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Direct complement fixation test with avian infectious bronchitis virus in chickens

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SUMMARY

The direct complement fixation test was performed to follow the antibody response in chickens infected with avian infectious bronchitis virus. Concentrated allantoic fluid (4 units) was used as an antigen and allowed to react with serially diluted antiserum in the presence of two complete units of guinea-pig complement for 3 hr. at 4° C. and ½ hr. at 37° C. before the addition of sensitized cells. Serum was unheated and used either fresh or within one month of storage at −30° C. Individual birds showed a rise and fall of complement-fixing antibody both after primary and secondary inoculations. The complement-fixing antibody was detected as early as the seventh day after primary inoculation. The highest complement fixation titre (1/32 to 1/64) was recorded from 14 to 21 days after inoculation with a subsequent gradual decline.

The results of the direct complement fixation tests have been correlated with the serum neutralization test. The neutralizing antibodies usually appeared by the 14th day but were not detected at a significant titre until the 21st day after primary inoculation. Serum neutralizing antibodies were still present at high titres even after 7 weeks of infection but the complement-fixing antibodies had disappeared by that time.

INTRODUCTION

Chicken or turkey sera failed to fix guinea-pig complement in the presence of homologous antigen, when heated at 56° C. for 30 min. (Bushnell & Hudson, 1927; Rice, 1947; Rice, Magwood & Annau, 1960; Nitzschke, 1954, 1956; Brumfield & Pomeroy, 1957). This finding led Rice (1948) to use an indirect complement fixation test for demonstrating antibody in heated avian antisera. However, Nitzschke (1954, 1956) developed a direct complement fixation test using sera of hens immunized with three different viral agents, viz. Newcastle disease, fowl plague and swine influenza viruses. Results obtained with direct complement fixation and inhibition complement fixation were substantially the same but the direct complement fixation test was less sensitive in distinguishing the viruses. A very few unheated chicken serum samples in the presence of guinea-pig complement (1·8 units) showed very slight anticomplementary activity at low dilutions. Brumfield & Pomeroy (1957) employed sufficient amounts of guinea-pig complement to overcome the anticomplementary effects of unheated turkey or chicken sera. They

studied ornithosis, infectious bronchitis and Newcastle disease viruses of birds and showed that the fixation was specific due to antibody rather than to the combined anticomplementary effects. Brumfield, Benjamin & Pomeroy (1959) subsequently developed a modified form of direct complement fixation test for the study of ornithosis in turkeys, which was based on the addition of unheated normal chicken serum to the test. By this technique they showed that the titres of these sera could increase two- to eightfold as compared to the titres obtained in conventional direct complement fixation tests. Rice *et al.* (1960) adopted this modified complement fixation test in favour of the indirect test when detecting antibodies to salmonella antigens in turkey sera. Contrary to the above findings, Orlans, Rose & Clapp (1962*b*) could not demonstrate the fixation of guinea-pig complement by fowl antibody against bovine serum albumin, either with fresh antiserum or with heated antiserum in the presence of normal chicken serum. This paper describes an experiment in which the antibody responses of individual birds during the course of infection with avian infectious bronchitis virus were measured both by direct complement fixation and by serum neutralization tests. The findings provide further evidence for the specificity of the direct complement fixation test for infectious bronchitis.

MATERIALS AND METHODS

Virus

The strain of avian infectious bronchitis virus isolated by Dr H. P. Chu and designated Cambridge 163/57 was used in the present study.

Experimental infection in chickens

Chickens were obtained from the flock kept at the isolation farm at the School of Veterinary Medicine, and were known to be free from avian infectious bronchitis virus. Thirty-four 8-week-old chickens were divided into two equal groups and were kept in an isolation block. All birds were bled before infection. The first group was inoculated with 0·25 ml. of undiluted infectious bronchitis virus (titre of the virus $10^{6\cdot5}$ EID 50/ml.) in the sinuses and trachea. The second group received the same concentration of virus intravenously. Two chickens from each group were killed on the 2nd, 5th, 7th, 10th, 14th and 21st day after infection. In addition, four chickens were also bled on the 5th, 7th, 10th, 14th, 28th, 35th, 42nd and 49th day. Both complement fixation and serum neutralization tests were performed on all the serum samples. In order to study the secondary response a second dose of virus consisting of 0·4 ml. of the same concentration was given intravenously to the remaining birds of each group on the 49th day. Chickens were again bled on the 7th, 14th and 21st day following reinfection. A complement fixation test was performed on all the sera, and a serum neutralization test on two chickens from each group. The chickens were bled from the wing vein, using sterile all-glass syringes, before and after infection. The serum was usually taken from the clot on the same day as the birds were bled, and stored unheated at $-30°$ C.

Complement fixation tests

Sheep erythrocytes

Throughout the study one sheep whose cells were not fragile was used for both the preparation of haemolysin and indicator system. One volume of blood collected aseptically was added to 1·2 volumes of modified Alsever's solution (Bukantz, Rein & Kent, 1946).

Haemolysin

Two young rabbits were inoculated subcutaneously with 0·5 ml. of a 10% suspension of sheep red blood corpuscles and were observed for 10 min. for any untoward reaction. Neither of the rabbits showed any reaction, and 1 ml. of a 10% suspension of sheep red blood cells was then inoculated intravenously into each. This was followed by 1, 2, 2·5, 3 and 5 ml. amounts of a 20% suspension given intravenously at intervals of two days. Rabbits were bled for testing a week after the last injection and the antibody titres were found to be satisfactory. Serum was heated at 56° C. for 30 min. and preserved in equal volumes of sterile neutral glycerine, bottled in small ampoules and stored at 4° C. Minimum haemolytic dose (MHD) of haemolysin was determined. The highest dilution of haemolysin showing complete haemolysis was taken as one unit. The titre of the haemolysin was 1/16,000.

Sensitized cells

After washing three times, the sheep red cells were packed at 2000 rev./min. for 10 min. A 4% suspension of the cells was made in veronal buffer solution (pH 7·4) (Mayer, Osler, Beir & Heidelberger, 1946) and an equal volume of this suspension was mixed with an equal volume of dilute haemolysin containing 4 MHD. The mixture was shaken thoroughly and kept in a water bath at 37° C. for 20 min. The sensitized cells were always prepared fresh before use.

Complement

Young male guinea-pigs were first bled individually and their sera tested for lytic activity against sheep erythrocytes. Only those guinea-pigs whose sera did not have any lytic activity were used. Clear serum collected from six to eight guinea-pigs was pooled and preserved by the addition of an equal volume of a solution containing 12% sodium acetate and 4% boric acid in distilled water (Sonnenschein, 1930). The preservative was tested for traces of zinc with 0·1% sodium-diethyl-dithio-carbamate. The complement was titrated in the presence of antigen and normal fowl serum under the conditions of the tests. The highest dilution of complement showing complete lysis was taken as one unit of complement.

Antigen

Fertile eggs 9–10 days old were inoculated in the allantoic sac with 0·1 ml. of a 10^{-2} dilution of virus (titre of the virus $10^{6.5}$ EID 50/ml.) and were incubated at 37° C. Eggs dying within 24 hr. were discarded. The eggs were chilled after

48 hr. of virus multiplication, the allantoic fluids harvested aseptically, pooled and centrifuged at 3000 rev./min. for 15 min. to remove the gross particles. The clear allantoic fluid was further centrifuged at 19,000 rev./min. for 1 hr. The resulting pellets were resuspended in a small quantity of veronal buffer and subjected to vibration at 1·5 amperes (MSE ultrasonic power unit) for 1 min. The suspension was then distributed in about 1·5 ml. volumes and kept frozen at −30° C. In practice the pellet yield from 1500 ml. of allantoic fluid obtained from 150 eggs was resuspended in 15 ml. of veronal buffer. The optimal concentraction of antigen was determined in the presence of positive serum by the chess board titration method. It was observed that the antigen in a dilution of 1/8 was quite suitable for the actual complement fixation test.

Sera

Sixteen normal chickens were bled in order to study the anticomplementary activity of their sera. The tests were done on fresh unheated sera and also after storage at 4° C. and −30° C. Twofold dilutions of each fresh unheated serum were made ranging from 1/8 to 1/128. Diluted sera in 0·2 ml. volumes were distributed in seven different rows of tubes and 2 units of complement in 0·2 ml. were added to each followed by 0·2 ml. of diluent in place of antigen. The system was incubated at 4° C. One row of the tubes was taken from the refrigerator at 1, 2, 3, 4, 6, 12 and 18 hr. and incubated at 37° C. for ½ hr. The system was further incubated for ½ hr. at 37° C. after the addition of sensitized cells. The results indicated that eight sera after 6 hr. and 11 after 12 hr. were anticomplementary. Similarly serum samples after 1 month of storage at 4° C. and −30° C. were tested as described above except that the system was incubated at 4° C. for 3 hr., a period at which none of the fresh unheated sera were anticomplementary. It was shown that after storage for 1 month at 4° C. seven of the 16 sera were anticomplementary, but after storage at −30° C. none of the sera were anticomplementary.

Test proper

Serial twofold dilutions of unheated chicken sera in 0·2 ml. volumes were made in round-bottomed tubes starting from a 1/8 dilution, 0·2 ml. of antigen (4 units) was added followed by 0·2 ml. of complement (2 units). The suitable controls of antigen, antiserum and complement were included. The system was kept in the refrigerator at 4° C. for 3 hr. with occasional shaking followed by further incubation for ½ hr. in a 37° C. water bath. Sensitized cells in volumes of 0·2 ml. were added to each tube and the results were read after 30 min., when the antigen, antisera and complement controls showed complete haemolysis. Positive and negative sera were used to ensure that the sensitivity of the successive tests were uniform. The highest dilution of the serum showing more than 50 % fixation was taken as the end titre of the serum. The results were read visually.

Serum neutralization test

Initial dilutions of 1/2 and 1/5 of heated sera (56° C. for 30 min.) were made. Serial twofold dilutions of 1/5 sera were subsequently made to 1/320. Constant

amounts of virus (0·05 ml. containing 100 EID 50) were mixed with equal volumes of the diluted sera. The mixture was kept at 4° C. for 1 hr. and then inoculated in 0·1 ml. volume into the allantoic sac of each of five 9-day-old fertile eggs which were incubated at 37° C. for 8 days. Death and dwarfing of the embryos was regarded as the indication of infection by the virus. Those eggs dying within 24 hr. after infection were discarded. The highest dilution of serum showing more than 50 % virus neutralization was taken as the titre of the serum. Positive and negative serum controls were always included in the experiments. Whenever there was a deviation in the titre of the positive control serum by more than one tube the test was repeated.

RESULTS

The antibody responses in chickens infected with infectious bronchitis virus both by respiratory and intravenous routes have been demonstrated both by direct complement fixation and serum neutralization tests as shown in Tables 1 and 2 and

Table 1. *Complement-fixing and neutralizing antibody titres in sera of chickens killed at various times after intratracheal and intranasal inoculation of avian infectious bronchitis virus*

Days after infection	Chicken no.	Antibody titre: Complement fixing	Antibody titre: Neutralizing
0	601	< 8	< 2
2	604	< 8	< 2
2	605	< 8	< 2
5	606	< 8	< 2
5	607	< 8	< 2
7	609	8	< 2
7	667	< 8	< 2
10	611	16	< 2
10	657	16	< 2
14	676	64	2
14	669	32	< 2
21	608	64	5
21	671	16	5

Figs. 1–4. It was found that none of the sera before infection showed complement-fixing or neutralizing antibody titres. However, complement-fixing antibody was clearly shown in most of the sera by the 7th day after virus inoculation, though neutralizing antibody could not be detected before the 14th day. There was a significant rise of the complement-fixing antibodies after the 7th day of experimental infection, reaching its highest titre between the 14th and 28th day, while neutralizing antibody reached its peak after the 28th day and remained more or less constant up to the 49th day, the maximum period tested. The complement-fixing antibody tends to disappear after the 28th day and was negative in most of the sera collected on the 42nd and 49th day after infection. After a second inoculation there was a rise both in complement-fixing and neutralizing antibody titres.

Table 2. *Complement-fixing and neutralizing antibody titres in sera of chickens killed at various times after intravenous inoculation of avian infectious bronchitis virus*

Day after infection	Chicken no.	Antibody titre: Complement fixing	Antibody titre: Neutralizing
0	600	< 8	< 2
2	602	< 8	< 2
2	603	< 8	< 2
5	656	< 8	< 2
5	683	< 8	< 2
7	655	8	< 2
7	659	8	< 2
10	661	16	< 2
10	663	32	< 2
14	672	32	2
14	654	32	< 2
21	658	32	5
21	669	32	5

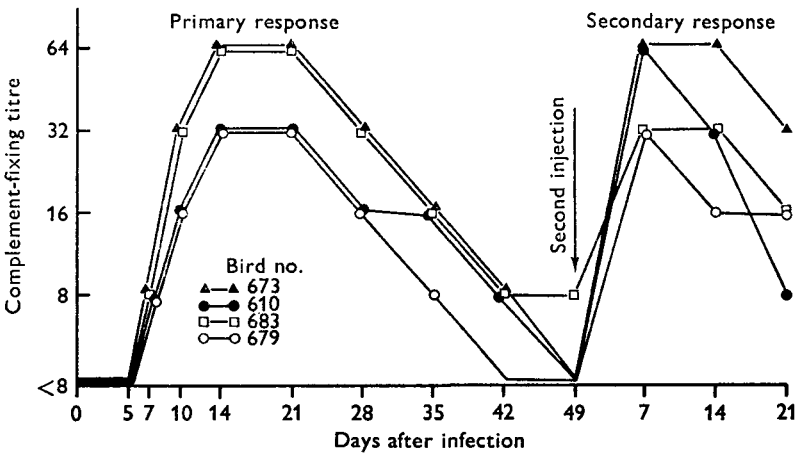

Fig. 1. Development of complement-fixing antibodies in chickens infected in the respiratory tract with avian infectious bronchitis virus.

DISCUSSION

In the present study it has been observed that the concentration of the antigen, dosage of complement, collection and storage of chicken serum and the period of interaction between antigen and antibody are all critical for the successful application of the direct complement fixation test. Allantoic fluid as an antigen without concentration failed to fix complement in the presence of homologous antibody. However, the allantoic fluid when centrifuged at high speed yielded a suitable antigen for the complement fixation test. Nitzschke (1954) also used six times concentrated allantoic fluid infected with Newcastle disease virus as an antigen for the direct complement fixation test. Brumfield & Pomeroy (1957) also

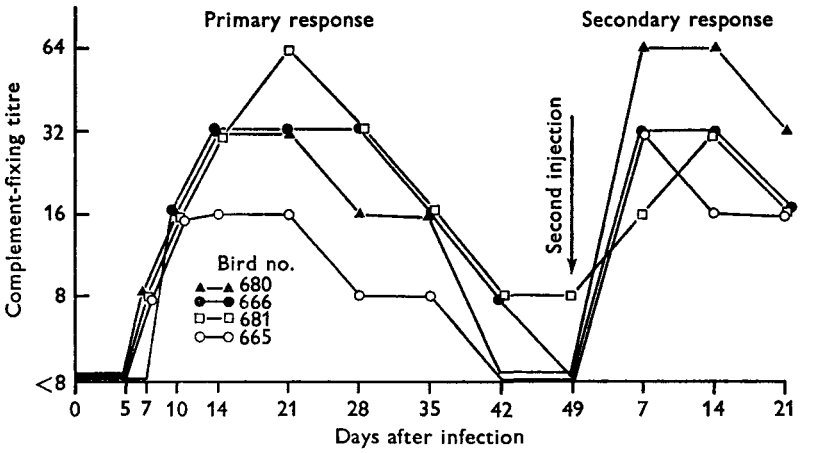

Fig. 2. Development of complement-fixing antibodies in chickens infected intravenously with avian infectious bronchitis virus.

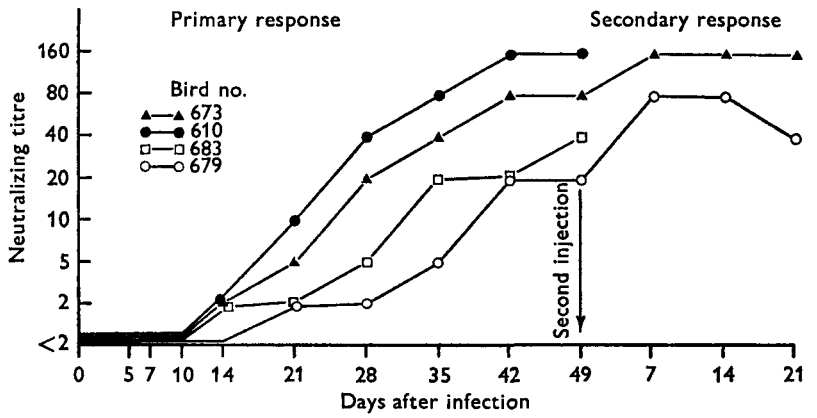

Fig. 3. Development of neutralizing antibodies in chickens infected in the respiratory tract with avian infectious bronchitis virus.

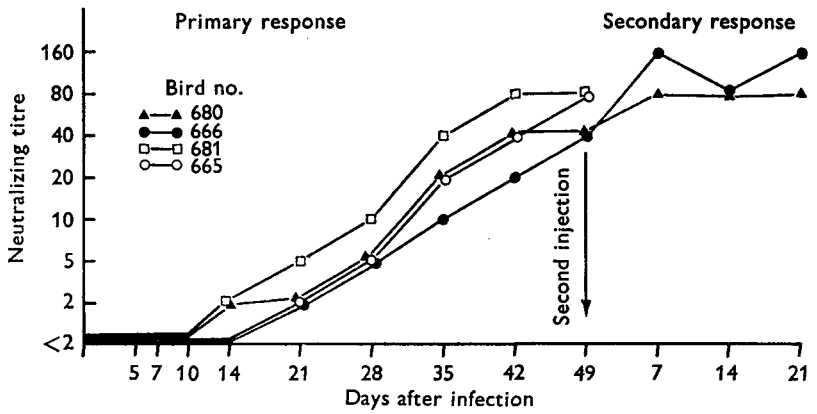

Fig. 4. Development of neutralizing antibodies in chickens infected intravenously with avian infectious bronchitis virus.

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noticed that the allantoic fluid harvested from eggs infected with infectious bronchitis virus, when used without concentration, failed to give reliable results.

None of the unheated chicken sera in the presence of two complete units of guinea-pig complement showed any anticomplementary activity when tested fresh or after storage for a month at −30° C. Similarly Brumfield & Pomeroy (1957), Tsubahara, Kataoko & Kato (1961), Harada & Matumato (1962) and Kato & Horiuchi (1965) did not find any anticomplementary activity with those unheated sera which were kept frozen at −20° C. before use. Orlans, Rose & Clapp (1962*a*), however, found that even fresh fowl serum in a dilution of 1/100 inhibits the lysis of sensitized cells by guinea-pig complement. In the present experiments appreciable anticomplementary activity was observed with certain sera when stored at 4° C. Nitzschke (1954) also found that, with chicken sera which had been stored for a long period at 2° C., four out of ten showed clear anticomplementary activity. The optimum time for the fixation of complement in the presence of unheated chicken serum and antigen was found out because most of the unheated chicken sera after long storage at 4° C. were so anticomplementary that specific fixation could not be interpreted. The period of 3 hr. at 4° C. followed by incubation at 37° C. for $\frac{1}{2}$ hr. was found to be ideal for the test.

Brumfield & Pomeroy (1957) employed direct complement fixation with the sera of birds immunized with three viral agents, ornithosis, infectious bronchitis virus and Newcastle disease virus. Immune sera against heterologous antigens within the three systems gave negative results. They showed that the fixation was specific due to antibody rather than anticomplementary effects. Orlans *et al.* (1962*b*) could not use direct complement fixation tests with soluble antigen–antibody systems. They attributed their failure to the possibility that particulate antigen or antibody produced during the course of infection might provide better conditions for C^1 fixation than the soluble antigen. In the present investigation the specificity of the test is shown by following the antibody response in the individual birds infected with avian infectious bronchitis virus. None of the 34 sera collected before infection showed any complement-fixing or neutralizing antibodies. The rise and fall of the complement-fixing titres in the individual birds both after primary and secondary inoculation suggest that the development of complement-fixing antibody is associated with infection rather than any non-specific reaction. The results of the complement fixation tests were further correlated with serum neutralization tests. It was shown that the complement-fixing antibodies appeared earlier than serum neutralizing antibodies. It is presumed that the direct complement fixation test will provide a useful additional serological test for the study of infectious bronchitis.

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