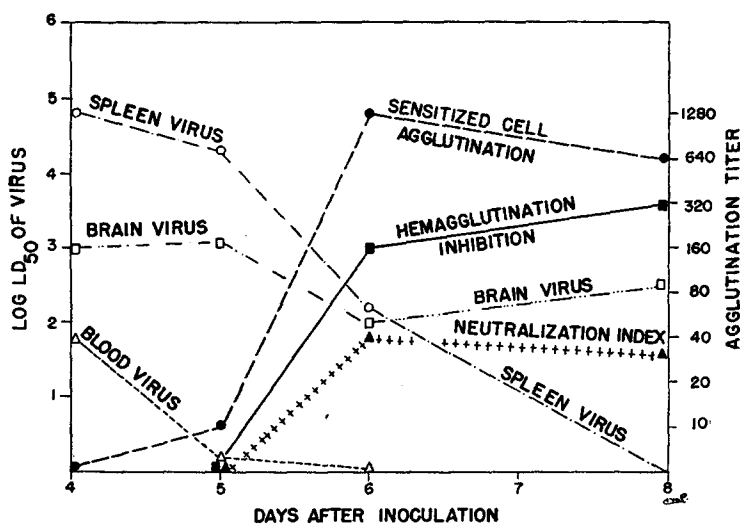
The Development of Antibody during the Course of an NDV Infection.—In parallel with the investigation of the rise and fall of virus titers (1), the same experimental groups of chickens were studied from the point of view of antibody production.

Antibodies were measured by the extent to which the serum (*a*) neutralized the infectivity of NDV in chick embryo tests; (*b*) inhibited the NDV agglutination of chicken RBC; and (*c*) agglutinated "sensitized" chicken RBC. The last test is based upon the fact that chicken RBC which have been sensitized by absorption and elution of NDV are agglutinated by the serum of

¹ For the micro Kjeldahl determinations, we are indebted to Dr. Manfred M. Mayer, Department of Bacteriology, The Johns Hopkins School of Hygiene and Public Health, Baltimore.

chickens recently convalescent from a Newcastle infection (3). The relationship of sensitized cell agglutinin to other antibodies has not been clearly established, but their appearance does coincide temporally with the development of protective antibodies (4).

Antibody production was studied in two experiments in which the B strain was given intramuscularly and in one experiment in which this strain was given intracerebrally. The complete data were presented in Tables IV and VI in the accompanying paper (1). Text-fig. 1 shows the pattern of the fall in virus titer after intramuscular infection in a typical experiment, plotted on the same graph as the rise in serum antibody. It was found that antibody



TEXT-FIG. 1. Development of serum antibody in relation to the decline in virus titers after intramuscular inoculation of the avirulent (B) strain of NDV.

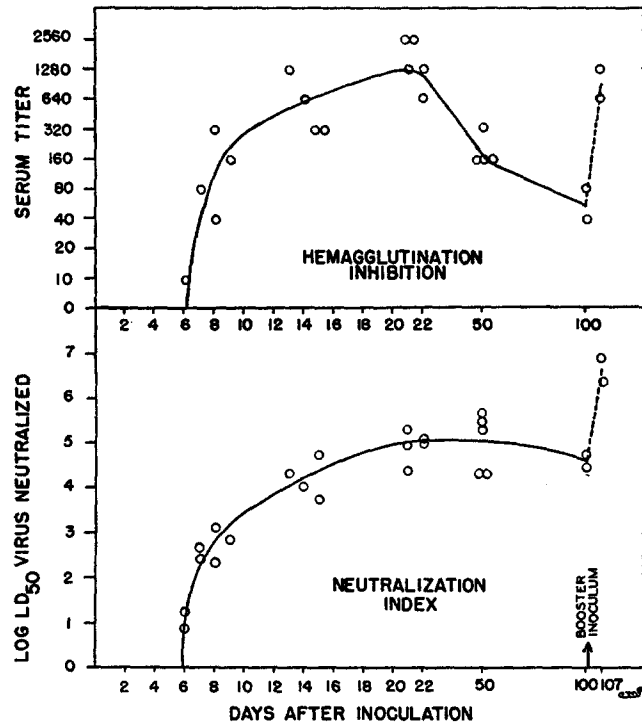
appeared in the serum in significant titer on day 6 when there still was a high virus content demonstrable in the viscera (spleen, lung, and rectum) and in the brain. On the 8th day, although circulating antibody had been available for 2 days, virus persisted in the brain tissue.

In the birds convalescent after intracerebral inoculation, serum antibody appeared in low titer on day 6 and fairly high titer on day 7. Virus was detectable in the spleen through day 7 and in the brain through day 9 despite the presence of serum antibody. It may be noted that the brain virus titers were irregular after antibody became available on day 6.

Persistence of Antibody in the Serum.—The persistence of antibodies over a period of 100 days was followed quantitatively in a group of birds convalescent from an intramuscular B strain infection. The detection of antibodies by both

the hemagglutination inhibition and embryo neutralization techniques revealed a disparity in the late convalescent titers which led us to question the assumption that a single antibody was being measured.

The two antibody systems paralleled each other closely in the phase of acute rise after infection (Text-fig. 2). However, the hemagglutination inhibition curve fell after the 3rd week, reaching low levels at 100 days, while the neutralization index remained elevated through 100 days. A booster dose

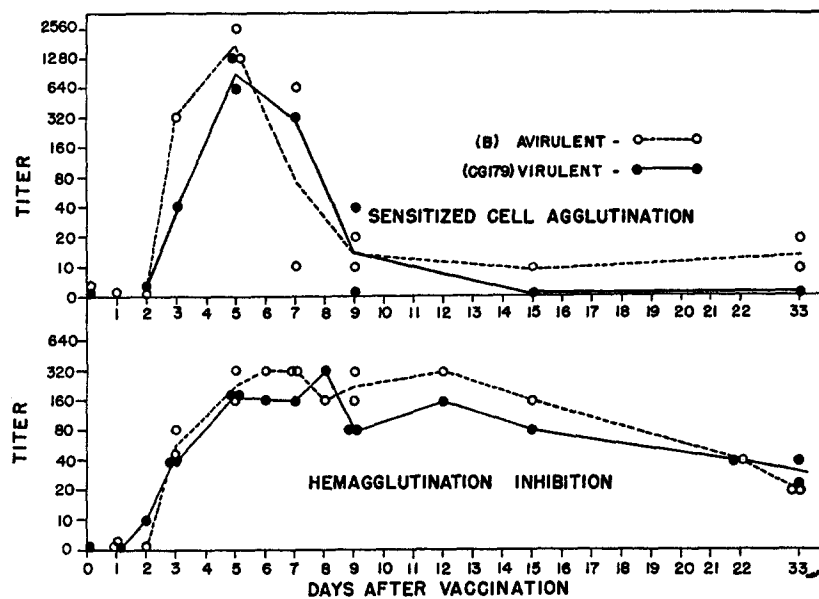


TEXT-FIG. 2. Antibody titer as measured by hemagglutination inhibition and neutralization of infectivity, determined in a group of chickens after infection with the avirulent (B) strain of NDV.

of the B strain (10^8 LD₅₀ of virus) was given intravenously as 0.5 ml. of undiluted allantoic fluid on day 100. Samples taken 7 days after the booster dose showed that both measurements had risen, but the neutralization index had exceeded its previous top titers, whereas the hemagglutination inhibition titer had only regained its original high level.

The discrepancies demonstrated between the two measurements indicated the possible duality of the antibodies. Studies with neutralizing and hemagglutination inhibition antibodies of influenza have given rise to similar suggestions (5-8).

Response to Vaccination with Virulent and Avirulent Strains.—In order to determine whether the variation in virulence of the CG 179 and B strains could be explained by a difference in their antigenic character, a controlled vaccination experiment was performed. Two groups of chickens were each given 4 ml. of formalized dead vaccine intravenously and bled at selected intervals. The CG 179 strain inoculum contained 0.20 mg. of nitrogen and the B strain 0.22 mg. of nitrogen. All vaccinated birds became solidly immune to challenge with 10^6 LD₅₀ of CG 179 virus inoculated *via* the intramuscular or intracerebral route.



TEXT-FIG. 3. Response of chickens to NDV vaccination with formalized CG 179 and B strains.

Text-fig. 3 shows the serum antibody response of the vaccinated birds as measured by hemagglutination inhibition and by sensitized cell agglutination. It is evident that the antibody appeared at the same time and reached the same height after both the CG 179 and B strain vaccines. The early appearance of significant amounts of antibody (on day 3) should be stressed. The curve of the rise and fall of the sensitized cell agglutination is the typical pattern found after a benign NDV infection (4).

In order to detect possible serological differences between the antisera of the CG 179 and B strains, cross-neutralization tests were performed on sera obtained from vaccinated birds on the 12th and 22nd days. Table I shows the resultant neutralization indices. There was marked crossing of the two viruses with some tendency to homologous preference, especially in the B strain.

The similarity in the antigenic properties of the two strains was thus established by (a) an identical antibody response following vaccination with equivalent amounts of antigen; and (b) a virtually complete crossing of the two systems in embryo neutralization tests.

Comparison of Rates of Antibody Production in Vaccinated and in Infected Birds.—During an acute infection with NDV, serum antibody has been shown to develop simultaneously with the decline in tissue virus titers. However, the time of appearance of antibody may be modified by altering the method of administration of the antigen. The rise of serum antibody, as measured by hemagglutination inhibition and sensitized cell agglutination, was studied

TABLE I
Cross-Neutralization Tests between the CG 179 and B Strains of NDV

| Antiserum | | Virus | |
|----------------------------------|--------|--------|------|
| Length of time after inoculation | Strain | CG 179 | B |
| <i>days</i> | | | |
| 12 | CG 179 | 2.7* | 1.4 |
| | B | 2.6 | 3.2* |
| 22 | CG 179 | 2.6* | 2.4 |
| | B | 2.8 | 3.7* |

* Homologous crossing.

All figures represent log LD₅₀ virus neutralized.

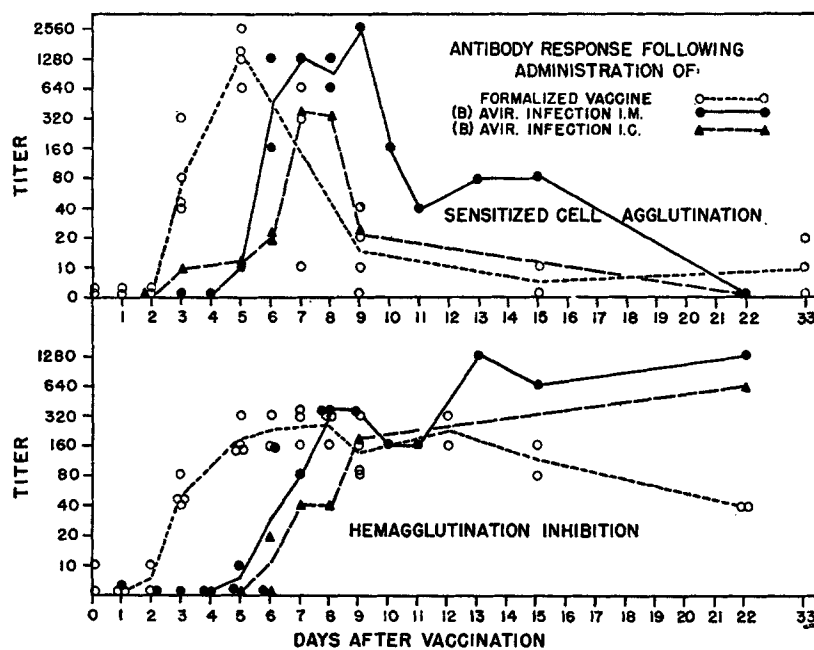
after vaccination and after intramuscular and intracerebral infection with NDV. (See Text-fig. 4.)

The quantity of virus antigen used in the vaccine was approximately the same as that calculated to be present at the height of an NDV infection, assuming all tissues of an infected bird to contain a concentration of 10⁸ logs of virus per 0.1 gm. of tissue at maximal levels. It was found that the peaks of extraneural titers were attained on day 3 after intramuscular infection and on day 4 after intracerebral infection (1). In the experimental infections, the levels of antibodies rose 3 days after peak antigen titers were reached, *i.e.*, antibodies rose on day 6 following intramuscular infection and day 7 following intracerebral infection. Thus, there was a constant latent period of 3 days between the maximal concentration of antigen and the appearance of measurable antibody.

Effect of Vaccination and Passive Immunization on the Course of a CG 179 Infection.—Attempts were made to alter the virulent CG 179 infection by modifying the quantity of antibody available during the early phase of the disease.

Two chickens vaccinated with the B strain, as described above (Text-fig. 3),

were challenged 23 hours later with 10^6 LD₅₀ of CG 179 intramuscularly. The birds remained completely asymptomatic and no virus was detected in undiluted samples of blood taken 2, 4, and 6 days after challenge. At the time of challenge there was no demonstrable serum antibody but hemagglutination inhibition antibody did appear in the serum of control birds on day 3. The early appearance of antibody due to the prior vaccination presumably was sufficient to cause protection.



TEXT-FIG. 4. The rapidity of antibody response after vaccination compared to the response following intramuscular and intracerebral infection with the avirulent (B) strain of NDV.

The same principle of protection was demonstrated by the passive administration of antibody during the early phase of the infection. A group of chickens was inoculated with 10^5 LD₅₀ of CG 179 strain intramuscularly and then treated with 1 ml. of hyperimmune NDV serum intravenously at intervals during the incubation period (Table II). Complete protection was obtained when the antiserum was introduced simultaneously with the virus, varying degrees of partial protection occurred when it was given 1 and 2 days after the infectious inoculum, and slight protection on the 3rd day. The three birds that died in the last group were already symptomatic when the antiserum was given. When death occurred, antiserum did not seem to have prolonged the course of the illness. All control birds inoculated with virus alone died between the 3rd

TABLE II

Modification of Virulent (CG 179 Strain) NDV Infection by Passive Antiserum Administration

| Time of administration of antiserum after virus inoculation | No. of birds receiving antiserum | Condition of birds at end of experiment | | |
|---|----------------------------------|---|-----------|------|
| | | Asymptomatic | Paralyzed | Dead |
| Day 0 | 3 | 3 | | |
| " 1 | 3 | 2 | 1 | |
| " 2 | 3 | 1 | 1 | 1 |
| " 3 | 4 | 1 | | 3 |

TABLE III

Comparative Neutralizing Titers of Serum and Brain Tissue against NDV

| Experiment No. | Preparation | Length of time after inoculation | Condition* | Neutralization index† | | |
|----------------|--|----------------------------------|------------|-----------------------|-------|---------------|
| | | | | Serum | Brain | CNS antibody‡ |
| | | <i>days</i> | | | | |
| 1 | B intramuscularly | 8 | N | 1.6 | 0.0 | 0 |
| | B " | 22 | N | 5.0 | 0.3 | 0 |
| | B " | 49 | N | 4.3 | 0.2 | 0 |
| | B vaccinated | 22 | N | 3.4 | 0.0 | 0 |
| 2 | B intracerebrally (3.5 logs inoculum) | 22 | N | 5.1 | 1.9 | + |
| | B intracerebrally (1.8 logs inoculum) | 21 | N | 4.4 | 1.0 | ± |
| | B intramuscularly and allantoic fluid intracerebrally | 21 | N | 5.3 | -0.2 | 0 |
| | B intramuscularly, control | 21 | N | 4.9 | 0.0 | 0 |
| 3 | B " young bird | 14 | P | 4.0 | 1.7 | + |
| | B " " " | 14 | N | 3.5 | -0.3 | 0 |
| 4 | CG 179 intramuscularly and antiserum on day 2 | 15 | P | 4.4 | 2.8 | + |
| | CG 179 intramuscularly and antiserum on day 2 | 15 | N | 3.7 | 1.5 | + |
| | CG 179 intramuscularly and B intramuscularly, "interference" | 20 | P | — | 2.0 | + |
| | B intramuscularly, control | 15 | N | 4.7 | 0.4 | 0 |

* Condition of bird at time of sacrifice: N = normal; P = paralyzed.

† Figures represent log LD₅₀ virus neutralized.

‡ A brain neutralization index of 1.0 log is considered positive.

and 6th days. Thus, the introduction of passive antibody during the incubation period, at a time when virus had already attained a high titer in the central nervous system (1), seemed to protect the birds. This protection failed once symptoms had begun.

Appearance of Antibody in Brain Tissue.—The quantitative relationship between antibody titers in the serum and in the central nervous system was studied in birds convalescent from NDV to determine the significance of brain antibody in recovery from an acute infection. The results of neutralization tests using $10^{-0.5}$ log crude brain suspension are shown in Table III.

It was found in Experiment 1 that birds convalescent from an intramuscular infection with the B strain did not develop significant titers of antibody in brain tissue in spite of a high titer in their circulating blood. One bird vaccinated with formalized virus had no brain antibody.

Experiment 2 measured antibody in two preparations after intracerebral inoculation of the B strain. Assuming a neutralization index of 1 log to be significant, there was definite antibody in the brain inoculated with $10^{3.5}$ LD₅₀ of virus and questionable antibody in the brain inoculated with $10^{1.8}$ LD₅₀. A control bird given B strain intramuscularly and another bird given B strain intramuscularly and also inoculated with normal allantoic fluid intracerebrally, were both negative.

In Experiment 3, the titer of brain antibody was determined in young birds, weighing 300 to 500 gm., inoculated intramuscularly with 5 logs of the B strain. Six birds were so inoculated and half became paralyzed. Of the two birds in the group selected for this study, the one that was paralyzed was found to have a significant neutralization index in the brain, whereas the one that was asymptomatic lacked such neutralizing antibody.

In Experiment 4, two birds were given CG 179 infections intramuscularly and the lethal outcome was aborted by the administration of antiserum on day 2. Both showed significant titers of brain antibody. One bird given CG 179 intramuscularly was simultaneously inoculated intramuscularly with concentrated purified B strain in an "interference" experiment. The brain of this animal contained antibody. A control bird given B strain intramuscularly was negative.

Thus, antibody was consistently found in the central nervous system of those birds which had severe infections as measured by obvious signs and pathological picture. Antibody was not demonstrable in benign infections with the B strain given intramuscularly but it is possible that the inaccuracy of the method would not have allowed detection of small quantities.

DISCUSSION

When an inoculum of NDV is introduced into a host, it simultaneously serves as an infectious agent and an antigenic stimulus. The factors affecting the capacity of two different strains of the virus to multiply and attain in the central nervous system titers sufficiently high to result in the production of symptoms are discussed in the accompanying paper (1). The present study is concerned with the temporal relationship between the virus' destructive process and the

host's antigenic response. The delicacy of this balance has been pointed out in two sets of experiments: (a) a fatal CG 179 infection was aborted, *i.e.* changed to a non-fatal B type infection, by the artificial provision of antibody slightly earlier than it would have appeared in the natural course of the disease; and (b) a B strain infection was transformed into a paralytic disease either by using the intracerebral route of inoculation or by using younger chickens. Similar fine adjustments between the number of infectious units and the number of antigenic units inoculated have been shown by Schlesinger working with Western equine encephalomyelitis in mice (9). This phenomenon accounted for a "paradoxical" type of response in which, under certain circumstances, the host survived a large virus inoculum but succumbed to a minimal challenge. Comparable findings have been demonstrated for yellow fever by Theiler and Hughes (10) and for rabies by Habel and Wright (11).

In our system it was shown that the antigenic response to the CG 179 and B strains was approximately equal. The earliest detection of circulating antibody coincided temporally with the decline of virus titers in convalescing NDV-infected birds. The order in which tissues became free of virus, blood, viscera, and brain, reflected the availability of circulating antibody to these tissues. The temporary persistence of the virus infection in brain tissue in the presence of significant quantities of circulating neutralizing antibody should be stressed. When serum antibody became measurable, it was indicative of an excess. Therefore, antibody bound to virus antigen may well have existed prior to the 6th day of infection. After vaccination, antibody appeared on the 3rd day, indicating the potential time of its appearance after an infectious inoculum. Since it has been demonstrated that the B strain multiplied more slowly in the central nervous system than did the CG 179 strain (1), this time difference may play a role in the lack of virulence of the B strain.

The significance of antibody found in brain tissue and its quantitative relationship to serum antibody has been the subject of much study. A serum/brain/spinal fluid antibody ratio on the order of 300/3/1 has been demonstrated with bacterial antigens (12) and equine encephalomyelitis (13-14) and yellow fever viruses (15).

Results with NDV infection indicate that there is a similar wide ratio between brain and serum antibody titers. No antibody was measurable in the brain after an asymptomatic avirulent infection or after vaccination, but significant titers were found in preparations with marked central nervous system involvement in which recovery occurred. The latter preparations were characterized in general by higher virus titers and by greater evidence of histological damage to the central nervous system (10). Whether the neutralizing antibody in the central nervous system was the result of *in situ* production or of an accumulation of antibody from the blood stream is of con-

siderable interest. Certain parallels exist between the present experimental results and Schlesinger's studies on equine encephalomyelitis in which the mechanism of the local antigenic booster response following intracerebral inoculation was investigated (9). In the NDV-chicken system, those situations which were accompanied by marked activity of the virus in the central nervous system presumably produced a sufficiently large amount of local antigen to act as a local stimulant to antibody production. The effect was to narrow the serum/brain antibody ratio. Local concentrations of neutralizing antibody have also been found in the central nervous system in poliomyelitis (16) and yellow fever infections (15). Both vaccinated and infected birds were solidly immune to intracerebral challenge with the CG 179 strain irrespective of brain antibody titer. The challenge may be thought to serve in itself as an effective antigenic stimulus.

It should be pointed out that the distribution of antibody has been studied heretofore entirely in mammals. The effect of testing the problem in an avian host in itself cannot be evaluated. The role played by the host in determining the outcome of an acute infection with NDV has been examined here from the point of view of the development of circulating antiviral substances as a response to the viral antigen. The possibility of other defense mechanisms on the part of the host has not been explored.

SUMMARY

Circulating antibody appeared in the convalescing NDV-infected chicken concomitantly with the disappearance of virus from the tissues. The antigenic response to the CG 179 and B strains was demonstrated to be approximately equal.

The neutralization test in the embryo and the hemagglutination inhibition technique yielded parallel results in the measurement of antibody early in convalescence, but late in convalescence the hemagglutination inhibition titers were relatively lower. This disparity indicates the possible duality of the antibodies.

There was a wide ratio between the neutralizing antibody titers found in the brain and in the serum after an asymptomatic infection with NDV. The antibody level in the brain appeared to be related to the extent of virus growth and damage in the central nervous system.

It appeared likely that a major factor in determining the virulence of the CG 179 strain was the more rapid attainment in the central nervous system of high virus concentration which outstripped the defense mechanisms of the host.

The authors wish to express their appreciation to Miss Ruth Libert for technical assistance.

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