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COMPLEMENT FIXATION REACTIONS WITH NEWCASTLE DISEASE VIRUS

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COMPLEMENT FIXATION REACTIONS WITH NEWCASTLE DISEASE VIRUS

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INTRODUCTION

Until 1937 the complement fixation test for the detection of neurotropic viruses was of little value. Since that time great strides have been made in developing a specific complement fixation test not only for this group of viruses, but for other viral and rickettsial infections. The factor causing the most trouble in setting up a complement fixation test for neurotropic viruses has been, and still remains, to prepare an antigen that is specific for a given homologous antibody. Neurotropic viruses establish themselves in the central nervous system and the greatest source of antigen can be found in the brains of those species affected. Normal brain material contains lipid materials. These substances are capable of exhibiting anticomplementary action or non-specific fixation in the complement fixation test. Much work has been done to remove these lipid substances from normal and infected brain material. Some of the methods used to accomplish this extraction are long and tedious and even the "simplified" methods involve considerable time and effort.

It was found in this laboratory that the brains of Syrian hamsters infected with Newcastle disease virus, did not exhibit any interference reactions when used in the complement fixation test, together with specific antibody. Since Syrian hamsters are readily obtained, easily kept, and highly susceptible to hamster adapted Newcastle disease virus, an investigation into the complement fixation reactions of Newcastle disease virus was undertaken.

For brevity, Newcastle disease virus will, hereafter, in this paper, be referred to as NDV.

REVIEW OF THE LITERATURE

An infection of fowl caused by a filterable virus was reported by Doyle (1926). This disease was given the name of NDV. From the Dutch East Indies came a report of an infection of fowl which in later years was shown to be similar to NDV (Kraneveld 1926). In 1940, poultry raisers in California described a type of nervous disorder in their flocks. These outbreaks revealed a respiratory disorder which was at first believed to be due to infectious bronchitis. This disease was named "avian pneumoencephalitis" (Beach 1942). Later it was demonstrated that this new disease in California was immunologically identical to Newcastle disease, the difference between the California disease and that occurring elsewhere being in the mortality rates. The disease in most parts of the world is characterized by high mortality, while in the United States the average mortality is five to 10 percent (Beach 1942). In survivors, the egg production remains low for a period of one to three months. The virus was first identified in the United States by Stover (1942).

Newcastle disease affects the central nervous system as well as the respiratory organs. Cases with involvement of the central nervous system usually continue for a longer period of time than those which are essentially respiratory. Nervous system disorders are characterized by ataxia, partial or complete paralysis of one or both legs, incoordination of the neck muscles and tremor of the head (Beach 1942). All these symptoms are seldom exhibited by any one bird but are usually randomly manifested in a flock.

There has been much discussion over the relationship of NDV either to neurotropic or respiratory viruses. Burnet (1942) observed reactions common to both Newcastle disease virus and influenza viruses. Both agglutinated chicken red blood cells in similar titers and multiplied in the same manner. Cunha, et al (1947) reported that "the resemblance to the influenza viruses must be extremely remote, from the point of view of morphology. With the present knowledge, a valid basis for classifying the Newcastle virus with the viruses of influenza is not obvious".

Bang (1948) was of the opinion that "Newcastle disease virus resembles those of the encephalitis group in its ability to spread throughout the developing egg and embryo, but it is similar to influenza virus in that high concentrations are found in the allantoic fluid before death". Reagan (1947) found that intracerebral inoculation of monkeys with hamster adapted NDV resulted in symptoms similar to the ones caused by poliomyelitis. He found that egg adapted Newcastle virus upon intracerebral inoculation did not produce any such symptoms. Wenner (1949) also reported a central nervous system disorder in monkeys intracerebrally inoculated with Newcastle disease virus.

Newcastle disease virus also has its public health significance. An epidemic of seventeen cases of conjunctivitis in Israel was reported (Yatom 1945). The infection almost exclusively attacked women who had contact with fowl affected with Newcastle disease. No direct spread from man to man was noted. The epidemic stopped as soon as the diseased birds were removed from the flock and destroyed. Ingalls (1949) reported two authentic

cases of Newcastle disease affecting the conjunctiva of man. In both cases the individuals were exposed to fowl which had Newcastle disease. Identification was made by hemagglutination-inhibition tests and by virus isolation through passage in embryonated chicken eggs. Howitt, et al (1948) gathered information on a mild central nervous system infection that took place in Tennessee. All sera taken from the infected persons were tested by the neutralization test against Eastern and Western equine encephalomyelitis and St. Louis encephalomyelitis. No reactions with these viral antigens were noted. Twenty out of twenty-five sera tested against NDV were positive. Although no virus has been isolated, there seemed to be some opinion that the Newcastle disease virus of fowl is the agent responsible for many of the atypical central nervous system infections in man that have been reported during the past few years. Like in the fowl, the manifestations are neurological in the young and influenza-like in the adult.

The first successful application of the complement fixation test in work with neurotropic viruses was accomplished by Howitt (1937). Essentially her method of obtaining antigenic material for work with the neurotropic viruses consisted of the following: brains of infected guinea pigs and mice were lyophilized, extracted with ether, brought up to original volume with ether, centrifuged at 2400 rpm for fifteen minutes and then dried again. Physiological saline was used as a diluent for the antigen when tests were to be made. Freezing and thawing five or six times in a CO_2-alcohol bath and again centrifuging at 2400 rpm for fifteen minutes completed the preparation. In this way Howitt was able to differentiate the viruses used.

In these tests the sera were produced by experimentally infecting animals. False positives resulting from Wassermann positive human sera did not affect her results.

Casals (1941) objection to Howitt's (1937) work was that the resultant antigen, although not anticomplementary was of very low titer. He reported that "supernatants of brain emulsions, when centrifuged at 10,000 to 30,000 rpm for one hour and especially if filtered through a Seitz pad, showed no anticomplementary action". Casals also found that the supernatant fluid when filtered without first being centrifuged, was anticomplementary and could not be used in the complement fixation test. An anticomplementary antigen can be diluted to a point where it no longer interferes with the test. However, since these neurotropic viruses have such low titers, it is likely that the antigen would be diluted beyond usable antigenicity before the disappearance of anticomplementary activity.

Havens, et al (1943) stated that the clinical and pathological picture of these neurotropic diseases were not distinct enough to warrant a diagnosis of a specific disease. Serological studies are required for positive identification, except in the case of rabies and poliomyelitis. Havens' group worked on the following viruses: St. Louis encephalomyelitis, Japanese B encephalomyelitis, Eastern and Western equine encephalomyelitis, West Nile and lymphocytic choriomeningitis. Syrian hamsters were found to be susceptible to all but lymphocytic choriomeningitis. Centrifugation at high speeds was used to remove anticomplementary material and other substances that gave non-

specific reactions in the complement fixation test. Immune serum was prepared by intraperitoneal inoculation of active virus into hamsters. None of the antigens were anticomplementary, no antigens fixed complement in the presence of normal inactivated serum, none of the antisera fixed complement with antigens from normal brain tissue after undergoing the same preparation as did the infected brain material.

A problem that arose in working with these viral agents was that there was danger of the laboratory worker becoming infected. Casals (1942) used ultraviolet light to inactivate these highly virulent viruses. The avirulent antigens he prepared had the same antigenicity, specificity, and lack of anticomplementary effect as did the fresh antigens. Although specialized standardized equipment must be used, the final product seems worth the effort. This safe antigen may be lyophilized and stored for periods up to two years without any decrease in titer. Casals (1945) also used heat to prepare avirulent antigens from infected mouse brains. A temperature of 60° for thirty minutes rendered the material safe for routine laboratory usage. This method does lower the titer of the antigen somewhat. It is, however, a simple procedure for producing avirulent antigens of a high enough titer for diagnostic work.

There are other methods available for producing antigens that are not anticomplementary, for work with these neurotropic viruses. Wolfe, et al (1946) prepared a rickettsial antigen from embryonated chicken yolk sacs. DeBoer (1946) reported that this method is applicable to the preparation of

any infected tissue rich in antigen. The preparation includes freezing, drying, extraction with lipid solvents, drying and centrifugation and being restored to volume before use. Espaňa (1948) modified DeBoer's method. This modification consists of using benzene for the lipid extraction. The antigen is lyophilized and upon rehydration requires no centrifugation. Casals (1949) recently prepared an antigen by means of successive extractions of infected brain tissue with acetone and ethyl ether. This antigen does not react with Wassermann positive sera.

Newcastle disease, a naturally occurring virus in fowl, was adapted to the Syrian hamster by Reagan (1948). A California strain of this virus was carried intracerebrally in hamsters, until the incubation period of the disease decreased from two to six days to approximately twelve hours. After twelve serial passages, the hamster adapted virus was non-pathogenic for chickens when inoculated intramuscularly or subcutaneously. Reagan (1949) infected hamsters with NDV by intranasal and intracerebral routes. In hamsters the incubation period is longer following intranasal instillation than following intracerebral inoculation. Hamsters infected by either route showed symptoms of irritability followed by involuntary motor reactions and paralysis. The hamster is also susceptible to other types of neurotropic viruses (Lennette 194[illegible]) (Havens, et al 1943). Sanders (1948) reported that hamsters, intracerebrally inoculated with the Lansing strain of poliomyelitis virus, showed signs of a polio-like infection but that poliomyelitis virus was not recovered. The Syrian hamster is a good experimental animal for use in producing neurotropic virus antigens.

MATERIALS AND METHODS

The Newcastle disease virus hamster-adapted strain was obtained from Dr. R. Reagan, University of Maryland, College Park, Maryland. The virus was from the 311th intracerebral serial hamster passage. Four additional passages were made at this laboratory to provide a supply of antigen for the serological tests that were made.

Newcastle disease infected allantoic fluid was obtained by inoculating embryonating chicken eggs with an egg adapted strain of the virus. Upon death of the embryo the allantoic fluid was harvested.

Preparation of Hamster Brain Antigen

Hamsters were inoculated intracerebrally with 0.06 ml. of a 10 percent suspension of Newcastle disease infected hamster brain. The incubation period varied from three to seven days. Symptoms of the viral infection included ruffled fur, conjunctivitis of one or both eyes, irratibility, spastic paralysis and death. The animals were sacrificed when in the acute stage of the disease. The brains were aseptically removed, placed in sterile tubes, one brain in each tube, sealed and stored at -40° C.

When required, each brain was triturated in a sterile mortar, using alundum as an abrasive and physiological saline as the diluent. The resulting 10 percent suspension was then centrifuged in a horizontal centrifuge at 2,500 rpm for ten minutes. The supernatant fluid was removed from the sediment with a sterile five ml. pipette attached to a rubber bulb. A sample of the super-

natant fluid was placed in thioglycholate medium for a sterility test.

The supernatant fluid was initially tested for viral activity by the hemagglutination test. The technique used was that recommended by the School of Public Health, University of Michigan:

Ten tubes were set up in a rack. One and eight tenths ml. of physiological saline was placed in the first tube and 0.5 ml. in tubes two to 10 inclusive. Two-tenths ml. of brain suspension was added to tube number one, and the contents of the tube were thoroughly mixed. Five-tenths ml. from tube number one was then transferred to tube number two and this procedure repeated in all 10 tubes. Five-tenths ml. was discarded from the last tube. The dilutions in tubes one through 10 were two-fold, ranging from 1:20 to 1:5,120. The indicator system, consisting of 0.5 ml. of 0.5 percent chicken red blood cells, was added to each tube with the exception of the first tube which was a dilution tube. The total volume in each tube used in the test was one ml. The addition of blood cells produced virus dilutions of 1:40 to 1:10,240. The tubes were shaken and put aside at room temperature for one hour.

The end-point was determined by the pattern formed by the cells at the bottoms of the tubes. Positive agglutination of red cells produced a thin diffuse layer of cells covering the bottom of the tube. Negative agglutination showed tightly packed discs of cells at the bottom of the tube. Doubtful agglutination was reported when a ring of packed cells appeared at

the bottom of the tube. The end-point was reported as the greatest dilution in which agglutination occurred.

Antigens prepared in this manner and possessing viral activity in the hemagglutination test were then stored in sealed ampoules at -40° C. until used in the complement fixation test (Table I.). Normal hamster brain suspensions were prepared in the same manner for controls.

Preparation of Newcastle Disease Infected Allantoic Fluid

The shells of eight to 10 day old embryonating chicken eggs were pierced using an electric drill. Care was taken not to puncture the shell membrane. The holes were drilled above the air sac and alongside the allantoic cavity. Metaphen, 1:10,000 dilution, was applied over the areas of the shell that were drilled to prevent bacterial contamination of the embryos. The shell membrane was then punctured with a sterile needle. Using a sterile 0.25 ml. syringe with a 27 gauge needle, 0.1 ml. of egg adapted NDV, contained in allantoic fluid, was injected into the allantoic cavity. The eggs were then returned to the incubator. All embryos dying in eighteen hours or less following injection were discarded. Embryos dying forty-eight hours after inoculation were stored overnight at 4-8° C. before harvesting the allantoic fluid. A sterile 10 ml. syringe with an 18 gauge needle was used to harvest the allantoic fluid. The material was collected under a hood to prevent contamination. The yields of allantoic fluid ranged from five to nine ml. per egg. The allantoic fluid from normal eight to 10 day old chicken embryos was collected as control antigen.

The hemagglutination test was used for the initial detection of NDV. Four serial egg passages were required to increase the titer of the virus to high hemagglutinating activity (Table I.).

Preparation of Antisera in Rabbits

Rice (1948) showed that chicken serum can not be used in the complement fixation test. Therefore, rabbits were used for the production of antiserum.

Rabbits whose ages varied from three months to one year were injected with NDV hamster-adapted brain suspension, normal hamster brain suspension, egg-adapted Newcastle virus allantoic fluid, and normal allantoic fluid respectively. The rabbits were injected every third day intravenously, using the marginal ear veins. Three injections each of 0.25, 0,50, 0.75, and 1.0 ml. respectively were made using a one ml. syringe with a 24 gauge needle.

Following the final injection the animals were allowed to rest for one week before bleeding. A preliminary bleeding was made to determine if the rabbit serum contained antibodies to the antigen. If the preliminary titrations showed the serum to have low titers, further injections of one ml. were made. The rabbits were bled by heart puncture, using a 20 ml. syringe with an 18 gauge needle. Care was taken to prevent hemolysis of the blood by first removing the needle and then slowly expelling the blood from the syringe into the sterile tube used for collection. The tubes were slanted,

allowed to clot and left at room temperature for thirty minutes. The serum was removed from the clot after rimming the clot and centrifuging at 2,500 rpm for fifteen minutes. The serum was inactivated at 56° C. for 20 minutes and then stored at -40° C. Reinactivation at 56° C. for 10 minutes and sterility tests, in thioglycholate medium, were performed on all serum prior to use in the complement fixation test.

The Complement Fixation Test

The methods employed were essentially those of Casals (1941). Preparation and titration of the reagents are as follows:

Hemolytic System:

1) Saline

Eight and a half grams of dry chemically pure sodium chloride was dissolved in one liter of distilled water. The saline solution was then autoclaved at fifteen pounds pressure for twenty minutes. This sterile solution was kept at 4-8° C. when not in use.

2) Sheep erythrocytes

Sheep blood was obtained from the Michigan Department of Health. Sterile Alsever's solution was added in an amount equal to the volume of the blood collected. The blood was stored at 4-8° C. for as long as three weeks without the cells hemolyzing or becoming excessively fragile. The Alsever's solution was made as follows:

Dextrose .....10.25 grams
Sodium citrate ..... 4.00 grams
Sodium chloride ..... 2.10 grams
Citric acid..... 0.28 grams
Water (distilled)..... 500 ml.
Autoclave five minutes at 10 pounds pressure.

The red cell suspension was washed three times using three volumes of sterile saline solution and sedimentation of the cells at 1,200 rpm for ten minutes. The final wash was centrifuged for twenty minutes to insure packing of the cells. The supernatant fluid of the final wash was always free from color, the same being true for the diluent of the suspension of red cells used in the test.

3) Antisheep hemolysis

Glycerinized antisheep hemolysin* in 1:100 dilution was called the stock solution. This stock solution was prepared from the original product by adding 0.1 ml. of glycerinized hemolysin to 9.9 ml. of sterile saline. Storage of this diluted stock solution was at 4-8° C. for not longer than seven days.

Hemolysin was titrated once a week. For the titration a dilution of 1:500 was made from the 1:100 stock solution, by diluting one ml. of the stock solution with four ml. of sterile physiological saline. In a series of 10 tubes higher dilutions were prepared as follows:

0.2 ml. hemolysin (1:500)..... 1:500
1.0 ml. hemolysin (1:500) + 1.0 ml. saline..... 1:1000
0.2 ml. hemolysin (1:500) + 0.4 ml. saline 1:1500
0.2 ml. hemolysin (1:500) + 0.6 ml. saline..... 1:2000
0.2 ml. hemolysin (1:500) + 0.8 ml. saline..... 1:2500

*Sharp & Dhome, Philadelphia, Penn.

0.2 ml. hemolysin (1:1000) + 0.4 ml. saline.....1:3000
0.2 ml. hemolysin (1:1000) + 0.6 ml. saline.....1:3500
0.2 ml. hemolysin (1:2000) + 0.2 ml. saline.....1:4000
0.2 ml. hemolysin (1:2000) + 0.3 ml. saline.....1:4500
0.2 ml. hemolysin (1:2500) + 0.2 ml. saline.....1:5000

The contents of each tube were mixed thoroughly and 0.2 ml. of the diluted hemolysin transferred to a clean tube.

A 1:30 dilution of guinea pig complement was prepared with sterile saline solution and 0.2 ml. added to the tube containing the diluted hemolysin. Two-tenths ml. of a two percent sheep erythrocyte suspension and 0.4 ml. of physiological saline were also added to each tube. The tubes were shaken and incubated in a water bath at 37° C. for one hour. One hemolysin unit was the highest dilution that exhibited complete hemolysis. Two units of hemolysin were used in all tests and the hemolysin was so diluted that 0.2 ml. contained two units. For example, if one unit equalled 0.2 ml. of a 1:4000 dilution of hemolysin, two units, therefore, would equal 0.2 ml. of a 1:2000 dilution.

4) Complement

The complement was obtained from guinea pigs. The guinea pigs were fasted for twelve hours prior to bleeding to prevent their sera from containing excessive quantities of lipid materials. They were bled in groups of 10 using a 10 ml. syringe and a 20 gauge needle. Five to 10 ml. of blood was drawn from each animal. Care was taken to prevent hemolysis by removing the needle from the syringe before expelling the blood into sterile tubes. The blood from each guinea pig was placed in a separate tube and allowed to

clot at room temperature. After 30 minutes the clot was rimmed and centrifuged at 2500 rpm for 10 minutes. A 0.2 ml. sample of serum was taken from each tube and a screen titration was made to determine the complement titer. Only serum which titered 1:40 or higher was used in the final pooled complement.

During the early part of the work the complement was preserved with Green's (1938) preservative. However, unsatisfactory complement resulted and other methods of preservation were found more satisfactory. During the greater part of the investigation one ml. volumes of complement were shell frozen in a CO_2-alcohol bath, hermatically sealed in ampoules and stored at -40° C. No significant decrease in titer was observed in complement preserved in this manner.

Complement was titrated each day a test was to be made according to the following method: Dilutions of 1:10, 1:20, 1:30, 1:40, 1:50, and 1:60 of complement were prepared. To 0.2 ml. of each dilution, 0.4 ml. of physiological saline was added. The tubes were then incubated for one hour in a 37° C. water bath. The sensitized cell system was made up 15 minutes prior to its use. It consisted of equal volumes of a two percent sheep cell suspension and two units of hemolysin, each contained in 0.2 ml., and were mixed in independent tubes and incubated in the water bath. Four-tenths ml. of the sensitized cells was added to each tube of the diluted complement. Finally, the tubes were read at the end of an additional 30 minute incubation period. The unit of complement was taken as the highest dilution that

showed complete hemolysis. Two units of complement were used in the final complement fixation tests. For example, the highest dilution showing complete hemolysis (1:40) was considered as one unit, then two units would be contained in 0.2 ml. of 1:20 dilution.

A complement titration in the presence of each antigen used in the investigation was made every two weeks. Two-tenths ml., containing two units of antigen, 0.2 ml. of each complement dilution, and 0.2 ml. of physiological saline were mixed together and the test conducted in the same manner as the daily titration. The daily complement titration was performed at the same time. Anticomplementary or procomplementary activity of the antigen could be demonstrated by comparison of the end points observed in the two titrations. Table II shows the effect of the antigen-complement titrations.

Titration of Antigen

Two units of antigen were used in the complement fixation tests. In order to determine this unit, the antigens were titrated as follows:

Two-fold dilutions of antigen, ranging from full strength to 1:128, were made. Two tenths ml. of each antigen dilution was added to 0.2 ml. of heated antiserum, diluted 1:10, and two units of complement contained in 0.2 ml.

A hemolytic control of the serum consisted of 0.2 ml. of antiserum and 0.4 ml. of physiological saline. Any hemolytic activity of antigen was demonstrated by preparing 0.2 ml. of full strength antigen, 0.2 ml. of antiserum,

Table I

Hemagglutination Titrations

Virus	Tube dilution	2 1:40	3 1:80	4 1:160	5 1:320	6 1:640	7 1:1280	8 1:2560	9 1:5120	10 1:10240	Control
Newcastle allantoic		≠	≠	≠	≠	≠	≠	≠	≠	-	-
Newcastle hamster brain		≠	≠	≠	≠	-	-	-	-	-	-

≠ = agglutination - = absence of agglutination

Table II

Complement Titrations

Antigen	2 unit dilution of antigen	Tube dilution	1 1:10	2 1:20	3 1:30	4 1:40	5 1:50	6 1:60	Cell control	Hemolysin control
None	-		-	-	-	4≠	4≠	4≠	4≠	-
Normal allantoic	1:4		-	-	-	-	4≠	4≠		
Newcastle allantoic	1:8		-	-	-	-	4≠	4≠		
Newcastle hamster brain	1:16		-	-	-	4≠	4≠	4≠		
Normal hamster brain	1:4		-	-	-	-	4≠	4≠		

4≠ = complete fixation - = hemolysis

and 0.2 ml. of physiological saline substituted for the complement. A red cell control was made by adding 0.2 ml. of the two percent cell suspension to 0.8 ml. of physiological saline. Four complement control tubes were prepared as follows: Two-tenths ml. of two units of antigen used in the titration was added to each of the four tubes. Five-hundredths, 0.1, 0.15, and 0.2 ml. each of the two-unit complement dilution was added to each tube respectively. Finally saline was added to bring the total volume to one ml. in each of the tubes.

All tubes were incubated for one hour in a 37° C. water bath. The sensitized cell system was made up 15 minutes prior to its use. It consisted of equal volumes of a two percent sheep cell suspension and two units of hemolysin, each contained in 0.2 ml., and were mixed in independent tubes and incubated at 37° C. Four-tenths ml. of the sensitized system was added to each tube of the diluted antigen. The tubes were read at the end of an additional thirty minute incubation period. The unit of antigen was taken as the highest dilution showing no hemolysis.

Antigen was also titrated in the presence of normal inactivated rabbit serum to determine if any non-specific activity was present. The normal rabbit serum was substituted for the heated antiserum and the test performed as was outlined in the preceding paragraphs. Table III gives the results obtained in the antigen titrations.

Table III

Antigen Titrations

Antigen	Antiserum	Dilutions								Controls (ml. complement)							
		Full strength	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1 .05	2 .1	3 .15	4 .20	5	6	7	8
Newcastle hamster brain	Newcastle allantoic	4+	4+	4+	4+	4+	4+	2+	-	4+	-	-	-	4+	-	-	4+
Normal hamster brain	Normal hamster brain	4+	4+	4+	4+	2+	-	-	-	4+	-	-	-	4+	-	2+	4+
Newcastle allantoic	Newcastle allantoic	4+	4+	4+	4+	4+	3+	2+	-	4+	-	-	-	4+	-	-	4+
Normal allantoic	Normal allantoic	4+	4+	4+	4+	-	-	-	-	4+	-	-	-	4+	-	-	4+
Newcastle hamster brain	Normal rabbit	3+	2+	-	-	-	-	-	-	4+	-	-	-	4+	-	-	4+
Normal hamster brain	Normal rabbit	-	-	-	-	-	-	-	-	4+	-	-	-	4+	-	-	4+
Newcastle allantoic	Normal rabbit	3+	3+	2+	-	-	-	-	-	4+	-	-	-	4+	-	-	4+
Normal allantoic	Normal rabbit	-	-	-	-	-	-	-	-	4+	-	-	-	4+	-	-	4+

4+ = Complete fixation - = Hemolysis
1, 2, 3, 4, = Complement controls 5 = Cell control
6 = Hemolytic control 7 = Serum control
8 = Hemolytic control

Serum Titrations

Two-fold dilutions of antiseru, ranging from full strength to 1:128, were prepared. Two-tenths ml. of the serum dilutions, 0.2 ml. each of antigen and complement, containing two units was added to a tube. A complete series of control tubes was made with each titration. The tubes were incubated at 37° C. for one hour in a water bath. Four-tenths ml. of the sensitized cells, previously incubated for fifteen minutes was added to each tube. All of the tubes were incubated for an additional 30 minutes and read. The final end point was taken at the highest dilution of serum showing no hemolysis.

Table IV

Titration of Sera

Antigen	Antiserum	Dilutions								Controls (ml. complement)							
		Full strength	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1 .05	2 .1	3 .15	4 .2	5	6	7	8
Newcastle allantoic	Newcastle hamster brain	4+	4+	4+	4+	-	-	-	-	4+	-	-	-	4+	-	-	4+
Newcastle hamster brain	Newcastle allantoic	4+	4+	4+	4+	4+	4+	3+	-	4+	-	-	-	4+	-	-	4+
Normal allantoic	Newcastle hamster brain	-	-	-	-	-	-	-	-	4+	-	-	-	4+	-	-	4+
Normal allantoic	Newcastle allantoic	4+	4+	4+	4+	4+	4+	4+	-	4+	-	-	-	4+	-	-	4+
Normal allantoic	Normal allantoic	4+	4+	-	-	-	-	-	-	4+	-	-	-	4+	-	-	4+
Normal hamster brain	Normal hamster brain	4+	4+	4+	4+	4+	4+	4+	-	4+	-	-	-	4+	-	-	4+
Newcastle allantoic	Normal hamster brain	-	-	-	-	-	-	-	-	4+	-	-	-	4+	-	-	4+
Normal hamster brain	Newcastle allantoic	-	-	-	-	-	-	-	-	4+	-	-	-	4+	-	-	4+

4+ = Complete fixation - = Hemolysis
1, 2, 3, 4 = Complement controls 5 = Cell control
6 = Hemolytic control 7 = Serum control
8 = Hemolytic control

RESULTS

Hemagglutination tests on Newcastle disease infected allantoic fluid (Table I), showed that a high titer virus could be obtained by egg passages. Hamster infected brain material did not produce comparable hemagglutination titers. However, the titer of the hamster infected brain material was sufficiently high to warrant its use in the complement fixation tests. The hemagglutination titers were of value in screening antigens. Hemagglutination-inhibition tests were also found to be useful in screening antisera. The materials exhibiting the highest titers were used in the complement fixation tests.

In the complement fixation test it was necessary to titrate complement to find the two-unit dilution that was used in the test. Too much or too little complement will affect the sensitivity of the complement fixation titrations. Complement was titrated in the presence of antigen to discover any anticomplementary activity. None of the antigens used in the study exhibited any anticomplementary action, when two units of antigen were used in performing the titrations. Full strength normal allantoic antigen showed anticomplementary action, however, at a dilution of 1:2 this activity was not demonstrated. All the antigens, with the exception of Newcastle disease infected hamster brain, produced an increase in the complement activity by one dilution tube. Newcastle disease infected hamster brain material did not affect the complement titration (Table II).

Four complement control tubes were set up in all antigen and serum titrations. These controls were used to indicate if too much or too little complement was used in the test. They also served as an indicator of anti-complementary activity. Hemolysis was absent in the dilution containing 0.05 ml. of complement if the concentration of complement used in the test was not too high. The second dilution, containing 0.1 ml. of complement was partially or completely hemolyzed, indicating that enough complement was used in the test. Finally, the dilutions containing 0.15 and 0.2 ml. of complement were completely hemolyzed. Since these complement control tubes contained antigen and no antiserum, it was possible to detect anticomplementary activity. Although two units of antigen were always used in the test proper, full strength hamster brain suspensions, both infected and normal, were used at various times, in the complement control tubes. At no time was the brain suspension found to possess any anticomplementary activity.

Antigen titrations (Table III) were used to establish antigenicity and also to determine the two unit dilution of antigen that was used in the tests. High titers of Newcastle disease infected hamster brain and Newcastle disease infected allantoic fluid were obtained, by their respective methods of preparation. Antigens were tested in the presence of inactivated normal rabbit serum. This was done as a control, since certain animal sera will show non-specific reactions. No fixation of any significance was found between normal rabbit serum and the antigens used throughout the work.

In the titration of the sera (Table IV), antigen and antiserum prepared

from the same animal species were not used in the same titration. For example, Newcastle disease hamster infected brain antiserum was titrated with Newcastle disease infected allantoic antigen. This was done to prevent organ specific reactions taking place. Both brain material and allantoic material are capable of eliciting an antibody response in rabbits. Since tests were made for the presence of Newcastle virus antibodies, other antigen-antibody reactions in vitro was to be avoided. Adequate controls were made to demonstrate the lack of non-specific reactions taking place in any of the titrations.

The complement fixing titers of Newcastle disease infected hamster brain and Newcastle disease infected allantoic fluid were markedly lower than the corresponding hemagglutination titers. Normal hamster brain was found to be a good antigen, while normal allantoic fluid was found to be a weak antigen. A high titer was obtained with one normal allantoic antigen and Newcastle disease infected allantoic antiserum. This difference in titer between normal allantoic antiserum and the Newcastle disease allantoic antiserum was probably due to the individual rabbits response to the allantoic fluids. A non-specific reaction could have also taken place.

Throughout the study it was difficult to obtain enough rabbits to obtain duplicate samples of antisera. Each animal's response to a particular antigen is varied. A comparison of titers between sera of two or more animals injected with the same antigen, would have been very useful in this type of work.

DISCUSSION & CONCLUSIONS

Learning the theory and technics of the complement fixation test was the main purpose of this study. Upon reviewing the literature on complement fixation reactions of the neurotropic viruses, difficulty with hamster brain antigens was anticipated. Much work has been published on the methods of extracting anticomplementary substances from normal and infected brains of different animal species, including the hamster. At no time was anticomplementary activity observed during the course of this experimental work. No special treatment of the brain material in any way was attempted. Perhaps Havens et al (1943) and other workers assumed that because other animal brain material (mice, guinea pigs, monkeys, and dogs) was found to be anticomplementary, hamster brain should also be anticomplementary. In the review of the literature, no data were found on the extent of anticomplementary activity of hamster brain materials. Since normal hamster brain material as well as the Newcastle disease infected brain suspension did not exhibit anticomplementary action, it appears that the NDV was not the agent responsible for the "disappearance" of anticomplementary activity. The results obtained indicate that by using hamster adapted neurotropic virus antigens, many of the problems now encountered in the diagnosis of the encephalitides might be overcome.

The rabbit was shown to be a good animal for the production of Newcastle virus antiserum. Normal rabbit serum did not exhibit any non-specific action with any of the antigens used. The complement fixation test is specific for the antibodies of NDV when tested with allantoic and hamster brain adapted NDV antigens.

The complement fixation test for the diagnosis of Newcastle disease is not practical and the hemagglutination-inhibition test now used, which is an easier test to use, does an excellent job in the laboratory diagnosis of this disease.

SUMMARY

1) It was found that Newcastle disease virus infected allantoic fluid and suspensions of infected hamster brain were good antigens for use in the complement fixation test. The rabbit was found to be a good experimental animal for the production of complement fixing Newcastle virus antiserum.

2) No non-specific action was shown by normal rabbit serum when used in the complement fixation test with Newcastle disease infected allantoic fluid, normal allantoic fluid, suspensions of Newcastle infected hamster brain, and normal hamster brain.

3) No anticomplementary activity was exhibited by normal or infected hamster brains.

BIBLIOGRAPHY

1) Doyle, T.M. 1927 A hitherto unrecorded disease of fowls due to a filter-passing virus. Jour. Comp. Path. & Ther. 40: 144-169.

2) Kraneveld, F.D. 1926 Over een in Ned.-Indië heerschende Ziete onder het Pluimvee. Nederlandsch-Indische Bladen voor Diergeneeskunde; Deel 38, Alf. 5: 448-450.

3) Beach, J.R. 1942 Avian Pneumoencephalitis. Proc. 46th Annual U.S. Livestock Sanitary Assoc., 203-223.

4) Stover, D.E. 1942 A respiratory-nervous disorder of chickens. Amer. Jour. Vet. Res., 3: 207-213.

5) Burnet, F.M. 1942 The affinity of Newcastle disease virus to the influenza virus group. Austr. Jour. Exptl. Bio. & Med., 20 (2): 81-88.

6) Cunha, R., Weil, M., & Beard, D. 1947 Purification and characters of the Newcastle disease virus (California strain). Jour. Immun., 55 (1): 69-87.

7) Bang, F.B. 1948 Studies on Newcastle disease virus. Jour. Exptl. Med., 88 (2): 233-240.

8) Reagan, R. 1947 Response of monkeys to poliomyelitis after injection with Newcastle disease virus. Proc. 51st Annual Livestock Sanitary Assoc., 55-62.

9) Wenner, H.A. and Lash, B. 1949 Choriomeningitis encephalitis following inoculation of Newcastle disease virus in Rhesus monkeys. Proc. Soc. Exptl. Bio. & Med., 70: 263-265.

10) Yatom, J. 1946 Conjunctivitis caused by virus of Newcastle disease. Jour. Am. Med. Assoc., 132 (8): 169 (in foreign news letters).

11) Ingalls, W.L. & Mahoney, A. 1949 Isolation of the virus of Newcastle disease from human beings. Amer. Jour. Pub. Health., 39 (6): 737-740.

12) Howitt, Beatrice, Bishop, L.K., & Kissling, R.E. 1948 Presence of neutralizing antibodies of Newcastle disease virus in human sera. Amer. Jour. Pub. Health., 38 (9): 1263-1272.

13) Howitt, B. 1937 Complement fixation reaction in experimental equine encephalomyelitis, lymphocytic choriomeningitis & St. Louis encephalomyelitis. Jour. Immun., 32: 235-250.

14) Casals, J. & Palacios, R. 1941 Complement fixation test in the diagnosis of virus infections of the central nervous system. Jour. Exptl. Med., 74: 409-426.

15) Havens, W.P., Watson, D.W., Green, R.H., Lavin, G.I., & Smadel, J.E. 1943 Complement fixation with the neurotropic viruses. Jour. Exptl. Med., 77: 139-153.

16) Casals, J. 1942 Inactivated antigens for complement fixation tests with central nervous system virus infection. Proc. Soc. Exptl. Bio. & Med., 49: 501.

17) Casals, J. 1945 Heated avirulent antigens for complement fixation tests with certain encephalitis viruses. Science., 102: 618.

18) DeBoer, C.J., & Cox, H.R. 1946 Specific complement fixing diagnostic antigens for neurotropic virus diseases. Jour. Bacty., 51: 613.

19) Wolfe, Van der Scheer, Clancy & Cox 1946 A method for the preparation of complement fixing antigens in a study of experimental Tsutsupamshi disease (scrub typhus). Jour. Bacty., 51: 247-253.

20) Espana, C. & Hammon, W. McD. 1948 An improved benzene extracted complement fixing antigen applied to the diagnosis of the arthropod-borne virus encephalitides. Jour. Immun., 59: 31-43.

21) Casals, J. 1949 Acetone-ether extracted antigens for complement fixation with certain neurotropic viruses. Proc. Soc. Exptl. Bio. & Med., 70 (2): 339-343.

22) Reagan, R., Reginold, L., Lillie, M., Hauser, J., & Brueckner, A. 1948 Response of the Syrian hamster to the virus of Newcastle disease. Proc. Soc. Exptl. Bio. & Med., 68 (2): 293-295.

23) Reagan, R., Lillie, M., Smith, D., & Brueckner, A. 1949 Comparison of Newcastle disease virus in hamsters exposed by intracerebral injection and intranasal instillation. Proc. Soc. Exptl. Bio. & Med., 71 (2): 293-294.

24) Lenette, E. 1941 Susceptibility of Syrian hamsters to viruses of St. Louis and Jap. B. encephalitis. Proc. Soc. Exptl. Bio. & Med., 47: 178-181.

25) Sanders, F.K. 1948 A poliomyelitis-like agent in hamsters inoculated with Lansing virus. Fed. Proc., 7 (1): 309 (abstract).

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