ATTEMPTS TO ADAPT AVIAN ENCEPHALOMYELITIS VIRUS TO SUCKLING MICE WITH PRELIMINARY OBSERVATIONS ON SERODIAGNOSTIC METHODS

by

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INTRODUCTION

Avian encephalomyelitis (AE) was first observed by Jones (23) in 1932 and reported as "an encephalomyelitis in the chicken", and later she (24) suggested the term "epidemic tremor". In 1936 the name "avian encephalomyelitis" was adopted because many birds failed to manifest tremors (43, 55, 61). AE has become quite prevalent within the past ten years and has assumed considerable economic importance, especially within the broiler and egg producing industries.

Jones (23), Van Roekel et al. (62), and Olitsky and Bauer (43) have shown the etiological agent of AE to be an infectious, filterable virus in the range of 20 to 30 millimicrons in diameter.

Natural outbreaks of the disease have been reported only in chickens and pheasants (34), but turkeys, pigeons, guinea fowl, and ducklings have been infected experimentally (27).

Eradication and control of AE is hindered by lack of a readily available method of diagnosis. At the present time there are three methods of diagnosis: 1. a typical history, with the absence of gross lesions but microscopic changes of degeneration, inflammation, and proliferation in the central nervous system; 2. reproduction of the disease syndrome in baby chickens or chicken embryos; and, 3. serum neutralization tests. All three diagnostic procedures are expensive, time consuming, and are not practical for use in routine diagnosis.

It has been thought that antigen and homologous chicken antibodies will not consistently fix complement, and therefore, give false negative results (50). Brumfield and Pomeroy (7) have modified the direct complement fixation (DCF) test to permit the detection of infectious bronchitis, Newcastle disease, and ornithosis antibodies in chicken serum. Benedict and O'Brien (3) reported

a modification of the passive hemagglutination (PHA) test for the detection of antibodies of ornithosis in turkey serum.

The development of a rapid and accurate method of diagnosing AE would greatly facilitate determination of the incidence of the disease, evaluation of vaccination, and eradication. In an effort to utilize a serological test, efforts were made to adapt the AE virus to the common laboratory mouse. It was postulated that antigens or antibodies suitable for the complement fixation test could be produced by this procedure. The serum neutralization (SN) test could be employed with greater accuracy because the mice would not be susceptible to intercurrent infections and the possibility of specific parental antibodies interferring with the results would be eliminated. The DCF test, using chicken brain tissue antigens and homologous or turkey antibodies, and the PHA test were used in an effort to find a serological test for the diagnosis of AE.

REVIEW OF THE LITERATURE

History

Jones (23), in 1932, described a condition which she called "an encephalomyelitis in the chicken". In a later paper (24), she used the term, "epidemic tremor". In 1938, Van Roekel et al. (61) suggested that the condition be called "infectious avian encephalomyelitis" because many of the birds failed to develop any tremors. In 1939, a special American Veterinary Medical Association committee on poultry disease nomenclature (55) adopted the binomial form "avian encephalomyelitis". During the first few years, AE was reported only in the eastern states but by 1955 the condition had been reported in 43 states, Canada, and Australia (45).

Characteristics of the Virus

The etiological agent of AE is tentatively classified as a member of the Polio group because of its neurotropic character and other resistance (9, 27). The disease is thought to be noncontagious as direct contact, feeding a suspension of infective material or management had no marked effect on the contraction of the disease (23, 27). However, a limited number of reports seems to indicate that direct contact may be a factor in the spread of the disease (27, 34, 52, 62). The naturally occurring disease appears to be mainly egg transmitted (31, 58, 59, 60).

For experimental infection, intracranial inoculation gives the most constant results, but infection following intravenous, intraperitoneal, subcutaneous, intramuscular, intranasal, intraocular, and wing web inoculations has been reported (17, 27, 52). Jones (23), Van Roekel et al. (61), Olitsky (42), and Feibel et al. (17) have shown that the etiological agent will pass through the Berkefeld filter N and Seitz 1 and 2 disc filters, but is retained by the Berkefeld filter W. Olitsky and Bauer (43), employing a gradocol membrane, determined that the particle size was 20 to 30 millimicrons. This is within the range of the etiological agents of the other virus encephalitides such as St. Louis encephalitis and equine encephalomyelitis, eastern and western strains.

The AE virus has remained infective for chickens after incubation in 50 percent glycerine at room temperature for 69 days (23), in a frozen state for 438 days (17), or at 4°C. for 836 days (44). Initial isolation of the virus from naturally infected flocks has proved very difficult (28, 59). Upon experimental repassages, the percent of infected birds increases and the incubation period decreases. The infective titer in chickens varies from 10^{-4} to 10^{-7}

(27, 42, 44, 51). Vitamin deficient diets, simultaneous intraperitoneal and intracranial injections, or adjuvants such as protamine zinc insulin, five percent soluble starch, Higgins india ink, and ten percent fresh testicular extract have been used with little success in an attempt to increase the virulence (27).

The virus of AE could not be serially propagated in chicken embryos until 1956. Then Jungherr et al. (28) reported the adaptation of the virus by intraocular injections, and Willis and Moulthorp (63) reported the adaptation by injections into the yolk sac. Natural infections have been reported in young chickens and pheasants (23, 27, 34). Milder infections have been observed in adult chickens (58, 59, 60). The number of susceptible experimental hosts is limited to chickens, turkeys, ducklings, pigeons, guinea fowl, and young pheasants (27, 34, 62). White mice, guinea pigs, rabbits, mature pheasants, and sparrows are refractory (27, 42, 62). Tissue cultures have not been efficient enough to yield large quantities of potent virus (31).

Immunological Studies

Olitsky (42) first reported the presence of antibodies in the serum of infected chickens. The presence of neutralizing antibodies in the serum of convalescent birds, both experimentally and naturally infected, has been reported by Jungherr and Minard (27). With the advent of the adaptation of the virus to the chicken embryo, SN tests can be conducted in only nine days, while a similar test in day old chickens required 14 to 28 days. The difficulty with the SN chicken embryo method has been in obtaining embryos that are uniformly susceptible. This variation in susceptibility is probably due to parental immunity, developed from mild infections during hatching season, natural exposure as young chickens, or vaccination (58, 59).

Agglutination. Bacterial agglutination tests (27) and hemagglutination tests in the cold (17) have been unsatisfactory for the diagnosis of AE. Boyden (5) and Stavitsky (56, 57) described a PHA test for the detection of purified protein antigens and specific antibodies. Benedict and O'Brien (3) attempted to adapt this procedure to the diagnosis of psittacosis, but encountered difficulties arising from nonspecific hemagglutination.

Complement Fixation. Complement apparently does not unite with antigen and specific chicken antibody combinations. Rice (50) postulated that in this combination there is no room for complement due to the small size of the aggregate. Bushnell and Hudson (10) suggested that the inability of the antigen-antibody combination to fix complement might be due to a heat labile factor. Consequently the DCF, as such, has been of little value in the diagnosis of chicken diseases.

A modification of the DCF test, called complement fixation inhibition or indirect complement fixation (ICF), was adapted to test serum not reacting to direct complement fixation (50). This method is time consuming, and obtaining the quantities of known complement fixing antiserum necessary for the test is difficult.

The DCF test was modified by Benedict and McFarland (4) to detect the presence of the antibodies of ornithosis in turkey serum. Neal and Davis (38) compared the DCF and ICF tests and found 85 percent agreement. Brumfield and Pomeroy (7) later modified the DCF test to permit the detection of infectious bronchitis, Newcastle disease, and ornithosis antibodies in chicken serum.

The application of a DCF test to the study of neurotropic viruses has been complicated by the fact that frequently infected nervous tissue is the only source of the virus, and antigens prepared from such tissues are often anticomplementary. Such antigens can only be used when they are diluted or

some of their components have been removed (13). Casals and Palacios (13) suggest the addition of two percent, normal rabbit serum (NRS) to an infected brain suspension, followed by alternate freezing and thawing until a white precipitate is formed. After centrifugation, the supernatant has lost most of its anticomplementary properties. An alcohol, ether extract of an infected brain suspension has been suggested as a method of removing anticomplementary components (20, 21).

An antigen extracted with alcohol and water was employed by Jungherr and Minard (27). This antigen added to convalescent chicken serum failed to fix complement. Rabbit immune serum could not be used as a source of antibodies as it was impossible to remove the basic brain antibodies by absorption.

Immunity

Schaaf and Lamoreux (52) have postulated that infection either by natural or experimental methods will result in the production of progeny that are less susceptible to AE than the progeny of unexposed hens. They, therefore, recommend the vaccination of young pullets prior to the beginning of production or of older hens which are in molt.

In a study of 59 flocks, some with history of AE, it was discovered that only four produced chicken embryos which permitted uniform virus growth to titers of 10^{-5}. Virus neutralizing substances were demonstrated in adult serum, serum of two day old chickens, and in the yolks of embryos which gave virus titers of $10^{-1.8}$ or lower (58, 59).

Signs

Jones (24) characterized AE as a fine tremor of the head and neck, which may extend over the entire body. Ataxia is concurrent with or subsequent to

the tremors, only rarely preceding them. Van Roekel et al. (61) contend that the ataxia is the most prominent sign and that tremors occur less frequently. A summary of signs follows: 1. a dull expression in the eyes and a weak cheep; 2. ataxia or incoordination of the leg muscles; and, 3. perhaps a tremor of the head and neck. Tremors very rarely precede the ataxia. The ataxia may progress to paralysis of the legs and death may result from starvation. As long as the birds can get to the food and water, they will continue to eat, but seldom develop into profitable birds. Occasionally the course of the disease may be so rapid that a bird appears normal one day and the next is somnolent, comatose, or dead. Jungherr and Minard (27) have used the terms "clonic" type and "ataxic or paralytic" type to describe the signs of the disease.

In adult birds there is usually only a temporary decrease in egg production and hatchability with an occurrence of AE in the progeny (45, 52, 58, 59, 60). As the birds resume normal production, the incidence of AE in the progeny decreases and the suitability of the chick embryos for propagation of the virus is greatly reduced (59, 60).

Pathology

AE in experimental or natural infections causes no gross lesions. Microscopic changes are quite evident in the brain stem, medulla, and the anterior horn of the spinal cord, especially in the lumbo-sacral region (45). The extent of pathological changes depends upon the acuteness and duration of the disease. A swelling of the neuron and its nucleus is the first microscopic evidence of infection. This is followed by displacement of the nucleus and a clearing of the Nissl bodies. The cytoplasm becomes progressively eosinophilic until it appears as a pinkish red mass, or disappears completely (42). Perivascular cuffing may be present (24, 26, 34). Microscopic lesions in the

ventriculus, proventriculus, pancreas, heart, striated muscle, spleen, liver, and kidney have been reported (24, 27, 42). These lesions consist of small hyperplastic lymphoid islands composed of lymphocytes with a few monocytes, myelocytes, and some cellular debris. Feibel et al. (17) reported that intraocular inoculation causes the development of an opacity of the lens. Peckham (46) and Bridges and Flowers (6) have reported the occurrence of lens opacities in flocks that had been affected by AE three to four months previously.

To diagnose AE, differentiation must be made from gout, avitaminosis A, avitaminosis E, riboflavin deficiency, perosis, neural lymphomatosis, equine encephalomyelitis, Newcastle disease, pleuropneumonia-like organism (PPLO) encephalitis[1], bacterial and mycotic osteomyelitis, botulism, arsenical poisoning, nitrophenide poisoning, lead poisoning, DDT poisoning and nitrofurazone poisoning (14, 15, 25, 26, 35, 36, 41, 44, 45). Diagnosis is most often accomplished by the absence of gross lesions, microscopic examination of the spinal cord, especially of the lumbo-sacral region, hemagglutination and hemagglutination inhibition tests for Newcastle disease, SN and animal inoculation for equine encephalomyelitis, culture for PPLO, bacteria and molds.

MATERIALS AND METHODS

Two strains of virus were obtained from Ostendorf (45) labeled, first passage from the original received from Dr. M. C. Morrissette (Van Roekel strain, VR) and original material received from Dr. Kermit Schaaf, Kimber Farms Inc., (KF strain), Niles, California. Each suspension of this material was inoculated intracranially into ten birds obtained from a local hatchery. When the birds were moribund, the brains were removed asceptically and placed in

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shaker bottles (17) according to strain. This formed the pool of infective material from which most of the experiments were initiated. The KF strain of virus was not used in this study due to a shortage of experimental animals.

Preparations of Suspensions

A technique similar to that described by Feibel et al. (17) and Ostenderf (45) was used. Sterile prescription bottles or screw top culture tubes containing broken glass were used as receptacles for the brain material and sufficient buffered saline was added to make a 30 percent suspension. This mixture was shaken manually until the brain material was sufficiently triturated to pass through a 27 gauge needle. During the trituration period, the material was frozen and thawed several times to facilitate breaking down the nerve cells (45). When sufficiently triturated, the material was stored in a mechanical freezer at -20°C. Prior to inoculation, the material was thawed and diluted, with buffered saline or adjuvant, to make a 10^{-1} suspension.

Buffered saline was prepared as follows: (19)

Solution I.

Sodium Chloride	50.0 Gms.
Monobasic Potassium phosphate (KH_2PO_4)	5.53 Gms.
Dibasic sodium phosphate (Na_2HPO_4 plus 12 H_2O)	14.31 Gms.
Distilled water	to make 1000 cc.

Solution II.

Four percent carbolic acid

For use.

One part of solution I and one part of solution II are added to eight parts of distilled water. The pH of 7.0 remains constant when sterilized by boiling.

A commercial starch suspension was prepared by adding ten grams of commercial starch (Argo plain) to ten cubic centimeters of buffered saline in a shaker bottle. This was boiled in a hot water bath until a paste was formed, then sterilized at 15 pounds of pressure for thirty minutes. One hundred

twenty four cubic centimeters of sterile, buffered saline was then added, making a dilution of 7.46 percent. This mixture was triturated until it would pass through a 27 gauge needle, then stored in a mechanical refrigerator at -20°C. For inoculation, two cubic centimeters of this suspension were mixed with one cubic centimeter of the stock brain suspension, giving a final dilution of approximately five percent commercial starch suspension and 10 percent brain suspension.

A 7.46 percent soluble starch solution was prepared by adding ten grams of soluble starch (Difco) to 134 cubic centimeters of buffered saline and sterilizing the solution at 15 pounds of pressure for thirty minutes. This solution was stored in a mechanical refrigerator at -20°C. For inoculation, two cubic centimeters of this solution were mixed with one cubic centimeter of the stock brain suspension, giving a final dilution of approximately five percent soluble starch solution and 10 percent brain suspension.

All stock suspensions were checked for bacterial contamination by inoculating blood agar plates and thioglycollate broth tubes at the time of preparation and again after removal of a quantity for dilution. All diluted suspensions were checked prior to inoculation.

Source of Experimental Animals

The chickens used in these experiments were obtained from two sources. Most of the birds, cockerels of a leghorn cross, were obtained from a local hatchery. White leghorn cockerels were obtained from Kansas State College Poultry Farm. All chickens were identified by numbered wing bands.

Adult cockerels, which had been used as uninoculated controls in previous experiments, were used to produce hyperimmune serum.

A colony of white mice maintained by the Department of Pathology was

used to produce young mice for the adaptation studies.

Method of Inoculation

Stock brain material was removed from the freezer and diluted with buffered saline or an adjuvant (Table 1). A one quarter to one cubic centimeter capacity tuberculin syringe fitted with a 3/4 inch, 26 or 27 gauge needle, was used for inoculation of 0.03 to 0.05 cubic centimeter of brain material into young birds. A one inch, 22 gauge needle was used for birds over eight weeks of age. Mortality from intracranial inoculation was very low, with most young chickens, two to 14 days of age tolerating up to 0.1 cubic centimeter without permanent damage. In the beginning of this study, two series of controls were maintained. One series was inoculated intracranially with 0.03 to 0.05 cubic centimeter of buffered saline and the other series was uninoculated. The former were eliminated when it was determined that the buffered saline produced no microscopic lesions. The inoculated and control birds were maintained in isolation.

Adults used for the production of hyperimmune serum were inoculated intraperitoneally with a 10^{-1} infective brain suspension which had been centrifuged at 2000 RPM for 30 minutes in an International Horizontal type centrifuge. The initial dose was 0.5 cubic centimeter. The birds were reinoculated with 1.0 cubic centimeter every 10 to 14 days for the next two months and seven days prior to all bleedings.

In the studies on adaptation of the virus to mice, serial mouse passages (i.e. mouse to mouse) or zig zag passages (i.e. normal to abnormal to normal host) were used (Table 1). Litters consisting of five or more two to four day old mice were used in this study. All pregnant females were placed in separate cages until the young were born. To conserve on space and equipment,

Table 1. Mouse adaptation studies.

Series number : Potent : Virus	: Control	: Adjuvant	: Number : mouse : passages	: Chicken sub- : passage made : after series	: Remarks
1	2	cortisone acetate	4	4	75 micrograms, injected IM 12 hours prior to and 12 hours after virus inoculation.
3	4	5% commercial starch	12	4, 5, 6, 11	adjuvant mixed with the brain suspension prior to inoculation.
5	6	5% soluble starch	6	4, 5, 6	adjuvant mixed with the brain suspension prior to inoculation.
7	8	10% soluble starch	3	1	adjuvant mixed with the brain suspension prior to inoculation.
9	10	5% commercial starch	3	3	adjuvant mixed with the brain suspension prior to inoculation. IP injection of 0.5 c.c. of brain suspension, centrifuged at 2000 RPM for 30 min.
11	12	5% soluble starch	4	zig zag passages	adjuvant mixed with the brain suspension prior to inoculation
13	14	5% commercial starch	3	1	5% starch suspension, IC left side of brain, 12 hrs. before inoculation of brain suspension in right side of brain. 48 hrs. later, brain suspension was inoculated in left side of brain.
15	16	5% commercial starch	7	zig zag passages	same as above except that zig zag pattern was added.

IM – intramuscular IP – intraperitoneal IC – intracranial

mice of the same age were grouped in the same series and passage at time of inoculation. Controls of the first passage of each series were inoculated with buffered saline plus adjuvant. Thereafter, all controls were inoculated with a normal brain tissue suspension plus adjuvant. When young mice were removed from the mother, she was marked, and returned to the colony. This was done to eliminate the possibility of parental immunity interferring with the results.

The method used for inoculating mice was similar to that used for chickens. The two to four day old mice could not tolerate a dose in excess of 0.05 cubic centimeter and at 0.03 cubic centimeter most mice developed an immediate shock reaction, but rapidly recovered. The over-all mortality of inoculated mice was 15 percent.

Serological Procedures

Preparation of Antigens. For details concerning variations in the preparation of the antigens see Table 2. Antigens one to five inclusive were extracted by the method described by Benedict and O'Brien (2). A one percent sodium lauryl sulfate solution was added to the infective brain suspension and incubated at room temperature for 24 hours. Barium chloride was added to precipitate the lauryl sulfate, which was then removed by centrifugation.

Antigen six was prepared by the method described by Casals and Palacios (13), except that two percent normal chicken serum (NCS) was used instead of two percent NRS. The brain suspension was frozen in a dry ice chest, then thawed in cold running water. This was repeated seven times, until a white precipitate was formed and centrifuged from the suspension. One half of the supernatant was filtered through number 1, Whatman white, filter paper, in an effort to remove all traces of the precipitate. Both filtered and unfiltered portions were stored at -20°C.

Table 2. Antigens for complement fixation.

Antigen	Method of Preparation	Original Suspension	Antigen Variation
1a	Benedict & O'Brien (2)	10^{-1}, centrifuged 2000 RPM's for 30 minutes	centrifuged only
1b	" "	" "	centrifuged and inactivated at 56°C. for one hour
1c	" "	" "	centrifuged, add 5% normal chick serum (NCS), used immediately
1d	" "	" "	centrifuged, add 5% NCS, incubate at room temperature for 20 minutes
1e	" "	" "	centrifuged, add 5% NCS, incubate at 37°C. for 30 minutes
1f	" "	" "	centrifuged, add 5% NCS, incubate at 56°C. for one hour
1g	" "	" "	centrifuged, dialized in physiological saline for 72 hours
2a	" "	sediment from antigen 1	centrifuged only
2b	" "	" "	centrifuged, add 5% NCS, used immediately
2c	" "	" "	centrifuged, add 5% NCS, incubate at 37°C. for 30 minutes
2d	" "	" "	centrifuged, add 5% NCS, incubate at 56°C. for one hour
3a	" "	10^{-1}, not centrifuged	centrifuged only
3b	" "	" "	centrifuged, add 5% NCS, incubate at 37°C. for 30 minutes

Table 2. (Con't.)

Antigen	Method of Preparation	Original Suspension	Antigen Variation
3c	Benedict & O'Brien (2)	10^{-1}, not centrifuged	centrifuged, add 5% NCS, incubate at 56°C. for one hour
4a	" "	30%, centrifuged at 2000 RPM's for 30 min.	centrifuged only
4b	" "	" "	centrifuged and inactivated at 56°C. for one hour
4c	" "	" "	centrifuged, add 5% NCS, incubate at room temperature for 20 minutes
4d	" "	" "	centrifuged, add 5% NCS, incubate at 37°C. for 30 minutes
4e	" "	" "	centrifuged, add 5% NCS, incubate at 56°C. for one hour
5a	" "	30%, not centrifuged	centrifuged only
6a	Casals & Palacios (13)	10^{-1}, centrifuged at 2000 RPM's for 30 min.	centrifuged only
6b	" "	" "	centrifuged, filtered through filter paper
6c	" "	" "	centrifuged, add 5% NCS, incubate at 37°C. for 30 minutes
6d	" "	" "	centrifuged, filtered, add 5% NCS, incubate at 37°C. for 30 minutes
7a	_____	10^{-1}, centrifuged at 3000 RPM's for one hr.	filtered through sintered glass filter
8a	Nigg, Millman, and Bowser (40)	10^{-1}, centrifuged at 2000 RPM's for 30 min.	boiled in water bath

Table 2. (Concl.)

Antigen	Method of Preparation	Original Suspension	Antigen Variation
9a	Hillman & Nigg (20)	10^{-1}, centrifuged at 2000 RPM's for 30 min.	diluted five times in boiling water
10a	Durmester & Gentry (8)	10^{-1}, centrifuged at 2000 RPM's for 30 min.	centrifuged only
10b	" "	" "	centrifuged and filtered through sintered glass filter

Antigen seven was a 10^{-1} infective brain suspension, centrifuged at 2000 RPM for 30 minutes and filtered through a sintered glass filter under 12 pounds of vacuum.

As suggested by Nigg et al. (40), antigen eight was a 10^{-1} suspension, held in a frozen state (-20°C.) for two to three months. Prior to use the suspension was boiled in flowing steam for thirty minutes and centrifuged at 2000 RPM for 30 minutes.

For antigen nine, a 10^{-1} infective brain suspension was centrifuged at 2000 RPM for 30 minutes. To one volume of the supernatant, five volumes of anesthetic ether was added, agitated, and incubated at 4°C. for 24 hours. This ether was removed, fresh ether added, and the incubation period repeated. The ether of both washings was combined and evaporated in a water bath at 56°C. to one volume. Five volumes of boiling saline were added to the concentrate and the antigen was stored at -20°C. (20, 21).

Antigen 10 was a 10^{-1} suspension, centrifuged at 2000 RPM for 30 minutes. Ninety-two volumes of supernatant were added to eight volumes of celite 512, thoroughly mixed, and filtered through number 1, Whatman white, filter paper.

Ninety-seven volumes of this filtrate were added to three volumes of celite 512, mixed thoroughly, and refiltered. This filtrate was filtered through a sintered glass filter, then stored at -20°C. (8).

Antigen 11 was a 10^{-1} suspension of infective brain material, centrifuged at 2000 RPM for 30 minutes, and inactivated at 56°C. for one hour.

Antibodies. Antiserums used were obtained from: 1. chickens repeatedly inoculated with infective AE suspensions; 2, experimentally infected chickens and turkeys bled in a moribund state; 3. naturally infected chickens bled in a moribund state; and 4. chickens believed to be free of AE. The blood was drawn and allowed to clot for not more than two hours. The clot was rimmed and centrifuged at 2000 RPM for 60 minutes. The serum was removed immediately and stored at -20°C. The clot was placed in the refrigerator and allowed to shrink for 24 hours, then the remaining serum was removed and also stored at -20°C.

Direct Complement Fixation Test. Complement for the DCF tests was obtained by bleeding adult guinea pigs which had been starved for 24 hours and collecting the serum. Hemolysin was obtained from the Department of Pathology. Sheep red blood cells were collected in Alsever's solution from sheep maintained by the Department of Pathology for this purpose.

Antigens one through 10, described under Preparation of Antigens were used. See Table 2 for details. Source of antiserum was described under Antibodies.

The techniques described below are those recommended by the Committee on a Standardized Complement Fixation Test for Anaplasmosis of the United States Livestock Sanitary Association (USLSA), (49) and modified according to the procedures of Brumfield and Pomeroy (7), Benedict and McFarland (4) and Neal and Davis (38).

Complement was titered both with and without the addition of normal chicken sera, and the exact unit of complement necessary for both groups was determined. Hemolysin was then titered according to the method recommended by the USL3A (49). Antigen was titered (49) using two units of complement and antiserum from known infected or unexposed birds. All antigens were tested for anticomplementary properties. In conducting the test proper (49) two units of antigen, two units of complement, antiserum from known infected chickens, and antiserum from chickens free from AE were used.

Passive Hemagglutination Test. Antigens one, 10 and 11, described under Preparation of Antigens, were used. Source of antiserum was described under Antibodies.

Sheep RBCs were collected in Alsever's solution, washed in buffered saline, pH 7.2, and centrifuged at 2000 RPM for ten minutes. This washing was repeated three times. The cells were then resuspended at 2.5 percent concentration in buffered saline at pH 7.2. One volume of a 1:20,000 dilution of tannic acid in buffered saline, pH 7.2, was added to one volume of diluted sheep RBCs and the mixture incubated at 37°C. for 10 minutes. After centrifugation at 2000 RPM for 10 minutes, the tanned RBCs were washed in buffered saline, pH 7.2, and diluted to a 2.5 percent concentration in buffered saline, pH 6.4. To one volume of 2.5 percent tanned cells, four volumes of antigen were added. This mixture was incubated at room temperature for 15 minutes and centrifuged at 2000 RPM for 30 minutes. The sensitized RBCs were washed and resuspended in saline containing 1:100 NRS or NCS to the original volume. All reagents were washed once with unsensitized sheep RBCs. The technique for the hemagglutination test is that described by Stavitsky (56). The test serum was serially diluted twofold until the highest dilution desired was obtained. One half cubic centimeter of each serial dilution was added to

one half cubic centimeter of sensitized RBCs and incubated at room temperature for three hours prior to reading.

Controls used in the PHA test were: 1. sensitized cells and negative serum; 2. sensitized cells and known positive serum; 3. tanned RBCs treated with saline in place of antigen and test serum; and, 4. unsensitized RBCs in 1:100 NRS or NCS and test serum.

Tissue Preparation

Spinal cords of all chickens and mice used for the adaptation studies and half of the brains of a representative number were identified and fixed in a 10 percent buffered formalin solution. Other tissues were collected when indicated. After a minimum fixation period of 24 hours, each tissue was prepared for histopathological studies. The spinal cord was sliced transversely through the lumbar region anterior to the lumbo-sacral sinus (45). Sections from various levels of the cord were collected for study. The spinal cords of mice of the same series and subpassage were collected for sectioning. Tissues were dehydrated in alcohol, cleared in xylol, embedded in paraffin and sectioned three to six microns in thickness. They were stained with hematoxylin and eosin (33) for microscopic examination.

Titration of the Virus

Chickens, two to three months of age, were inoculated intracranially with 0.03 cubic centimeter of the VR strain of virus suspension. The virus suspension was serially diluted tenfold, beginning at 10^{-1}, and continued until the highest dilution desired (10^{-15}) was reached. See Table 3 for details.

Table 3. Titration.

Test No.	Highest Dilution	Source of Chickens	Number of Chickens
1	10^{-6}	Local hatchery	70
2	10^{-10}	K.S.C. poultry farm	110
3	10^{-9}	K.S.C. poultry farm	36
4	10^{-15}	Local hatchery	51

RESULTS

Mouse Adaptation Studies

Series in which starch was used as an adjuvant, a small creamy white, necrotic foci involving the meninges and underlying cerebral cortex was observed in the cerebral hemisphere of most mice at the site of injection. This reaction was found in both the controls and in mice inoculated with virus material. No signs of encephalomyelitis appeared to be associated with this occurrence.

Details of series one, seven, nine, 11 and 13 are found in Table 4, details of series three in Table 5, details of series five in Table 6, and details of series 15 in Table 7.

Serology

Complement Fixation. All of the antigens showed some instability i.e. gave false positive or negative results to DCF tests. The same serum would give both positive and negative results. The results of antigen titration and DCF tests are found in Table 8.

Table 4. Results of series 1, 7, 9, 11, and 13.

Number	Signs of E.	Histopathological evidence of E.	Remarks
1	none	none	discontinued due to continued contamination.
7	none	none	discontinued due to limited supply of mice.
9	none	none	passed only three times, with long intervals between passages.
11	none	none	discontinued when no evidence of E. was found after the fourth chicken passage (zig zag method).
13	none	none	discontinued due to limited supply of mice.

E. – encephalomyelitis

Table 5. Results of series 3.

Serial Number	Subpassage	Signs of AE	Histopathological Evidence of AE	Remarks
1,2,3	all	none	none	
4	a,b,d,e	none	none	
4	c	4/13 hs	slight perivascular cuffing	initiated series 5, a-i and 4 mouse to chick passages from 4c.
5	a,b,d	13/37 icn, ppar	perivascular cuffing, edema, early stage neuronal degeneration	initiated 3 mouse to chick passages. from 5a,b,d.
5	c,e,f, g,h,i	none	none	e,f,g,h,i inoculated 6-14 weeks after a,b, c,d.
6	a	5/17 slight icn, hs	slight hyperemia	from serial passage 5a,b,d.
6	b,c	4/11 slight hs	none	inoculated 10 days after 6a.
6	d	none	none	from serial passage 5a,b,d.
6	e,f,g	none	none	from serial passage 5a,b,d 6-14 weeks after 6a.
7-12	all	none	none	

hs – hypersensitivity icn – incoordination ppar – posterior paralysis

Table 6. Results of series 5.

Serial Number	Subpassage	Signs of AE	Histopathological Evidence of AE	Remarks
1,2	all	none	none	
3	a	2/10 slight ien, tr	slight perivascular cuffing	initiated 4a,b,c from 3a.
3	b,c	none	none	inoculated 2–4 weeks after 3a.
4	a,b	8/11 slight hs	none	
4	c,d	2/18 brief hs	none	inoculated 3–4 weeks after 4a,b.
5	a	6/12 slight hs	none	10 day delay in repassage from 4a,b.
5	b,c	none	none	2½ month delay in repassage from 5a.
6	all	none	none	no further passages due to a shortage of mice.

ien – incoordination tr – tremors hs – hypersensitivity

Table 7. Results of series 15.

Source of chicken passage	Zig zag passages	Chicken passages	Evidence of AE	Remarks
pooled material	3	3	none	killed 2 chickens after 14 days, 3 retained 45 days, then killed.
pooled material passed once through chickens	2	2	none	killed 2 chickens after 14 days, 3 retained 45 days, then killed.
pooled material passed twice through chickens	1	1	none	retained 45 days, then killed.
pooled material passed three times through chickens	1	1	none	control birds inoculated with known AE virus showed signs of AE at 5 days and death by 8 days.

Table 8. Antigen titration and direct complement fixation tests.

Antigen	Titration of Antigen	Results of the Test Proper
1a	NAC, 2/2 negative	not tested
1b	NAC, 1/4 positive	KPS, 3/3 negative KNS, 1/1 negative
1c	NAC, 2/6 positive	KPS, 2/4 positive KNS, 1/1 positive
1d	NAC, gave positive reactions to both positive and negative serum	not tested
1e	NAC, 1/3 positive	KPS, 2/2 negative KNS, 2/2 negative
1f	NAC, 2/2 negative	not tested
1g	NAC, gave positive reactions to both positive and negative serum	not tested
2a	AC	not tested
2b	AC	not tested
2c	AC	not tested
2d	AC	not tested
3a	NAC upon initial preparation but became AC after incubating several days at $-20^{\circ}C$.	not tested
3b	AC	not tested
3c	AC	not tested
4a	NAC upon initial preparation but became AC after incubating several days at $-20^{\circ}C$.	not tested
4b	AC	not tested
4c	AC	not tested

Table 8. (Concl.)

Antigen	Titration of Antigen	Results of the Test Proper
4d	AC	not tested
4e	AC	not tested
5a	AC	not tested
6a	NAC, 1/4 positive	KPS, 2/5 positive KNS, 2/3 positive
6b	NAC, 1/3 positive	KPS, 2/2 positive KNS, 2/2 positive
6c	AC	not tested
6d	AC	not tested
7a	NAC, 1/2 positive	not tested
8a	AC	not tested
9a	AC	not tested
10a	NAC, 1/4 positive 2/4 positive to both positive and negative serum	not tested
10b	NAC, 1/2 positive	KPS, 3/4 positive KNS, 2/4 positive

NAC – non anticomplementary
AC – anticomplementary

KPS – known positive serum
KNS – known negative serum

Passive Hemagglutination. Non specific hemagglutination, encountered frequently in the PHA tests, obscured most of the results. The trials in which agglutination occurred in the controls were omitted from Table 9. The dilutions of antiserum in which the most hemagglutination occurred are shown in Table 9. No hemagglutination was present in dilutions below 1:8 with antigen 11 or below 1:32 with antigen 10.

Titration of the AE Virus. The results of titration of the AE virus are found in Table 10.

DISCUSSION

To eliminate the possibility of parental antibodies influencing susceptibility, only one litter from each female was used. A shortage of mice of appropriate age prevented rapid subpassage of the material throughout the adaptation studies. Weaned mice from the same colony were also provided for the rabies diagnostic laboratory which further limited the number of young mice available for AE inoculation.

Care was exercised to maintain a colony of mice free of disease, especially Theiler's disease or, "mouse encephalomyelitis". Mice exhibiting abnormal action were killed, the brain removed aseptically, suspended in diluent, and inoculated into mice. Inoculation of control mice revealed no evidence of a latent virus infection. A closed colony was used for this study to prevent the introduction of carrier diseases. A vigorous control program for the destruction of wild mice was followed during this study.

Mouse Adaptation Studies

Suckling mice are frequently more susceptible to infection than are older mice (30, 37) therefore, two to four day old mice were used in the adaptation

Table 9. Results of passive hemagglutination tests.

Antigen	Positive: Serum 1	Positive: Serum 2	Positive: Serum 3	Positive: Serum 4	Positive: Serum 5	Negative: Serum 1	Negative: Serum 2
filtered	1:128	1:64	1:128	1:256	1:64	1:16	1:32
Na lauryl sulfate extracted	none	none	none	none	not tested	no agglutination	1:8
10^{-1} centrifuged 2000 RPM/30 min. inactivated 56°C./1 hr.	not tested	1:16	1:32	1:16	1:8	1:32	1:64

Table 10. Results of titration of AE virus.

Test No.	Highest dilution inoculated	Highest dilution to produce 50% ataxia	M. I. $D._{50}$	Remarks
1	10^{-6}	10^{-6}	10^{-6} or higher	51/60 chickens affected in 45 days.
2	10^{-10}	none	0	reinoculated 10 birds and 5 controls 14 days later with potent virus. None were affected.
3	10^{-9}	10^{-9}	10^{-9} or higher	32/32 affected in 30 days.
4	10^{-15}	10^{-11}		39/49 affected in 30 days.

study. Serial and zig zag passage procedures (1, 32) were used in an attempt to adapt the VR strain of the AE virus for propagation in mice. Cortisone and related steroids have been shown to facilitate adaptation of several infective agents (16, 29, 53, 54). Ostendorf (45) used cortisone acetate as an adjuvant in an attempt to facilitate adaptation of the AE virus to mice. His preliminary results indicated the desirability of further investigation. Cortisone has a marked antiinflammatory action, inhibits the recall mechanism and depresses the total cellular response (17, 47, 48), as well as other known and unknown actions. It was postulated that in this instance the cortisone would inhibit the body defenses sufficiently to permit growth and adaptation of the virus (47). Bacterial contamination and complications in this series proved to be a constant source of error. Experience eliminated much of this, but not to the level maintained in following series. The higher level of bacterial infection could have been due to the reduction of the body defenses by the cortisone acetate. Since there was no evidence of an encephalomyelitis in series one and contamination was a problem, series one was discontinued after four passages (Table 4). Further experimentation with cortisone acetate as an adjuvant might be advisable.

Jones (23) employed a five percent soluble starch preparation as an adjuvant and increased the AE infection in 33 chickens about eight percent. Soluble starch was not available, necessitating the use of a commercial starch suspension in series three (Table 5) and series nine (Table 4). It was thought that the starch particles would delay the absorption of the virus and give it a chance to adapt and multiply (47). Most mice injected with starch (both controls and those receiving virus material) upon necropsy showed a small, cream colored, necrotic area involving the meninges and underlying cerebral cortex at the site of inoculation. As this also appeared in

controls, it apparently did not produce signs of encephalomyelitis in the mice.

In series three (Table 5), signs of encephalomyelitis were noted in four mice of passage 4c. A suspension of the four mice brains was immediately subpassed (5a, b, d) and 13 of 37 mice exhibited slight incoordination and posterior paralysis. Passage 5c was eliminated due to bacterial contamination. Passages 5e, f, g, h, i were inoculated with brain suspension from 4c, six to 14 weeks after 5a, b, d. These mice did not develop signs of encephalomyelitis. Passage 6a was inoculated within three days with material from 5a, b, d and five of 17 mice developed signs of encephalomyelitis. After a 10 day delay, passages 6b, c were initiated. Milder signs of encephalomyelitis were observed but no histopathological changes developed. In the subsequent six passages, which were delayed several months, evidence of encephalomyelitis was not observed. Passage 6a was serially subpassed six additional times before it was discontinued. Material remaining from passages 4c, 5a, b, d, and 6a, b, c was inoculated as soon as mice were available and carried for five to six additional, subserial passages without evidence of encephalomyelitis.

Soluble starch was employed as an adjuvant in series five (Table 6), seven and 11 (Table 4). Evidence of encephalomyelitis was noted in passages 3a, 4a, b, c, d, and 5a of series five. Passages 4a, b were subpassed quickly after harvesting the brain tissue from 3a. Approximately 75 percent of the mice exhibited signs of encephalomyelitis. Passages 4c and d were delayed three to four weeks. Only 10 percent of these mice were affected. Passage 5a was delayed 10 days and 6a for six days. No mice in passage six showed signs of encephalomyelitis. This series was discontinued only when brain material from passages 2, 3a, 4a, b, d, c, and 5a was exhausted.

A 10 percent, soluble starch solution was used as an adjuvant in series

seven in an attempt to increase the effectiveness of the starch by increasing the concentration of the solution. As no evidence of encephalomyelitis developed and as mice were needed in series three and five, series seven was discontinued after four passages.

Jungherr and Minard (27) reported the use of simultaneous intracranial and intraperitoneal injections in an attempt to increase virulence. Using this method, the weaned mice not needed for breeding replacement or rabies diagnosis were inoculated in series nine (Table 4). This series was discontinued after three passages with no evidence of encephalomyelitis noted.

A zig zag method for adapting a virus to an abnormal host has been used (1, 32, 45). It is postulated that a few of the virus particles will undergo slight mutation while in the abnormal host and that immediate repassage back to the normal host will allow these mutants to multiply. Successive zig zag passages should increase the amount of adaptation to the abnormal host. Signs of infection in the normal host may indicate a residual infective virus, although mutants do not necessarily produce signs in the normal host. The zig zag method was tried in series 11 and 15. Series 11 was discontinued after four complete zig zag passages, as no evidence of encephalomyelitis had been found (Table 4).

Colonel Randall[2] suggested prior inoculation of a two percent starch solution, followed in 12 hours by the virus material in the opposite cerebral hemisphere. Double inoculations 24 to 48 hours apart have been found effective in the adaptation of the human influenza virus to mice and in shortening the incubation period of the Lansing Strain of Poliomyelitis virus (18, 39).

2. Colonel Raymond Randall, Chief, Department of Hazardous Operations, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Division of Communicable Disease, Washington D. C., Personal communication.

The first inoculation was to desensitize the body defenses i.e. to allow the leucocytes and macrophages to react against the virus and thus deplete their numbers. The second inoculation was to be given before the production of antibodies was begun, thus overwhelming the weakened body defenses. These two procedures were combined for use in series 13 (Table 4) and for the mouse inoculation part of series 15 (Table 7).

The results of series three and five seem to indicate that the time lapse between passages materially affected the results. It appeared that a more rapid passage through mice might have enhanced the adaptation of the AE virus for propagation in mice. As adjuvants of both commercial and soluble starch produced results, additional research with these is indicated. A series of variations in the incubation period and/or use of mice of different ages, along with an adequate supply of experimental animals to permit rapid passage, might provide the combination necessary for successful adaptation.

Serological Tests

Little success was encountered with either the DCF or PHA tests, although both showed enough promise to encourage further work.

Complement Fixation Test. The preparation of a non anticomplementary antigen proved to be one of the most difficult problems encountered. Benedict and O'Brien (2) suggested the use of a one percent solution of sodium lauryl sulfate to extract the anticomplementary components. Utilizing this method, only the 10^{-1} infective brain suspension centrifuged at 2000 RPM for 30 minutes was found to be non anticomplementary (Antigen 1, Tables 2 and 8). Antigens 2, 3, 4, and 5 were used in an attempt to provide a more concentrated antigen but were anticomplementary. The greater concentration of brain tissue apparently provided too many anticomplementary components to be absorbed.

A higher percent of sodium lauryl sulfate is reported to cause excess protein denaturation (2).

The method suggested by Casals and Palacios (13) (antigen 6, Tables 2 and 8) provided non anticomplementary antigens, but the results of the tests run were very unreliable, for both positive and negative results occurred with the same serum. The filtered antigen (antigen 7, Table 6) was also non anticomplementary but the process was slow and such a very small amount of material was recovered and as a result only two tests were conducted. Both of these tests appeared very unreliable.

Celite 512 has been used in the studies of visceral lymphomatosis, as an absorbent for the particulate material (3). The positively charged particles are repelled by the celite. Although non anticomplementary, antigen 10 (Tables 6 and 7) gave little evidence of being a reliable antigen.

Brumfield and Pomeroy (7) reported that for the best results, the infective titer of the virus must be 10^{-7} or higher, and the serum diluted 1:8. This apparently puts the antigen and antibodies in a more favorable ratio.

Neutralizing antibodies may differ from complement fixation antibodies. According to Carpenter (11), in infections from encephalitic or other neurotropic viruses, most neutralizing antibodies appear two weeks after the onset of signs and remain in the circulation for years. Complement fixation antibodies, on the other hand, may appear later and are more transient, disappearing within a few weeks to three years. An exception to the above theory is found in lymphocytic choriomeningitis, in which the neutralizing antibodies appear after the complement fixation antibodies have declined (11). Serum collected from blood drawn at different stages of the disease process

was used. The results from the tests run were so erratic that no opinion could be made as to the presence of antibodies.

Antigens 1, 6, and 10 produced the most reliable results, although they were still not constant enough to be of value. The procedure followed, allowed a total volume of 2.5 cubic centimeters for the test (USLSA, 49). Brumfield and Pomeroy (7) recommended an alternate procedure in which the total volume is 1.0 cubic centimeter. This dilution might conceivably have given better results.

The passive hemagglutination (PHA) test, performed according to the recommendations of Benedict and O'Brien (3), proved very unstable as non-specific or spontaneous hemagglutination frequently occurred. The incidence of nonspecific hemagglutination was reduced but not eliminated by treating all solutions with unsensitized sheep RBCs. Further reduction of nonspecific hemagglutination might be accomplished by purification of the antigen, and permit routine use of this test. As the dilution of sera increased, the amount of hemagglutination also increased. This might indicate the presence of the prozone phenomenon.

Titration of the AE Virus. Part of the difficulty encountered in the titration of the virus was due to an underestimation of the infective titer. An infective titer of 10^{-4} to 10^{-7} was reported in literature (27, 42, 45, 51). Present studies indicated an infective titer of 10^{-9} or higher (Table 10).

In all trials except number two, at least 50 percent of the birds inoculated, with all dilutions were affected. In trial two, none of the birds developed signs of infection. The infectivity of the virus suspension was checked by the inoculation of day old chickens, obtained from a local hatchery. Signs of infection developed in ten days, indicating the viability of the virus. The control birds of trial two were inoculated 21 days later with a known potent

virus and exhibited no evidence of infection. Chickens obtained from the same source, and presumably from the same parentage, two months later, were successfully infected with dilutions as high as 10^{-9} (trial 3, Table 10). The chickens of trial two were apparently immune, but the reason for this immunity could not be determined.

SUMMARY AND CONCLUSIONS

An attempt to adapt the AE virus for propagation in two to four day old mice was made. Cortisone acetate, soluble starch solution and a commercial starch suspension were used as adjuvants. In one series using soluble starch (series 5) and one series using commercial starch (series 3), mice exhibited evidence of encephalomyelitis. The first signs of encephalomyelitis observed were very mild and a low percentage of mice affected. On immediate repassage of the brain material the signs and histopathological evidence increased. A delay in further repassages was associated with decreases in the incidence and intensity of the signs and histopathological changes. Signs of encephalomyelitis were not observed in subsequent subpassages.

Attempts to develop a serological test for the diagnosis of AE were unsuccessful. The DCF test was easier to perform, but obtaining non anti-complementary antigens and satisfactory complement fixing sera was difficult. From the limited number of tests performed, both the DCF and the PHA tests appear to offer promise but additional studies are needed.

Titration of the virus indicated that the M. I. $D._{50}$ was 10^{-9} or higher. This is higher than previously reported in the literature.

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ATTEMPTS TO ADAPT AVIAN ENCEPHALOMYELITIS VIRUS TO SUCKLING MICE WITH PRELIMINARY OBSERVATIONS ON SERODIAGNOSTIC METHODS

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Avian encephalomyelitis (AE), "epidemic tremor", has become more prevalent and assumed greater economic importance within the past ten years. The etiological agent of AE is an infectious, filterable virus which is spread mainly by egg transmission. Diagnosis and control of AE have been hindered by lack of an accurate and rapid method for diagnosis.

In this study an attempt was made to adapt the AE virus to the common laboratory mouse in an attempt to produce antigen and antibodies suitable for a direct complement fixation test or to provide an abnormal host suitable for serum neutralization tests. A strain of AE virus highly pathogenic for chickens was inoculated intercerebrally into two to four day old mice by serial and zig zag passage methods. In an attempt to facilitate adaptation, adjuvants of cortisone acetate, soluble starch and commercial starch were used. In one series using soluble starch and one series using commercial starch, mice exhibited evidence of encephalomyelitis. The first signs of encephalomyelitis observed were very mild and a low percentage of mice were affected. On immediate subpassage of the brain material the signs and histopathological evidence of encephalomyelitis was increased. A delay in further repassages was associated with decreases in the incidence and intensity of the signs and histopathological changes. Mice in subsequent subpassages did not develop signs of infection.

The direct complement fixation (DCF) test, using chicken brain antigens and homologous or turkey antiserums, and the passive hemagglutination (PHA) test were used in an attempt to develop a serological test for the diagnosis of AE. The preparation of non anticomplementary antigens for the DCF tests was difficult. Antigens prepared by extraction with sodium lauryl sulfate, absorption with celite 512 or by precipatation of anticomplementary factors by alternate freezing and thawing following the addition of normal chicken

serum, were non anticomplementary but gave false positive and negative results. Nonspecific or spontaneous hemagglutination, which occurred frequently in the PHA tests, was reduced but not eliminated by treating all solutions with unsensitized sheep red blood cells. From the results obtained the DCF and PHA tests appear to justify further studies.

Titration of the virus indicated that the M. I. $D._{50}$ was 10^{-9} or higher. This was higher than previously reported in the literature.