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GROUP A ARBOVIRUSES

Final Report

P. H. Coleman, D.V.M., Ph.D.
J.V. Formica, Ph.D.

December 1975

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20314
Contract Number DADA17-72-C-2161

Virginia Commonwealth University
Health Sciences Division
Richmond, Virginia 23298

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These investigations of Group A arboviruses were designed to isolate mutant strains of Eastern Equine Encephalitis (EEE) virus which could serve as a potential vaccine candidate strain and to characterize the Clone-15 strain of Western Equine Encephalitis (WEE) virus to determine its potential as a human vaccine virus.

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embryo cell culture vaccine as a candidate for human use. About 150,000 doses of final vaccine were prepared and stored in bulk. About 3,500 doses were lyophilized as a final package vaccine. Final packaged vaccine was free of detectable anaerobic and aerobic bacteria, mycotic, mycoplasmal, and murine and avian viral agents. The vaccine does elicit antibody production in laboratory animals. The final WEE vaccine contains 2 plaque types of virus; a large plaque which resembles the parental strain and a small plaque which is genetically unstable and gives rise to the large plaques. The vaccine appears to be safe in laboratory animals; it does not produce clinical evidence of disease in adult guinea pigs, hamster, or rhesus monkeys when inoculated both parenterally and into the nervous system. The vaccine does produce clinical disease in newborn mice and in young hamsters when inoculated into the nervous system.

Studies were undertaken to develop an attenuated vaccine candidate strain of EEE virus using both BeAn 221 strain and the Arth 167 strain. Studies with mutagenic chemicals and serial passages at various temperatures did not yield an isolate of virus which had lost its neurotrophic characteristics for laboratory animals. It was concluded that EEE virus is very stable virus and difficult to attenuate by the methods attempted.

SUMMARY

Investigations were undertaken to characterize the Clone-15 strain of Western equine encephalitis virus and to determine its potential usefulness as an attenuated virus. The clone-15 strain was found to be a safe and efficiaous virus when inoculated into laboratory rodents and primates. The virus grew well in cell culture systems and is an excellent candidate for a human and equine live-virus vaccine.

Several thousand doses of Final Clarified Vaccine were produced by growing the Clone-15 strain in chicken embryo cell culture. The vaccine was free of detectable bacterial, fungal, mycoplasmal, and murine and avian agents. The vaccine did not produce disease or pathology when inoculated peripherally or directly into the nervous system of adult Rhesus monkeys, 100 g hamster, weanling mice or adult guinea pigs. The vaccine did produce clinical disease and pathology in newborn mice and young (50 g) hamsters.

In purity testing the Final Clarified Vaccine was found to be genetically unstable. The vaccine contained two virus populations as evidenced by the production of large and small plaques in cell culture. The atypical, small-plaque material would give rise to large plaques when passed in cell culture. Although the Final Clarified Vaccine appears avirulent, there is a suggestion that the small plaque isolate may be more virulent (in hamsters) than the parential large plaque virus.

In other studies, attempts were made to isolate an avirulant strain of Eastern equine encephalitis (EEE) virus. Two strains of EEE virus were studied; the North American Arth 167 strain and the South American BeAn 221 strain. Both strains produce clinical disease and death when inoculated intracerebrally into laboratory animals. Studies were done using N-methyl-N^1-nitro-N-nitrosoquanidine both in vitro and in vivo to produce EEE viral mutants. No evidence of mutation was obtained based on viral temperature sensitivity at 30, 35, or 40 C or loss of virulence for animals. The EEE virus appears to be very genetically stable and not subject to mutation easily by the methods used.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animals, Resources, National Academy of Sciences-National Research Council.

The information reported here has been approved for public release; distribution unlimited. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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INVESTIGATIONS OF ATTENUATED STRAINS OF GROUP A ARBOVIRUSES

The purpose of this study is two-fold: First, to characterize the Clone 15 strain of attenuated Western equine encephalitis (WEE) virus to determine its suitability as a vaccine for man and animals; and second, to isolate a strain of Eastern equine encephalitis (EEE) virus which has lost its virulence characteristics for animals and man but retains it immunogenic properties. EEE and WEE vaccines are necessary in order to immunize researchers and diagnosticians working with these viruses, veterinarians and others working with infected animals and special groups such as military personnel who might be exposed to natural infections because of their assignments in areas of endemic or epidemic viral activity.

Theoretically, attenuated EEE and WEE viral vaccines should be superior to killed vaccines for several reasons. Such vaccines could provide protection within a few days after vaccination in contrast to the available killed vaccines which require several weeks or months before solid protection can be evoked. Attenuated vaccines should induce longer lasting immunity than inactivated vaccines without the necessity of frequent boosters. Furthermore, the development of living attenuated EEE and WEE vaccines could lead to the development of a "Group A" arbovirus vaccine. That is, the use of an immunizing regimen including multiple viruses (e.g. EEE, WEE, and VEE) could induce immunity to a wide spectrum of Group A arboviruses including o'nyongnyong and chikungunya, which are known to produce massive epidemics of disease in man. For these reasons, it is desirable to develop attenuated vaccines for EEE and WEE viruses.

STUDIES WITH THE CLONE 15 STRAIN OF WEE VIRUS B628

Introduction

The Clone-15 strain of WEE virus B628 was selected as a potential vaccine candidate virus. This strain was originally isolated from tissues of a nestling house sparrow, *Passer domesticus*, collected in Kern County, California in 1957 (1). The virus was passed four times in hamster kidney (Hk) cell culture and alternately grown in agar overlay and fluid chicken embryo cell culture (CECC) for a total of 30 passages (2).

During the 1960's Dunayevich *et al*. (3) reported on the selection of a clone (Clone 5; 10th CECC passage) of the B628 strain which was not pathogenic for young adult mice. Johnson (2) extended Dunayevich's work and selected a second clone (Clone-15; 30th CECC passage) from the B628 strain which was shown to be suitable as a living attenuated WEE vaccine in horses.

Dr. Johnson (4) grew the selected plaque virus (clone) in one additional fluid culture of CECC and used this material as his vaccine candidate virus. The vaccine candidate virus was subsequently passed in embryonated chicken eggs or cell cultures in the California Public Health Viral and Rickettsial Disease Laboratories and the resulting vaccine was shown to be efficacious in vaccinating horses (5). Additionally, material was submitted to Dr. Roca Garcia, Lederle Laboratories, Pearl River, New York, where a chicken embryo

vaccine was prepared and also tested in horses (6, 7). The Lederle chicken embryo virus was also tested for neurovirulence in monkeys, guinea pigs, and adult mice. No evidence of neurovirulence was detected (Appendices 1 and 2).

Because the Clone-15 strain of WEE virus B628 has been reported to have lost its neurovirulence for monkeys, guinea pigs, weanling mice, and hamsters; and because it does elicit protective antibody response, it was selected as the most likely candidate for a human vaccine for WEE virus.

Materials and Methods

Viruses:

The Clone-15 strain of WEE virus B628 (hereinafter referred to as WEE Clone-15 or WEE Cl-15) was used as a vaccine candidate virus in the present study. The Master Seed virus was obtained as a lyophilized preparation produced by Lederle Laboratories. For information concerning the production and testing of the Lederle Clone-15, WEE vaccine used as the Master Seed (Appendices 1 and 2), a flow-chart depicting the passage history of the virus is shown in Table 1.

For the current studies, about 1000 ml (200 vials) of the Master Seed was kindly supplied in February of 1973 by Drs H. Johnson and E. Lennette, California State Health Department, Berkley, California. About half (100 vials) of this material was stored at the U.S. Army Medical Unit, Frederick, Maryland. An ampule of this vaccine was used as a Master Seed for the production of the final vaccines. The remaining half of the Master Seed was kept in our laboratories for testing and gathering information on the purity, potency, and safety of the vaccine.

The identifications of the various WEE Clone-15 viral passages used in vaccine production testing are given below:

Master Seed	A lyophilized product obtained from Lederle (passage virus history is $Hk_4CECC_{31}CE_2CECC_2$).
Working Seed	One chicken embryo cell culture passage of the Master Seed.
Final Unclarified Vaccine -	One chicken embryo cell culture passage of Working Seed taken before centrifugation and Millipore filtration.
Final Clarified Vaccine -	Same as Final Unclarified Vaccine except that the virus was centrifuged at 10,400 g for 30 min and the supernatant filtered through a 0.45 micron Millipore filter.
Packaged Vaccine	Final Clarified Vaccine that was diluted 1:1000 and then lyophilized.
WEE Pl-148	A large, plaque strain of virus selected from serum neutralization breakthrough test of the Final Unclarified Vaccine.

WEE P1-149 A small, plaque strain of virus selected from a serum neutralization breakthrough test of the Final Unclarified Vaccine.

In challenge studies the Fleming strain of WEE virus, a highly virulent strain, was obtained as a lyophilized suckling mouse brain suspension from the Reference Reagents Unit, NIAID, NIH, Bethesda, Maryland. About 200 ml of the Fleming strain was grown in CECC and put in ampules in 3 and 10 ml quantities and stored at -70 C for use as a challenge virus in the present studies.

Animals:

All animals were obtained from commerical sources. Weanling (15-20 g) and pregnant mice were obtained from Flow Laboratories, Dublin, Virginia. Pregnant mice were allowed to litter in our facilities. Syrian hamsters (50-100 g) were obtained from Engle Laboratory Animals, Inc., Farmersburg, Indiana. Fertile, specific pathogen free chicken eggs were obtained from SPAFAS, Inc., Norwich, Connecticut, and incubated in our laboratory. Male Rhesus monkeys, 2 to 4 kg, were obtained from Primate Imports, Inc., Port Washington, New York. Adult guinea pigs (300-350 g) were obtained from J. C. Corell, Inc., Waynesboro, Virginia.

Cell Cultures:

Primary chicken embryo cell cultures (CECC) were prepared from decapitated 9- to 10-day-old chicken embryos. Embryos were minced with scissors, and trypsinized from 1-2 hours in 0.25% trypsin in isotonic glucose-KCl-NaCl solution. Trypsinized cells were filtered through eight layers of gauze and one to two layers of lens paper, and plated at a 1:200 dilution in disposable plastic culture bottles or plates. Growth medium consisted of Hanks' 199 containing 5% fetal calf serum, phenol red, and sodium bicarbonate with antibiotics.

For virus production, monolayers of CECC were refed Hanks' 199 containing 0.5% bovine albumin fraction V (Miles Laboratories) with antibiotics.

For titration studies, the agar-overlay plaque system was used. Infected monolayers were overlayed with Earle's balanced salt solution (EBSS) containing 2% newborn calf serum, 1% Noble agar, 1.65 mg/ml of lactalbumin hydrolysate and sodium bicarbonate with antibiotics. After 48 hours of incubation at 35-37 C, a second overlay containing 5 μg neutral red per ml was added. Monolayers were examined for plaques at 4 hours, and at 24 to 48 hours. All plaque titrations were done in duplicate and the viral titer expressed as plaque forming units (PFU) per ml. For harvest, plaques were "picked" utilizing a sterile Pasteur pipette. The harvested material was suspended in one ml of phosphate buffered saline, pH 7.2 - 7.6. containing 4.0% bovalbumin fraction V. In some cases, cells were monolayered in plastic plates (Linbro) and grown at 35 C under atmospheric conditions using Hanks' 199 containing 5% FCS, 20 μM Hepes buffer (Gibco) and antibiotics. These were overlayed as above, but with the addition of 20 μM Hepes/ml of overlay.

Plaque sizes were determined by measurement with a millimeter ruler at 24 hours or 48 hours. No appreciable increase in diameter was noted for those plaques monitored for up to five days.

Sera:

Two different sera were used in neutralization tests. One serum was obtained from the Reference Reagents Branch, NIAID as hyperimmune mouse ascetic fluid for WEE Fleming virus. This serum is considered to be free of antibodies to a broad spectrum of viruses. The second serum was obtained from the Communicable Disease Center, Atlanta, Georgia. This material was equine serum.

Other materials and methods:

Specific materials and methods used in purity, potency, and safety testing are listed under the appropriate section of results.

Results

Preliminary Studies:

Prior to the preparation of a final WEE Cl-15 vaccine, studies were undertaken with the original WEE, non-cloned virus (HK_5) and the Lederle prepared Master Seed virus ($HK_4CECC_{31}CE_2CECC_2$).

The HK_5 passage virus only titered $10^{2.5}$ plaque forming units (PFU) per ml when received. This material was passed two times in fluid CECC cells (24 hour harvest) in our laboratory and tested for titer and virulence. The results are shown in Table 2. The original B628 strain of WEE after two CECC passages titered 8.8 X 10^6 PFU per ml in agar overlay cultures of CECC. This virus also killed weanling mice and adult hamsters inoculated intracerebrally whereas the Master Seed and Final Vaccine failed to kill weanling mice or hamsters. From these results, it is clear that the WEE Cl-15 virus, which was derived from the B628 original, is significantly different from the original in terms of animal virulence. These results provide "markers" for determining that the virus has been attenuated as well as for studying reversion of the WEE Cl-15 virus to the original (wild) type.

In preliminary studies with the Master Seed virus (Lederle's vaccine), the virus was titrated in mice and tested for virulence in hamsters and guinea pigs. Three bottles of the Master Seed virus were reconstituted and individually tested. Suckling (1-3 day) mice, weanling (3-4 week) mice, golden hamsters (ca 100 g) and guinea pigs (ca 350 g) were inoculated either intracerebrally (ic) or intraperitoneally (ip) with the reconstituted vaccine. The results are shown in Table 3. Undiluted virus failed to kill weanling mice, hamsters, or guinea pigs when inoculated by either route. Conversely, the virus did produce clinical illness and death in suckling mice (SM) by either route of inoculation. In suckling mice, the virus titered from $10^{5.8}$ to $10^{6.1}$ fifty per cent lethal doses ($SMLD_{50}$) per ml; the animal death endpoints were well defined.

In other animal studies, the viremia level of the WEE Master Seed virus was determined in hamsters and guinea pigs inoculated either ic or ip with 0.1 ml (ca 10^4 $SMLD_{50}$) of virus. Following inoculation, whole blood was collected daily for seven days via cardiac puncture of ether anesthesized animals. The blood was allowed to clot at room temperature and the serum separated by centrifugation and stored at -70 C. For plaque assays, 0.05 ml of undiluted serum (in some cases, serum was diluted 1:2) was inoculated onto monolayers of CECC. The results are shown in Table 4.

Viremia could be detected in 4 of 9 guinea pigs inoculated ip; 3 of 6 guinea pigs inoculated ic; 11 of 12 hamsters inoculated ip; and 8 of 8 hamsters inoculated ic. The detectable viremias were very low -- less than two logs of virus in 0.05 ml of serum. Furthermore, many animals exhibited a viremia only on the day following inoculation and, thus, in these animals the viremia could be due to the persistance of the inoculum rather than to viral replication. However, in some cases (e.g. hamsters 121, 122, and 124), the viremia was not detected until the third or fourth day post-inoculation indicating an "infection" had occurred. In addition to these viremia studies, these animals were examined daily for signs of clinical illness and daily temperatures were recorded for each animal. There was no evidence of clinical disease in the animals nor was there any detectable febrile response following vaccination.

Six weeks after inoculation with the WEE Master Seed virus, the guinea pigs and hamsters were challenged with virulent WEE virus. The Fleming strain of WEE virus was used and animals were challenged with ca 10^3 weanling mouse fifty per cent lethal doses ($WMLD_{50}$) of virus administered ic. The results of these studies indicated that protection was excellent for at least six weeks against the challenge dose of virus used (Table 5).

In a similar protection study, a mouse protection extinction test was performed by vaccinating weanling mice with ten-fold increments of the WEE Master Seed administered either ic or ip. Six weeks later, the mice were challenged with 10^3 $WMLD_{50}$ of the WEE Fleming virus administered ic. The mice were protected with as little as 100 $SMLD_{50}$ of vaccine virus administered ic or 1000 $SMLD_{50}$ of virus administered ip (Table 5).

In a series of cell culture experiments, various methodologies were examined to gather preliminary data regarding the optimal procedure to use in producing a large batch of WEE vaccine seed virus and vaccine. Basically, CECC were planted at with either 5 X 10^5 or 3 X 10^6 cells per ml. Four different growth media were used: Eagle's MEM, BME, 199, or Hanks' BSS containing 0.1% yeastolate. For growth of the cells, 5% fetal calf serum was added to each medium. For maintenance of virus infected cultures, 2% bovine albumin was substituted for fetal calf serum. Cells were grown for 24 to 48 hours (until monolayered), washed three times, and inoculated with ca 10^5 PFU of the WEE Master Seed virus. Virus was absorbed for two hours at 36 C before the maintenance medium was added. Cultures were then grown at 36 C and at 12-hour intervals, two cultures were frozen and thawed, and pooled. The virus content of the cultures was then determined by plaque titration in CECC. In general, few differences were detected among the methods explored. The viral titers ranged from $10^{6.4}$ PFU per ml to $10^{7.6}$ PFU per ml. The highest titers were obtained in the Hanks' BSS and Eagle's MEM media. The maximum virus titer was achieved in 24 to 48 hours. Unexpected difficulty was encountered in those cultures planted with 3 X 10^6 cells per ml in that the cells did not adhere to the vessel surface in many instances.

Production of Vaccine:

During October and November of 1973 period, the principal investigator spent two weeks in the Vaccine Production Facilities, U.S. Army Medical Research Institute of Infectious Diseases, working with Dr. F. E. Cole, Jr.,

and his staff. During this period, we produced a "Working Seed Virus" and a "Final Vaccine Virus" with the WEE Cl-15 virus. Based on an estimated dose of 10^6 infectious units, about 1.5 million doses of Final Vaccine Virus were produced.

The Lederle prepared lyophilized virus was used as the Master Seed Virus for the preparation of the Working Seed Virus. Both the Working Seed Virus and the Final Vaccine Virus were grown in primary chicken embryo cells prepared from fertile eggs obtained from COFAL negative flocks, SPAFAS Inc., Norwich, Connecticut. Nine day decapitated embryos were minced and trypsinized for the preparation of roller bottle (stationary bottles for controls) cultures containing ca. 3 X 10^6 cells per ml. All media were antibiotic free. Cells were grown overnight at 35 C in Hanks' 199 medium containing 10% fetal calf serum. On the following day, the medium was decanted, the cell monolayer fed with Earle's 199 containing 0.5% human serum albumin (HSA) and the cultures incubated at 35 C for 24 hours.

After the 24 hour incubation period, the medium was decanted and the roller bottles inoculated with about 10^5 chicken embryo cell culture (CECC) PFUs per bottle and incubated one hour at 35 C. The inoculum was then decanted and the infected monolayers washed three times with 100 ml of serum free Hanks' balanced salt solution. Finally, bottles were fed with Earle's 199 containing 0.5% HSA and incubated at 35 C for 24 hours.

Control bottles of cells consisted of stationary CECC monolayers and were treated as above except that they were not inoculated with virus. After 24 hours of incubation, the Working Seed Virus and the Final Vaccine Virus were harvested by decantation. Samples of both were stored at -70 C without further clarification. The bulk of these preparations were partially clarified. Approximately 300 ml of Working Seed Virus was centrifuged at 1000 X g for 30 minutes followed by a second centrifugation at 10,400 X g for 30 minutes. Finally, the supernatant was pooled and filtered through a 0.45 micron Millipore filter. About 1050 ml of the Final Clarified Vaccine was prepared, 150 ml in samples for testing and 800 ml in bulk as the Final Vaccine Virus.

Packaging of the Final Clarified Vaccine:

In February of 1975 a lot of the Final Clarified Vaccine was packaged and lyophilized in the facilities of USAMRIID.

For the Packaged Vaccine it was decided that a "dose" of vaccine should contain between 10^5 - 10^6 chicken embryo cell culture PFU. In order to use an inoculation volume of 0.5 ml per dose, the Final Clarified Vaccine had to be diluted one thousand fold to obtain 10^5 - 10^6 PFU per 0.5 ml of Packaged Vaccine.

Three-1 ml vials of the Final Clarified Vaccine were thawed. One milliliter of the vaccine was then added to each of two-100 ml vials of Earle's 199 medium containing a final concentration of 0.5% HSA. After mixing, the two-100 ml vials were pooled and sufficient Earle's 199 with 0.5% HSA added to give a total volume of two liters.

The vaccine was then dispensed into sterile 25 ml vaccine vials, 5.4 ml per vial, using a sterile Cornwall syringe. Rubber stoppers were placed on each vial and the vials placed in a pre-chilled lyophilizer for freezing. Sixteen hours later a vacuum was applied to the lyophilization chamber and the material was dried for 72 h. Stoppers were mechanically seated on the vials in the lyophilizer. Metal seals were crimped onto each vial after their removal from the lyophilizer. Individual vials were tested for vacuum, labeled, packaged (25 per box), and stored at -20 C. Three hundred and forty-six vials were prepared. Twenty-one vials were transported to the Medical College of Virginia for sterility and potency testing. The remainder were stored at the USAMRIID. The CECC, PFU titer of the Packaged Vaccine is about $10^{5.3}$ per dose (0.5 ml). The Packaged Vaccine is labeled as follows:

WESTERN EQUINE ENCEPHALITIS VACCINE
LIVE. ATTENUATED LOT 1 Lyophil Run 1
Chick Cell Origin Mfg. Date FEB 75
Store at -20°C Add 5.4 ml water for inj. USP
CAUTION: New drug-Limited by
Federal Law to Investigational Use

Testing of Vaccine:

Studies were conducted to determine the neurovirulence, purity, potency and safety of the WEE virus vaccine. The procedures used were basically those outlined in the Food and Drug Administration's regulations for the manufacture of Rubella vaccine.

Prior to doing extensive purity, potency, and safety testing of the WEE virus Final Clarified Vaccine, several preliminary tests were conducted. The results of these tests are summarized in Tables 6 and 7. The results indicate that there was no gross bacterial contamination in the vaccine and that there was no evidence of a change in virulence or potency of the vaccine. As shown in Table 7, three of four monkeys inoculated intracerebrally with the virus did develop a febrile response. Furthermore, monkeys Nos. 3 and 4 developed a detectable, although low, level viremia and all four monkeys developed neutralizing antibody.

Potency Tests: The WEE Cl-15 Final Clarified Vaccine had a titer of ca 10^9 PFU/ml (titrations of 3 frozen vials = $10^{8.7}$, $10^{9.1}$, $10^{9.4}$) in chicken embryo cells, $10^{8.5}$ LD_{50}/ml inoculated intracerebrally in suckling mice, and $10^{6.8}$ LD_{50}/ml inoculated intraperitoneally in suckling mice.

Neurovirulence Tests in Rhesus Monkeys: For neurovirulence testing, the undiluted Final Clarified Vaccine preparation was used. Monkeys were weighed, prebled, and then inoculated under barbiturate anesthesia.

Because of a lack of infected monkey holding space, only 8 monkeys were used at one time. In all, twenty monkeys were inoculated with virus, and three served as uninoculated controls.

Each monkey received 0.5 ml of test sample inoculated (via trephination) into each thalmic region of each cerebral hemisphere, 0.5 ml inoculated intraspinally into the lumbar spinal cord enlargement, and 1.0 ml inoculated

intramuscularly into the gluteal muscle of the right leg. The monkeys were not administered cortisone acetate, but did receive 300,000 units of procaine penicillin in the right arm muscle. Monkeys were observed for 21 days, bled, and then euthanized with sodium barbital before autopsy. Organs were examined grossly and tissue specimens (brain and spinal cord) were preserved in 20% formalin for histopathological studies.

As shown in Table 8, only one monkey demonstrated clinical signs of illness. Monkey #12 had a mild paresis of the right arm for 4 days following inoculation. This paresis was most likely produced by brain trauma, considering the fact that the needle tract was found to extend into the brain stem. However, it is possible that the injection of penicillin, which was given in the right arm, was responsible for the clinical signs. Weight losses were not observed during the 21-day holding period. All monkeys were free of detectable neutralizing antibody prior to inoculation. The 20 virus-inoculated monkeys developed $\geq$ 2.9 $\log_{10}$ of antibody during the 21-day holding period.

At autopsy, no gross pathology was seen other than evidence in the skull and occasionally on the meninges of the trephining lesion. Histopathological studies were performed by Dr. W. Rosenblum, Chief, Division of Neuropathology, Medical College of Virginia. Tissues were stained with hematoxyin and eosin, phosphotungtic acid hematoxylin, and tricolor stains. Generally, little evidence of histopathology was noted except for perivascular cuffing (Table 8).

Safety Test: Various "safety tests" were carried out on the Final Unclarified Vaccine by the inoculation of animals. The results are shown in Table 9.

Twenty-four adult mice, weighing 15 to 20 grams each, were inoculated with 0.03 ml and intraperitoneally with 0.5 ml of undiluted Final Unclarified Vaccine. Mice were examined for 28 days. None of the mice exhibited clinical signs of disease during the observation periods, and none of the mice died. After 28 days, these mice were divided into three groups (8 mice per group). All three groups were inoculated intracerebrally with the virulent Fleming strain of WEE virus - one group receiving 30,000 $WMicLD_{50}$, one group receiving 3,000 $WMicLD_{50}$ and one group receiving 300 $WMicLD_{50}$ of Fleming virus. None of the vaccinated mice died, indicating that the Final Unclarified Vaccine had induced specific protection against WEE virus.

In other safety tests, 20 guinea pigs weighing about 350 gm each were inoculated intracerebrally with 0.1 ml and intraperitoneally with 0.5 ml of the undiluted Final Unclarified Vaccine. The guinea pigs were observed for 21 days, and except for one animal that died from anesthesia, no evidence of clinical disease was noted. After 21 days, 10 of the guinea pigs were euthanized and autopsied. No gross evidence of disease was noted. Brain material was collected for histopathological studies. The only histopathological lesion noted was evidence of mononuclear infiltrates of the meninges.

The remaining 9 guinea pigs were challenged with 10^5 $WMicLD_{50}$ of the virulent WEE Fleming virus via intracerebral inoculation. These animals were observed for 2 weeks. None developed clinical signs of disease.

Twenty 55-65 gram hamsters were also inoculated with 0.1 ml intracerebrally and 0.5 ml intraperitoneally with the undiluted Final Unclarified Vaccine. Beginning 2 days after inoculation, a number of hamsters were observed to develop clinical signs of disease. Signs consisted of "roughened" appearance, anorexia, matted fur, hair around eyes, and progressed to include paralysis of the hind limbs, prostration and death. Twelve of the twenty inoculated animals were affected. Animals were observed to be sick from the second day post-inoculation through the tenth day post-inoculation. Animals found dead in the cages (if not eaten) and paralyzed animals were collected and brain tissues examined for histopathological changes and assayed for virus content.

For virus isolation studies, brain tissue of six hamsters which had died during the first 7 days post-inoculation were individually triturated and 10% suspensions inoculated into monolayers of chicken embryo cells using the agar overlay system. Virus could only be detected in one specimen which had been harvested three days after inoculation. Histopathological examination of the brains of the sick and dead hamsters revealed areas of perivascular cuffing and monocytic infiltration of the meninges.

The 8 hamsters that did not become ill were observed for 21 days, sacrificed, and their brains collected. Histopathological studies of these hamsters did not reveal any marked evidence of encephalitis or myelitis.

Because we had not previously detected hamster deaths with the Lederle produced Master Seed Vaccine, we inoculated an additional lot of hamsters with the undiluted Final Unclarified Vaccine alone and after incubation with WEE Fleming antisera (obtained through the courtesy of the Research Reference Reagents Branch, NIAID, NIH); with the Master Seed Virus, and with a suspension of brain from the viral positive hamster in the first experiment. The results are shown in Table 10. In this study, only three of twenty hamsters inoculated with the final vaccine alone became sick and died, while none of those hamsters inoculated with the Final Vaccine plus WEE antisera became sick. Similarly, none of 10 hamsters inoculated with the Master Seed Virus died. Interestingly, 5 of 6 hamsters inoculated intracerebrally with a 10% suspension of infected hamster brain material died. These results indicate that the final vaccine may produce disease in a percentage of young hamsters and that the disease can be prevented by antisera. The failure of the Master Seed Virus to induce infection and disease may be because of its lower titer - approximately 10^6 PFU/ml for the Master Seed virus and 10^9 PFU/ml for the Final Unclarified Vaccine.

Sterility Tests: For bacterial and fungal sterility testing, samples of the Master Seed Virus, Final Unclarified Vaccine and the Packaged Vaccine were examined for mycoplasma, anaerobic and aerobic bacteria, fungi and *Mycobacteria tuberculosis*. Results are shown in Table 11.

For anaerobic and aerobic bacteria testing, fluid thioglycollate (Difco) and soybean casein digest (Difco) media were prepared as directed by the Bureau of Biologics, FDA. For fungal agent culture, fluid Sabouraud dextrose medium (Difco) was used. For *Mycobacterium* testing, Lowenstein-Jensen medium prepared from the Difco base and fresh eggs was obtained from the Virginia State Health Department, Richmond, Virginia. As an additional medium for the culture of anaerobic organisms, fluid peptone yeast extract glucose medium

(PYG medium) was obtained from Dr. P. Hylemon, Medical College of Virginia. The formula for PYG medium is as follows:

Difco Peptone	10 g
Difco Yeast Extract	10 g
Glucose	5 g

added to 1 liter of salt solution containing 0.002 g each of $CaCl_2$ (anhydrous) and $MgSO_4$; 0.1 g each of K_2HPO_4 and KH_2PO_4; and 0.02 g of NaCl. After autoclaving, 1 g of sterile cysteine and 4 g of sterile Na_2CO_3 were added and the final medium is dispensed into rubber stoppered bottles for use.

Five vials of Master Seed virus, 20 vials of Packaged Vaccine, and 80 ml each of the Working Seed and Final Unclarified Vaccine were tested. The ratios of inocula to media used were: 1 ml to 10 ml for soybean casein digest medium; 5 ml to 25 ml of Sabouraud dextrose medium and PYG medium; and, either 5 ml to 25 ml, or 1 ml to 10 ml for thioglycollate.

Lowenstein-Jensen agar slants were incubated at 37 C in 5% CO_2. All other cultures were equally divided; one-half were incubated at 35 C and one-half at 20±2 C. Cultures were kept for two weeks (except the Lowenstein-Jensen which was kept for 8 wk) and examined grossly for evidence of bacterial or fungal growth. In addition, after the incubation period, fluid cultures were centrifuged and "sediments" Gram stained and examined microscopically for bacteria. The results are shown in Table 11. No evidence of bacterial or fungal contamination was seen.

For mycoplasmal testing, the Final Unclarified Vaccine and uninoculated controls were tested for the presence of mycoplasma following the procedures outlined by the Bureau of Biologics, FDA. This test was done at the USAMRIID and the results indicate that mycoplasmas were not present in the Final Vaccine.

<u>Tests for Adventitious Agents</u>: In cell culture tests for adventitious agents, preliminary tests were conducted utilizing human diploid cells (WI-38), African green monkey kidney cells (BSC-1) and primary Rhesus monkey kidney cells obtained from Flow Laboratories, Rockville, Maryland. Monolayers of cells were inoculated with dilutions of virus and dilutions of virus preincubated (for 1 h at 37 C) with WEE serum (CDC, equine). Cultures were observed 14 days for evidence of viral cytopathic effects and for evidence of viral "breakthroughs" in serum neutralization tests. The results are shown in Table 12. The Final Unclarified Vaccine produced CPE in cultures of WI-38 and BSC-1 cells. The CPE was effectively neutralized when virus was treated with serum. Only one tube of WI-38 cells exhibited signs of virus "breakthrough." The "breakthrough" agent was found to be WEE virus. The primary Rhesus cells did not show specific viral CPE effects in either control or virus neutralization titrations. These cells did not stand up well under the conditions used, however, and it was difficult to fully ascertain the potential extent of virus damage to these cells.

For murine virus testing of the Final Unclarified Vaccine, a 20 ml sample of material was hand carried to Dr. John Parker, Microbiological Associates, Bethesda, Maryland. This material was used in mouse antibody

production by Dr. Parker. The results (see appendix 3) of Dr. Parker's tests indicated that the Final Unclarified Vaccine is free of 12 different murine viruses.

Tests for avian leucosis viruses were performed by Microbiological Associates (MBA), Bethesda, Maryland. Three different samples were submitted for testing: (1) Final Unclarified Vaccine, (2) Hanks' 199 media with human serum albumin (used in preparing the vaccine) and (3) Final Unclarified Vaccine which had been treated with rabbit anti-WEE serum (kindly supplied by Dr. G. Eddy, USAMRIID). Treatment of the virus was accomplished by incubating equal parts of vaccine and rabbit antiserum for 2 h at 37 C and then inoculating the mixture onto chicken embryo cell cultures, maintained for two weeks at 36 C in medium containing rabbit anti-WEE serum. After two weeks, the cells were harvested and inoculated onto fresh CECC maintained in the same fashion for two additional weeks.

Specimens submitted to MBA were treated by several methods and tested for the presence of leucosis antigens by the COFAL test of inoculated quail and chicken embryo cells. Specimens were negative for leucosis antigens (Appendix 4).

Reversion Tests: The Final Unclarified Vaccine was serially passed four times intracerebrally in suckling mice. Ten percent suspensions of infected suckling mouse brain material were used as inocula. At each passage level, the infected brain material was inoculated into weanling mice (15-20 g) intracerebrally. Evidence of reversion to a "virulent" form of virus was not detected. That is to say that the suckling mouse brain material did not kill weanling mice, even after four passages.

Plaque Size Variability in the Final Vaccine:

In other tests, one ml quantities of ten-fold dilutions of the Final Unclarified Vaccine were cultured in 75 cm^2 bottles of CECC. The bottles were overlaid with nutrient agar containing neutral red and observed twice daily for plaque development. Two distinct plaque types were observed. The predominant type averaged 0.75 to 1 mm in diameter at 48-72 hrs post-inoculation, and titered ca 10^9 PFU/ml. However, at high virus concentrations, a few plaques were observed to be 2-3 mm in diameter and appeared to titer about 3 X 10^6 PFU/ml. These two plaque types had not been observed in plaque titrations of virus where 25 mm^2 plates had been used and the neutral red indicator dye had been added to the plates 48 hrs after inoculation.

The discovery of two different plaque sizes in the Final Vaccine was an unexpected development that altered the course of the project. In order to determine that the results were real, the experiment was repeated several times with similar results. Typically, when the Final Unclarified Vaccine was diluted to contain 200 to 600 PFU per inoculum and seeded onto a large surface area of cells, 2-3 "large" plaques were observed in the field.

Initially, it was felt that the large plaques could be due to several foci of infectious virus grouped together to produce a coalescence of plaques which would appear as a single large plaque. Therefore, virus was

harvested from several large plaques and small plaques, and "cloned" in CECC. The large plaque reproduced large plaque types; the small plaque type gave rise to both large and small plaques.

The next factor investigated was the possibility of a "contaminating" adventitious agent. The Final Vaccine preparation and suspensions of large and small plaque material were tested in serum neutralization breakthrough tests utilizing virus dilutions and undiluted WEE-Fleming hyperimmune mouse ascitic fluid obtained from the Reference Reagents Branch, NIAID, NIH. This ascitic fluid had been tested against a variety of arboviruses and adventitious murine viruses and was known to be free of antibody for these agents. The ascitic fluid neutralized both the large and small plaque types (Table 13). Plaques that formed in the presence of immune ascitic fluids were found to be WEE virus. One large plaque type (PL-148) and one small plaque type (PL-149) were harvested for other studies.

The next experiments were designed to determine which plaque type was "atypical" for the WEE CI-15 vaccine. Therefore, the original (BHK_5CECC_1) WEE B628 virus (from which Cl-15 was derived); the Lederle produced Master Seed of WEE Cl-15, and the Final Unclarified Vaccine were plaqued in CECC and the plaque sizes compared. The results are shown in Table 14. From these results, it was found that the small plaque type was the "atypical" virus.

The results of the animal inoculation experiments raise some questions concerning the small plaque type. The Final Vaccine did not produce clinical infection in monkeys, guinea pigs, or weanling mice, but did produce sporadic disease in hamsters inoculated intracerebrally and intraperitoneally. Hamster illness and death was not seen when the Master Seed Virus was inoculated similarly; however, this may reflect a titer difference between the two viruses. Interestingly, virus isolated from the brain of infected hamsters was predominantly of the small plaque type, but some large plaques were also observed.

In order to determine if the development of two plaque types was an isolated occurrence or could be repeated, studies were undertaken to repeat the vaccine production procedure. The procedures used in preparing the Final Vaccine were followed. Briefly, 9 day chicken embryo cells were plated at 3×10^6 cells/ml, incubated overnight in Hanks' 199 with 10% fetal calf serum (Rehautin, Reheis Chemical Corp., Kankakee, Illinois). The cells were then washed, inoculated with about 10^3 infectious units of Master Seed Virus, and incubated in Hanks' 199 with 0.5% human serum albumin. Twenty-four hours later the virus was harvested and passed one additional time under similar conditions to produce a "Final Unclarified Vaccine." Again we were able to detect two plaque types in the "Final Vaccine," the material titered 7×10^8 small (2-3 mm) PFU and 1.7×10^9 large (5 mm) PFU per milliliter. Thus, it appeared that the development of the two plaque morphologies was characteristic of the WEE Cl-15 virus under the conditions used.

The stability of the large and small viral types was tested by serial passage of each plaque type through CECC agar overlay systems for three passages. Invariably, the small plaque variant produced both large and small plaques, while the large plaque virus appeared to produce only large plaques. However, the large plaque virus may simply mask the small plaques and, therefore, we cannot be sure that the large plaque virus reproduces true.

In other studies a large plaque virus (Pl-148) and a small plaque virus (Pl-149) were selected from serum neutralization breakthrough test. These viruses were passed in fluid CECC five times. The temperature of incubation did not appear to affect the development of plaques. Both Pl-148 and Pl-149 failed to plaque at 40 C; whereas both do plaque at 35 and 30 C (Table 15).

Finally, the virulence characteristics of these two viruses was examined in animals. The Final Unclarified Vaccine and the small plaque viruses produce death in hamsters. Also, there appeared to be some evidence of selection for hamster virulence in that small plaque virus isolated from hamster-brain material was more virulent for hamsters than the original vaccine. Both the Pl-148 and the Pl-149 viruses were passed through fluid CECC five times. Passage material was tested for virulence and for titer in suckling and weanling mice and 50-60 g hamsters. The results are shown in Table 16. Again, it appears as if the small plaque, Pl-149, virus may become more virulent for hamsters and weanling mice with passage. Conversely, the large plaque, Pl-148, virus appeared to retain its virulence characteristics through five passages.

Conclusions and Recommendations

Approximately 350 vials (3,500 doses) of the Packaged Vaccine has been prepared and about 70,000 ml (150,000 doses) of undiluted Final Clarified Vaccine are available if needed.

All of the results obtained indicated the WEE Cl-15 virus would be a reasonable candidate for a human and animal vaccine. Such a vaccine has potential advantages over a killed vaccine in that (1) less material would be required to produce large volumes of vaccine quickly, (2) the live virus initiates immunity much quicker than killed vaccines, and (3) presumably the live virus vaccine could produce a longer lasting immunity. Certainly, if an explosive epizootic and epidemic of WEE virus occurred similar to the 1971 Venezuelean equine encephalitis virus (VEE) epizootic in the U.S., an available live virus vaccine would be of great use.

The Final Vaccine produced in the present study is potent, free of detectable bacterial, fungal, and mycoplasmal contaminants and free of adventitious viral agents by the methods used in testing. The Final Vaccine does not appear to be neurovirulent for adult monkeys and guinea pigs, or weanling mice. However, the vaccine does contain some neurovirulence character for young hamsters and newborn mice. Inoculation of animals with the vaccine does elicit the production of protective antibodies. Thus, the WEE Cl-15 Final Vaccine appears to be similar in potency and neurovirulence characteristics to the TC-83 strain of Venezuelean equine encephalitis virus.

The most disquieting fact concerning the present lot of WEE Cl-15 Final Vaccine is the presence of two plaque variants in the vaccine. Although both the large and the small plaque viruses appear to be strains of WEE virus, clearly the small plaque mutant is virulent for hamsters, and it does have the propensity to easily give rise to the large plaque type--thus, indicating that the small plaque mutant is capable of "reversion" to the large plaque.

Because the Final Vaccine does contain two plaque variants of WEE virus, in the opinion of this investigator the vaccine should not be used

as a routine vaccine for man. Studies should be undertaken to develop a monotypic virus population for any vaccine being considered for human utilization. Possibly, the vaccine could be safely utilized in horses; however, additional studies in this species need to be initiated.

STUDIES WITH EEE VIRUS

Introduction

The need for an effective EEE viral vaccine for man has been known for many years. As early as 1941 Beard,et al. (8) reported on the vaccination of man with a killed EEE vaccine. Other killed vaccines for EEE virus have been developed by Randall et al. (9), Maurer et al. (10) and Lowenthal et al. (11). Many of the inactivated vaccines have been effective for the immunization of domestic animals; however, because of the need to use multiple injections, the poor immune response elicited, and difficulties of achieving completely inactivated virus, these vaccines havenot been entirely satisfactory for use in man.

The possibility of developing a strain of EEE virus suitable for use as a vaccine is supported by the fact that other Group A arboviruses have been successfully attenuated. An attenuated strain of Venezuelean equine encephalitis (VEE) virus has been developed as a vaccine for use in man and horses (12, 13, 14), and an attenuated strain of WEE virus has been developed and field tested in a limited number of horses (2, 5, 7). Both of these vaccines were obtained by serial passage in laboratory cell culture systems.

Other workers have shown that chemical mutagens can be used with certain Group A arboviruses such as Sindbis and Semliki Forest to induce mutant strains of these viruses (15, 16). Although these chemically induced mutants have not been studied for their vaccine potential, it is reasonable to assume that the methods employed might be useful in selecting a strain of EEE virus suitable as an attenuated vaccine. Thus, the present studies were initiated to attempt to select an avirulent strain of EEE virus which could serve as a vaccine candidate strain.

Materials and Methods

Viruses:

Two strains of EEE virus were chosen for preliminary studies: Arth 167-SPM and BeAn 221. The Arth 167-SPM and the BeAn 221 Strains of EEE virus were selected somewhat arbitrarily. The Arth 167-SPM virus was isolated by one of us (PHC) in 1962 and is known to be less virulent than the parent strain. This virus will infect weanling mice and hamsters without killing the animals and will produce protective antibodies in these animals (Coleman, P. H., unpublished data). The Arth 167-SPM virus does not produce protection in guinea pigs vaccinated with the virus. Thus, it is evident that this virus needs further study to determine its potential as a vaccine candidate strain.

The Arth 167-SPM isolate used in the present studies was obtained from the WHO Regional Arbovirus Reference Center for the Americas, CDC, Atlanta, Georgia. This isolate was originally made from a pool of mosquitoes,

Culeseta melanura, collected in 1950 in Louisiana (17). As obtained in our laboratory, the virus has been passed extensively: weanling mice, intracerebrally, 2; embryonated chicken eggs, 1; de-embryonated chicken eggs, 50; primary hamster kidney cell cultures, 15; and duck embryo cell cultures, 2. By clonal selection during the duck embryo passage a small plaque mutant was isolated. This mutant is known to have reduced pathogenesis for weanling mice inoculated peripherally or intracerebrally. In addition, the Arth 167-SPM isolate antigenically resembles other North American isolates when tested by Casals' hemagglutination test (18).

The BeAn 221 strain was selected because it is a South American isolate and, although it is known to be antigenically distinguishable from North American EEE strains, it does produce neutralizing antibodies against both South American and North American viruses (18). A South American isolate was selected because epidemiological studies in South America have shown that although EEE virus is prevalent, and both man and animals have antibody to the virus, there is no reported evidence of large scale clinical epidemics or epizootics of EEE disease in the areas of viral activity.

The BeAn 221 strain was obtained from Dr. R. Shope, WHO Arbovirus International Reference Center, Yale University. This isolate was originally obtained from sentinal monkeys in Brazil in 1955 (19). The virus had been passed 5 times intracerebrally in suckling mice.

Animals:

All animals were obtained from commercial sources. The ICR strain of mice (Flow Laboratories, Inc., Dublin, Virginia) was used throughout the study. Where suckling mice were used, pregnant mice were purchased and allowed to litter in our facilities. In addition, weanling ICR mice (18-20 g), adult guinea pigs (300 g), and Syrian hamsters (50-100 g) were used. Male Rhesus monkeys (2-4 kg) were obtained from Primate Imports, Inc., Port Washington, New York. All animals were fed the appropriate Purina feed ad libitum and provided water ad libitum.

Cell Cultures:

For chicken embryo cell cultures (CECC), fertile, specific pathogen free eggs were obtained from SPAFAS, Inc., Norwich, Connecticut. Primary CECC were prepared from 9-11 day embryos. Embryos were harvested aseptically, decapitated and delimbed. Embryos were minced with scissors, and trypsinized from 1-2 hours in 0.25% trypsin in isotonic glucose-KCl-NaCl solution. Trypsinized cells were filtered through eight layers of gauze and plated at a 1:150 dilution in disposable plastic culture bottles. Growth media consisted of antibiotic free Hanks' balanced salt solution (HBSS) containing 5% fetal calf serum, 0.1% yeastolate (Difco), phenol red, sodium bicarbonate. Cells were grown at 36 C.

For virus production, monolayers of CECC were rinsed three times with HBSS and inoculated with virus. After an absorption period of 1 hour, infected cells were maintained at 36 C with Scherer's Maintenance Solution (Microbiological Associates) containing 1% gelatin (Difco), 0.5% lactalbumin hydrolysate, sodium bicarbonate and without antibiotics.

For purification and titration studies, the agar-overlay plaque system was used. Infected monolayers were overlayed with Earle's balanced salt solution (EBSS) containing 2% newborn calf serum, 1% Noble agar, 1.65 mg lactalbumin hydrolysate per ml, and sodium bicarbonate without antibiotics. After 48 hours of incubation, a second overlay containing 5 μg neutral red per ml was added and the plaques examined after 24 hours. All plaque titrations were done in duplicate and the viral titers expressed as plaque forming units (PFU) per ml. For plaque purification studies, plaques were "picked" utilizing a sterile Pasteur pipette, and the harvested material was suspended in one ml of phosphate (0.015 M) buffered saline (0.15 M), pH 7.2-7.6, containing 4.0% bovalbumin fraction V (Armor).

In studies with N-methyl-N'-nitro-N-nitrosoquanidine 200 μg of the chemical was added to undiluted viral stocks. At selected intervals, samples of virus were removed and titrated in agar overlay CECC cultures. The amount of surviving virus was calculated as the virus titer in PFU.

Results

Both strains of EEE virus were "purified" by three plaque passages. The original stock viruses were inoculated initially into fluid CECC cultures to prepare about 100 ml of virus for plaque purification. Before plaque purifying the virus, preliminary studies were conducted to determine whether any differences could be seen in EEE viral plaque formation between a single overlay system containing neutral red and a double overlay system in which the neutral red is included in the second overlay only. In several studies it was found that both the Arth 167-SPM and the BeAn 221 strains gave about two-fold higher titers in the double overlay system. Thus the double overlay method was selected for plaque purification and titration of the viruses.

For the plaque purification of these two viruses, ten-fold dilutions of each of the CECC passaged viruses were inoculated in duplicate onto monolayers of CECC and agar overlayed. At that dilution which produced between 5 and 30 plaques, four plaques were "harvested" individually and stored in one ml of diluent at -70 C. Ten-fold dilutions of one of the harvested plaques were then prepared and inoculated into fresh CECC for agar overlay. Again four plaques were harvested, and the plaque purification procedure was repeated one additional time. Material harvested from the third plaque passage was inoculated into large fluid monolayer cultures and after 48 to 72 hours harvested, dispensed in small samples and stored at -70 C as the working seed virus preparation. The results of the plaque purification study are shown in Table 17. Both purified viruses were identified as EEE virus by neutralization.

Titration of the working seed virus in CECC and intracerebrally in newborn mice showed that the Arth 167-SPM had a titer of 4.7×10^7 PFU/ml and $10^{8.3}$ LD_{50}/ml in mice.

The BeAn 221 strain only titered 2×10^3 PFU/ml in CECC and less than $10^{3.5}$ $icLD_{50}$ in newborn mice. Therefore, a fresh working seed lot of this virus was prepared. Again the titer was low; only 6×10^3 PFU/ml in CECC. Thus, a new lot of the BeAn 221 working seed virus was prepared which titered a 10^7 PFU/ml in CECC.

Studies with the Arth 167 Small Plaque Mutant (SPM) Strain of EEE

Studies with Nitrosoguanidine:

Routinely, about 99% of the PFUs of the Arth 167-SPM virus could be inactivated by mixing 200 μg of nitrosoguanidine (NTG) with a cell culture suspension of virus. The mixture was incubated for up to 60 minutes at room temperature. Dilutions of the treated virus were plaqued on CECC in replicate cultures incubated at 36 and 42 C. After 48 hours, cultures were examined for differences in plaque number of plaque morphology and compared with untreated controls. Several hundred cultures were examined by this method without detecting any evidence of viral mutation. It appears that the individual EEE virions were either inactivated, or unaffected, or at least plaquing capabilities were not altered. Results of a typical experiment are shown in Table 18.

In other studies, virus was absorbed to monolayers of CECC and the cultures incubated at 35 C for either four or six hours in maintenance medium. After incubation, the medium was decanted and maintenance media containing 100 μg, 300 μg, or 500 μg of NTG per ml were added to the infected monolayers. After one hour, the NTG medium was decanted, fresh medium added, and cultures incubated at 36 C for an additional 6 to 20 hours. The harvested virus was titrated in overlayed cultures of CECC at 42 or 36 C. Again, no visual differences were noted in the plaque numbers or morphology of plaques between the two temperatures, but up to 5 logs of PFUs were inactivated in this procedure.

Studies with the BeAn 221 Strain of EEE Virus:

Because of the failure to produce a usable EEE vaccine with the Arth 167-SPM strain of EEE virus, studies were begun with the BeAn 221, South American Virus.

Initially, the virus was plaque purified by four passages at terminal dilution in CECC. Subsequently, the virus was passed in CECC cells, periodically plaqued at 30, 36 and 40 C and the plaques observed for "atypical" appearance. In addition, several experiments were conducted utilizing nitrosoguanidine in an attempt to develop an attenuated virus. We did not observe any obvious differences between plaques produced following nitrosoguanidine treatment and controls.

In animal virulence studies (Table 19), the eighth CECC passage of the BeAn 221 strain virus was inoculated into several species of laboratory animals. This virus titered about $10^{7.5}$ LD_{50}/ml when inoculated into newborn mice via either the intracerebral or intraperitoneal route.

In weanling mice, the virus titered $10^{6.5}$ LD_{50}/ml when inoculated via the intracerebral route, but only $10^{1.0}$ when inoculated intraperitoneally. Similarly, hamsters inoculated intraperitoneally with 10^6 mouse LD_{50} of virus developed encephalitis and died, while 300-350 gram guinea pigs were not affected following intraperitoneal inoculation of the same dose of virus. Thus, it appears that the BeAn 221 strain of EEE virus may be less virulent for animals than naturally occurring North American isolates.

The stock virus pool was tested on several occasions for evidence of its mutants by plaquing the virus at 30, 35, and 40 C. In general, no significant differences were noted in virus titers or plaque morphology (except plaques were smaller at 30 than 35 or 40 C). Similarly, no differences were noted in titers or plaque morphology when the virus was titrated at 30, 35, and 40 C using Nobel agar or Argose agar in the overlay media.

Consistently, different plaque sizes were demonstrable in culture vessels with large numbers of plaques. Invariably some plaques were smaller in size than the general population. Plaques harvested and passed produce parental type plaques indicating that the size variation seen is primarily a function of "crowding" with the virus and not a viral characteristic.

In other studies, the BeAn 221 stock virus was treated with nitrosoguanidine (NTG) and with 5-fluorouracil (5FU) to determine if mutants could be selected. The majority of our work in this connection was preliminary in nature to determine time, temperature, dose, and procedural effects. Results of a typical study with NTG is shown in Table 20. Basically, NTG reduced the titer of the EEE virus, but in the studies conducted to date, we were unable to demonstrate any significant alterations in plaque morphology of NTG treated material. 5-FU was similarly disappointing in preliminary tests.

In other studies the BeAn 221 virus was serially passed in 9-11 day old chicken embryonated eggs and in chicken embryo cell cultures. Periodically, material from selected passages was tested for plaque morphology, as well as virulence in weanling mice. No evidence of an attenuated virus was detected.

Conclusions and Recommendations

In attempts to develop a strain of attenuated EEE vaccine, we have concluded that the Arth 167-SPM virus is not suitable human vaccine candidate. This virus appeared to be attenuated in that it has lost some of its ability to "cross" the blood brain barrier of infected animals. However, when inoculated directly into the brains of animals, the virus appeared to as neurovirulent as are wild strains of virus. Furthermore, multiple attempts to further attenuate the virus with chemical mutagens and/or plaque selection proved futile. Therefore, studies were initiated utilizing the BeAn 221 strain of EEE virus.

After about 10 passages in cell culture, the BeAn 221 strain of EEE virus is not highly virulent for laboratory animals inoculated peripherally, but still retains virulence characteristics when inoculated intracerebrally.

Because of the need for an avirulent vaccine strain of EEE virus for use in man and animals, it is recommended that studies with the BeAn 221 virus be continued in an effort to select a suitable vaccine candidate.

Table 1: Passage History of the Western Equine Encephalitis Virus B628 Strain

Passage	Remarks
Isolated in Primary Hamster Kidney Cells. (Johnson) (HK_1)	Originally isolated in 1957 from tissues of a house sparrow, <u>Passer domesticus</u> collected in Kern County, California.
Passed Three Times in Primary Hamster Kidney Cells (Johnson) (HK_4)	
Passed 30 times in Primary Chicken Embryo Cell Cultures (Johnson) (HK_4CECC_{30})	The attenuated B628 (Clone-15) strain was obtained at this level
Passed One Time in Primary Chicken Embryo Cells (Johnson) (HK_4CECC_{31})	This material sent to Lederle
Passed Two Times in Fertile Chicken Eggs (Lederle) ($HK_4CECC_{31}CE_2$)	This material used to make equine vaccine
Passed Two Times in Primary Chicken Embryo Cells (Lederle) ($HK_4CECC_{31}CE_2CECC_2$)	This is the lyophilized product used as Master Seed Virus in the present studies.
Passed One time in Primary Chicken Embryo (Cofal) Cells (Coleman) ($HK_4CECC_{31}CE_2CECC_3$)	This is the Working Seed Virus
Passed One Time in Primary Chicken Embryo (Cofal) Cells (Coleman) ($HK_4CECC_{31}CE_2CECC_4$)	This is the Final Vaccine Virus

Table 2. Comparative Titers of WEE B628 Virus in Animals and Chicken Embryo Cells.

Virus	CECC (PFU/ml)	Hosts* Newborn Mice (LD_{50}/ml)	3-4 Week Mice (LD_{50}/ml)	Hamsters (100 g) deaths/inoculated
WEE B628 Orig. (HK_5CE_2)	8.8×10^6	1.4×10^6	7.2×10^3	6/ /6
WEE C1-15 Master Seed	3.4×10^6	1.2×10^6	0	0/ /6
WEE C1-15 Final Clarified Vaccine	9.0×10^8	2.0×10^8	0	0/ /6

*Newborn and weanling mice inoculated intracerebrally with 0.02 and 0.03 ml, respectively, of undiluted and ten-fold dilutions of virus. Hamsters inoculated with 0.1 ml intracerebrally of undiluted virus only.

Table 3. Virulence of WEE B628 Strain (Lederle) Vaccine.

HOST			DILUTIONS									TITER LOG_{10}
Bottle No.	Species	Route	UND	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	LD_{50}/ml
1	Mice 1-3 day	ic	8/8	8/8	8/8	8/8	6/8	0/8	0/8	0/8	0/8	6.0
		ip	8/8	8/8	8/8	8/8	5/8	0/8	0/8	0/8	0/8	5.9
	Mice (3-4 wk)	ic	0/8	0/8	0/7	0/7	0/8	0/8	0/8	-	-	
		ip	0/8	0/8	0/8	0/8	0/8	-	-	-	-	
	Hamsters (ca 100 g)	ic	0/7	-	-	-	-	-	-	-	-	
		ip	0/8	-	-	-	-	-	-	-	-	
	G. Pigs (ca 350 g)	ic	0/5	-	-	-	-	-	-	-	-	
		ip	0/6	-	-	-	-	-	-	-	-	
2	Mice (1-3 day)	ic	8/8	8/8	7/7	7/8	5/8	0/8	0/8	0/8	0/8	5.8
		ip	8/8	5/5	8/8	8/8	4/8	1/8	0/8	0/8	0/8	5.8
	Mice (3-4 wk)	ic	0/8	0/8	0/6	0/8	0/8	0/8	0/8	-	-	
		ip	0/8	0/8	0/8	0/8	0/8	-	-	-	-	
	Hamsters (ca 100 g)	ic	0/7	-	-	-	-	-	-	-	-	
		ip	0/8	-	-	-	-	-	-	-	-	
	G. pigs (ca 350 g)	ic	0/6	-	-	-	-	-	-	-	-	
		ip	0/6	-	-	-	-	-	-	-	-	

Table 3. Virulence of WEE B628 Strain (Lederle) Vaccine (cont..)

HOST			DILUTIONS									TITER LOG_{10}
Bottle No.	Species	Route	UND	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	$LD_{50/ml}$
3	Mice (1-3 day)	ic	8/8	8/8	*	8/8	6/8	1/8	0/8	0/8	0/8	6.1
		ip	8/8	8/8	8/8	5/6	5/8	0/8	0/8	0/8	0/8	5.8
	Mice (3-4 wk)	ic	0/8	0/8	0/8	0/8	0/8	0/7	0/8	-	-	
		ip	0/8	0/8	0/8	0/8	0/8	-	-	-	-	
	Hamsters (ca 100 g)	ic	0/8	-	-	-	-	-	-	-	-	
		ip	0/8	-	-	-	-	-	-	-	-	
	G. Pigs (ca 350 g)	ic	0/6	-	-	-	-	-	-	-	-	
		ip	0/6	-	-	-	-	-	-	-	-	

* Litter died.
- Not done.

Table 4. Detectable Viremia (PFU) in Guinea Pigs and Hamsters Following Inoculation with ca 10^4 $SMicLD_{50}$ of WEE B628 Lederle Vaccine

Animal No.	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Guinea Pigs inoculated ip							
11	0*	10	0	0	0	0	0
12	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0
22	8	0	0	0	0	0	0
31	6	0	0	0	0	0	0
32	0	5	0	Died (Anesthesia)			
33	0	0	0	0	0	0	0
Guinea Pigs inoculated ic							
71	2	2	0	0	0	0	0
72	0	0	0	0	0	0	0
81	0	0	0	0	0	0	0
82	2	0	0	0	0	0	0
91	1	0	0	0	0	0	0
92	0	0	0	0	0	0	0

Animal NO.	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Hamsters inoculated ip							
111	5	0	0	0	0	0	0
112	18	0	0	0	0	0	0
113	21	0	0	0	0	0	0
114	0	0	0	0	0	0	0
121	0	0	0	7	0	0	0
122	0	0	7	2	0	0	0
123	10	1	0	0	0	0	0
124	0	0	2	2	0	0	0
131	1	0	0	0	0	0	0
132	4	0	0	0	0	0	0
133	0	57	0	0	0	0	0
134	3	Died Trauma					
Hamsters inoculated ic							
141	7	0	0	2	0	0	0
142	4	17	0	0	0	0	0
143	26	Died trauma					
144	9	0	0	0	0	0	0
151	4	0	0	0	0	0	0
152	6	0	0	0	0	0	0
153	2	0	0	0	0	0	0
154	0	7	0	0	0	0	0

*Plaque forming units per .05 ml of serum.

Table 5. Challenge of Animals Six Weeks after Vaccination with the Lederle WEE Vaccine.

	Vaccinated with WEE B628		Challenged ic with 10^3 $WMLD_{50}$ of WEE Fleming	
Species	Dose	Route	Died	Survived
Guinea Pigs	10^4 $SMLD_{50}$	ip	0	8
	10^4	ic	0	5
	-	-	8	0
Hamsters	10^4	ip	1*	10
	10^4	ic	0	7
	-	-	10	0
Mice (weanling)	10^4	ip	1	9
	10^3	ip	8	2
	10^2	ip	10	0
	10^1	ip	10	0
	10^4	ic	0	10
	10^3	ic	1	9
	10^2	ic	0	10
	10^1	ic	9	1
	-	-	19	0

*Hamster No. 131

Table 6. Preliminary Testing of the WEE B628 Strain of Working Seed Virus of Final Unclarified Vaccine-Virus.

Test	Working Seed Virus	Final Vaccine Virus
Titration in CECC agar overlays	10^8 PFU/ml	10^9 PFU/ml
Growth on Blood Agar Plates (10 ml)	Negative	Negative
Growth in Thioglycolate (10 ml)	Negative	Negative
Pathogenesis in 20 adult mice inoculated intracerebrally	No symptoms	No symptoms
Pathogenesis in 4 Rhesus monkeys inoculated intracerebrally	No symptoms	No symptoms
Mycoplasma testing (Done at USAMRIID)	Negative	Negative

Table 7. Responses of Monkeys Inoculated Intracerebrally with 0.2 ml of the WEE B628 Final Clarified Vaccine Virus

Day tested	Monkey No 1 Temp.	V	Ab	Monkey No 2 Temp	V	Ab	Monkey No 3 Temp	V	Ab	Monkey No 4 Temp	V	Ab
0	103.4	-	-	102.6	-	-	103.2	-	-	103.4	-	-
1	102.2	-	-	102.4	-	-	103.5	-	-	103.0	-	-
2	102.0	-	-	102.2	-	-	104.2	13	-	104.4	10	-
3	102.8	-	-	102.4	-	-	104.6	-	-	104.8	2	-
4	102.6	-	+	102.0	-	+	103.0	-	+	104.4	-	+
5	104.2	-	+	102.8	-	+	102.8	-	++	106.5	-	+
6	105.2	-	++	102.4	-	++	104.6	-	++	105.2	-	++
7	104.2	-	++	102.4	-	++	103.6	-	++	103.2	-	++
8	103.6	nt	nt	102.6	nt	nt	103.4	nt	nt	103.6	nt	nt
9	103.4	nt	nt	102.6	nt	nt	103.4	nt	nt	103.2	nt	nt
10	103.6	nt	nt	102.4	nt	nt	103.6	nt	nt	103.8	nt	nt
14	103.2	-	++	102.4	-	++	103.4	-	++	103.4	-	++

Monkeys inoculated intracerebrally with 0.2 ml of undiluted Final Vaccine Virus on day 0. Venus blood specimens were collected prior to inoculation, for 7 days after inoculation and at 2 weeks after inoculation.
V= viremia, - = negative, numbers are PFU obtained in 0.1 ml of serum.
Ab = antibody, - = negative, + = at least 50% reduction in PFUs of the WEE 628 strain; ++ = greater than 90% reduction
nt = no test

Table 8. Neurovirulence Studies in Rhesus Monkeys Inoculated Intracerebrally, Intraspinally, and Intramuscularly with the Final Clarified Vaccine Preparations of WEE Cl-15 Virus.

Monkey Number	Weights (Kg)		Clinical Signs	Viral Neutralization		Histopathology
	Day 1	Day 21		Pre-serum	Post-serum (21 day)	
1 (control)	4.2	4.4	None	$<10^{1}$	$<10^{1}$	None, i.e. cerebrum, cerebellum & stem, basal ganglion - all negative
2	318	4.1	None	$<10^{1}$	$10^{3.0}$	Single mononuclear (MN) cuff, venial cerebrum pia. Cord, roots and nerve-negative.
3	3.5	3.8	None	$<10^{1}$	$\geq 10^{3.5}$	No evidence of histopathology noted.
4	4.5	4.2	None	$<10^{1}$	$\geq 10^{3.5}$	Very minimal MN infiltration of cerebellar meninges & a few perivascular MN infiltration around needle tract. Gleiosis. Area of necrosis around needle tract. Cord - negative
5	3.3	3.2	None	$<10^{1}$	$\geq 10^{3.5}$	Perivascular mononuclear infiltration, also in basal ganglion. Cord - negative
6	3.9	4.0	None	$<10^{1}$	$\geq 10^{3.5}$	Mild to moderate perivascular cuffing with MN infiltration in brain. Cord - negative
7	4.1	4.3	None	$<10^{1}$	$\geq 10^{3.5}$	Areas of necrosis and gleiosis. Needle tract - negative. Cord - negative
8	4.1	4.1	None	$<10^{1}$	$\geq 10^{3.5}$	Rusty cyst in right frontal area, which was not extensive and could be needle tract.

Table 8. Cont.

Monkey Number	Weights (Kg) Day 1	Day 21	Clinical Signs	Viral Neutralization Per-serum	Post-serum (21 day)	Histopathology
9	2.5	2.4	None	$<10^1$	$\geq 10^{2.9}$	Some necrosis around needle tract, No other pathology seen.
10	3.1	3.1	None	$<10^1$	$\geq 10^{2.9}$	Some necrosis around needle tract. No other pathology seen.
11	3.6	3.7	None	$<10^1$	$\geq 10^{2.9}$	Mild focal meningtitis (lymphocytic) in one side of cerebrum. Necrotic needle tract. Cord negative.
12	3.7	3.4	Paresis* rt. arm for 4 days PI	$<10^1$	$\geq 10^{2.9}$	Very mild perivascular lymphocytic cuffing, cord. Needle tract seen. Tract extended into brain stem.
13	3.8	3.7	None	$<10^1$	$\geq 10^{2.9}$	Evidence of needle tract in mid-brain. No other pathology.
14	3.2	3.3	None	$<10^1$	$\geq 10^{2.9}$	Minimal perivascular lymphocytic cuffing in brain stem. Needle tract not identified. Cord negative
15	2.9	2.8	None	$<10^1$	$\geq 10^{2.9}$	Necrotic needle tract. Cord negative
16	3.3	3.2	None	$<10^1$	$\geq 10^{2.9}$	Mild lymphocytic meningitis in area of cerebrum. Minimal lymphocytic cuffing of basal ganglia. Needle tract not identified. Cord negative
17	2.7	2.7	None	$<10^1$	$\geq 10^{2.9}$	Rare vessels show perivascular cuffing. Needle tract not identified. Cord negative.
18	2.6	2.8	None	$<10^1$	$\geq 10^{2.9}$	No lesions seen. Needle tract not identified.

Table 8 Cont.

Monkey Number	Weights (Kg) Day 1	Weights (Kg) Day 21	Clinical Signs	Viral Neutralization Pre-serum	Viral Neutralization Post-serum	Histopathology
19	3.2	3.4	None	$<10^{1}$	$\geq 10^{2.9}$	Probable needle tract detected. Rarefaction of one peduncle of cerebellum. Microgranuloma in cord.
20	2.6	2.6	None	$<10^{1}$	$\geq 10^{2.9}$	No lesions seen. Needle tract indentified.
21	2.8	3.1	None	$<10^{1}$	$\geq 10^{2.9}$	Necrosis in needle tract. Perivascular cuffing in dorsal pons. Cord negative.
22	2.8	2.8	None	$<10^{1}$	$\geq 10^{2.9}$	No lesions detected. Needle tract not idintified
23 (control)	2.4	2.4	None	$<10^{1}$	$\leq 10^{1}$	No lesions seen
24 (control)	3.6	3.7	None	$<10^{1}$	$\leq 10^{1}$	Not tested.

Table 9. Safety Testing of the WEE, Cl-15 Undiluted Final Unclarified Vaccine in Animals.

Species	No.	Inoculum Dose (ml)	Inoculum Route	Clinical Disease	Protection Results (ic challenge with WEE Flem)
15-20 g Weanling Mice	24	0.03 ml 0.5 ml	ic ip	None	Protected against 300-30,000 $WMLD_{50}$ *
350 g Guinea pigs	20	0.1 ml 0.5 ml	ic ip	None	Protected against 100,000 $WMLD_{50}$ **
55-65 g Hamsters	20	0.1 ml 0.5 ml	ic ip	12 developed paralysis and died	Not done

*Eight mice challenged with 300 $WMicLD_{50}$ 8 mice with 3,000 $WMicLD_{50}$ and 8 with 30,000 $WMicLD_{50}$. All mice challenged 28 days after inoculation with the Final Unclarified Vaccine.

**Nine guinea pigs were challenged by ic inoculation of 100,000 $WMicLD_{50}$ of WEE virus Fleming 21 days after inoculation with the Final Unclarified Vaccine.

Table 10. Hamsters (50-65 g) Inoculated witn 0.1 ml Intracerebrally and 0.5 ml Intraperitoneally with the Indicated WEE Virus.

Inoculum	Number of Hamsters	Results
ca. 10^9 PFU/ml of Final Unclarified Vaccine.	20	One died on the 6th, 8th, & 9th day post-inoculation.
ca. 10^9 PFU/ml of Final Unclarified Vaccine mixed with equal parts of WEE Fleming antisera	10	No clinical infection
ca. 10^6 PFU/ml of Master Seed Virus	10	No clinical infection
ca. $10^{1.4}$ PFU/ml of Final Unclarified Vaccine Hamster 1 Passage	6	5 died between 7th and 13th day post-inoculation.

Table 11. Bacterial and Fungal Sterility Testing of WEE 628, Cl-15 Master Seed, Working Seed, Final Unclarified Vaccine, and Packaged Vaccine*

Test Medium	Incubation Temperature	Master Seed (5 vials)	Working Seed (Bulk)	Unclarified Vaccine (Bulk)	Packaged Vaccine (20 vials)
Mycoplasma (tested by USAMRIID following standard Methods)	--	NT	--	--	NT
Fluid Thioglycollate Medium (Difco #569201 with .001% fresh Resazurin)	35	5 ml	10 ml	10 ml	10 ml
	20±2	5 ml	10 ml	10 ml	10 ml
Soybean-Casein Digest Medium (Tryptic Soy Broth, Difco #608012)	35	5 ml	10 ml	10 ml	10 ml
	20±2	5 ml	10 ml	10 ml	10 ml
Fluid Sabouraud Dextrose Medium (Difco #540993)	35	NT	10 ml	10 ml	10 ml
	20±2	NT	10 ml	10 ml	10 ml
Lowenstein-Jensen Medium (agar slants Difco)	37 (5% CO_2)	5 ml**	10 ml**	10 ml**	10 ml**
Fluid Peptone-Yeast Extract Glucose Medium (pre-reduced)	35	NT	5 ml	5 ml	NT
	20±2	NT	5 ml	5 ml	NT

* All tests were negative. No evidence of bacterial or fungal contamination by gross examination or by Gram stain of sediments from broth culture media.

** No evidence of growth after eight weeks of incubation.

Table 12. Safety testing of WEE, C1-15 Final Unclarified Vaccine in Cell Cultures Systems.

Cell Culture	Inoculum	Results* Viral Dilutions 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Human Diploid Cells (WI-38)	Virus	NT	NT	5/5	5/5	5/5	4/5	1/5	0/5
	Virus + serum***	1/5	0/5	0/5	0/5	0/5	NT	NT	NT
African Green Monkey Kidney Cells (BSC-1)	Virus	NT	NT	5/5	5/5	5/5	3/5	2/5	0/5
	Virus + serum	0/5	0/5	0/5	0/5	0/5	NT	NT	NT
Primary Rhesus Monkey Kidney Cells**	Virus	NT	NT	0/5	0/5	0/5	0/5	0/5	0/5
	Virus + serum	0/5	0/5	0/5	0/5	0/5	NT	NT	NT

*Results expressed as number of tubes showing cytopathic effects/ number of tubes inoculated after 14 days of incubation at 35 C.

**Rhesus monkey cells were observed for only 10 days because of deterioration of the inoculated controls.

***Immune serum used is WEE Fleming horse antiserum obtained from CDC, Atlanta, Georgia.

Table 13. Serum Neutralization Breakthrough Test with the WEE, Cl-15 Final Unclarified Vaccine

Viral Dilutions (10⁻)	Number of Plaques Observed			
	Control Serum		NIH-Mouse Hyperimmune ascitic fluid	
	large	small	large	small
3	NT		6*	10*
4	NT		0	12
5	TNTC		0	2
6	13	23	NT	
7	1	4	NT	
8	0	1	NT	

*Virus from plaques harvested from both large and small types were identified as WEE virus by subsequent neutralization. One large plaque type (subsequently labeled Pl-148) and one small plaque type (subsequently labeled Pl-149) were isolated from this plate.

NT=not tested; TNTC = too numerous to count.

Table 14. Comparison of Plaque Sizes in CECC Inoculated with WEE, Cl-15 Final Vaccine, WEE Cl-15 Master Seed, and the Original WEE B628 Virus.

Inoculum (Number of PFU)	Plaque Sizes (MM) at 48 - 72 hours		
	Final Unclarified Vaccine	Master Seed	Original
0 - 10	0.75 - 1.0	2 - 3	2 - 3
10 - 100	0.75 - 1.0	2 - 3	2 - 3
100 - 1000	0.75 - 1.0 and 2 - 3	Confluent	Confluent

Table 15 Effect of Temperature on Plaque Production, Using the Fifth Passage of P1-148 and P1-149 (at 48 hours)

Virus	Temperature and Plaque Size					
	40 C		35 C		30 C	
	> 1 mm	< 1 mm	> 1 mm	< 1 mm	> 1 mm	< 1 mm
P1-148 (large)	0	0	8×10^6	0	9×10^6	0
P1-149 (small)	0	0	2×10^6	4×10^6	1.6×10^7	4×10^7

TABLE 16 Virulence of P1-148 and P1-149 in Animals

Virus type	CECC Passage	Host		Inoculum		Results	
		Species	Age or Wt.	Route	Dilution	Deaths/ inoculated	Titer (per ml)
		Mice	2-4 g	ic	10^{-1}-10^{-9}	--	$10^{8.1}$
P1-148	5	Mice	15-20 g	ic	und.	0/8	--
		Hamsters	50-60 g	ic & ip	und.	0/20	--
		Mice	2-4 da	ic	10^{-1}-10^{-9}	--	$10^{7.3}$
P1-149	2	Mice	15-20 g	ic	und.	0/8	--
		Hamsters	50-60 g	ic & ip	und.	0/6	--
		Mice	2-4 da	ic	10^{-1}-10^{-9}	--	$10^{7.8}$
		Mice	15-20 g	ic	10^{-1}-10^{-8}	--	$10^{2.5}$
P1-149	5	Hamsters	50-60 g	ic & ip	und.	20/20	--
		Hamsters	50-60 g	ip	und.	1/6	--
		Hamsters	50-60 g	ic	10^{-1}-10^{-5}	--	$10^{2.5}$
Final	-	Mice	2-4 da	ic	10^{-1}-10^{-9}	--	$10^{9.1}$
Unclarified	-	Mice	15-20 g	ic	und.	0/8	--
Vaccine	-	Hamsters	50-60 g	ic	und.	6/20	--
Control	-	Hamsters	50-60 g	ip	und.	2/20	--

Table 17. Plaque Purification of EEE Virus in Chicken Embryo Cell Culture Systems

	Number of Plaques*						
Passage Number	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Arth 167-SPM							
1	dead	dead	dead	dead	dead	TNTC	28/41**
2	dead	TNTC	98/87	12/15**	--	--	--
3	dead	TNTC	80/NA	15/19**	--	--	--
BeAn 221							
1	dead	dead	dead	dead	TNTC	29/12**	--
2	TNTC	36/23	7/8**	--	--	--	--
3	5/11	3/4**	--	--	--	--	--

*TNTC = Too Numerous To Count; -- = No Plaques; NA = Not Available
**Dilution from which plaques were selected.
28/41 = counts on two different bottles

Table 18. Effect of NTG on the Arth 167-SPM Strain of EEE Virus

	Time in Minutes	Virus Titers
Experiment number 1 NTG was solubilized in buffered saline and the NTG solution was added to the virus to give a final concentration of 200 μg/ml	0	4.7×10^7
	5	1.5×10^6
	10	1.4×10^7
	15	2.8×10^6
	30	7.3×10^7
	60	4.8×10^5
Experiment number 2 NTG powder was added directly to the virus solution to give a final concentration of 200 μg/ml	0	3.6×10^7
	5	1.4×10^7
	10	1.5×10^8
	15	5.0×10^6
	30	6.5×10^5
	60	1.5×10^5

Table 19. Animal Virulence and Cell Culture Titer of EEE BeAn 221 virus. ($Sm_5CEPP_4CECC_4$)

Host	Inoculum		Titer
	Route	Dose	
Mice (2-4 day)	intracerebral	serial dilution	$10^{7.5}$ LD_{50}/ml
Mice (2-4 day)	intraperitoneal	serial dilution	$10^{7.3}$ LD_{50}/ml
Mice (15-20 g)	intracerebral	serial dilution	$10^{6.5}$ LD_{50}/ml
Mice (15-20 g)	intraperitoneal	serial dilution	$10^{1.0}$ LD_{50}/ml
Hamsters (90-110 g)	intraperitoneal	$10^{6.0}$ Mouse LD_{50}	20/20*
Guinea pigs (290-310 g)	intraperitoneal	$10^{6.0}$ Mouse LD_{50}	0/8
Chicken embryo cell culture	---	serial dilution	1 x $10^{7.0}$ PFU/ml

* Number dead/number inoculated

Table 20. Treatment of BeAn 221 Virus with Nitrosoguanidine (NTG)

Treatment**	Plaques*** 10^{-3}	10^{-4}	10^{-5}	Titer PFU/ml	Plaques (at 72 h)
Untreated Control	TNTC	TNTC*	9, 10, 10	9.5×10^6	2-3 mm
Treated in vitro with 100 µg NTG for 1 h	TNTC*	12, 12, 10	1, 0, 3	3.2×10^5	2-3 mm
Treated in vivo with 100 µg NTG for 1 h	TNTC	22, 20, 29*	--	2.5×10^5	2-3 mm

* Plaques harvested and retitered in CECC agar overlay systems and also retreated with NTG and retitered. No significant characteristic change in plaque morphology or temperature sensitivity seen.

** In vitro treatment: Undiluted stock virus was treated with NTG for one hour at room temperature. The virus is then serially diluted and titered. In vivo treatment: Monolayers were inoculated with undiluted virus stock. Four hours later, the maintenance medium was removed and replaced with maintenance medium containing 100 µg of NTG, then cells were reincubated at 35 C. After one hour, the drug was removed, the cells were washed and reincubated with fresh maintenance medium for 20 hours. After reincubation, the infected fluid was titered in CECC agar overlay systems at 30, 35, and 40 C.

*** Triplicate tests run.

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APPENDIX
1

Viral and Rickettsial Research December 9, 1963

Dr. A. W. Moyer **Dr. H. R. Cox**

Confirmation of the attenuation of the strain WEE clone 15, received from Dr. Harald N. Johnson

This strain, which arrived here as chick embryo tissue culture material, received one egg passage in this lab. A batch of seed virus was prepared for further studies and for the preparation of experimental vaccine requested by Dr. Harald N. Johnson.

Experimental Vaccine Preparation - Using seed virus prepared in this lab, two batches of experimental vaccine were produced in 7-day eggs inoculated via the yolk sac with 0.2 ml inoculum; each embryo received about 10^3 MLD_{50} (estimated by I.C. titration in 1-2-day suckling mice). Approximately 36 hours after inoculation, the embryos started to die and by the 48th post-inoculation hour, 50% were dead, at which time the live and dead embryos were harvested and made into a 10% suspension. Heads were discarded. The material was centrifuged for 25 minutes at 1,500 RPM; the supernatant became the final experimental vaccine. Bacteriological sterility was tested in thioglycollate medium. The material was desiccated after being dispensed into 20.0 ml ampoules in amounts of 5.0 ml each. Bacteriological sterility was again tested. Fresh and desiccated materials were titrated in suckling and young adult mice inoculated intracerebrally. The fresh material showed a titer of 10^6 MLD_{50} per 0.02 ml and the desiccated, 5.2×10^5 MLD_{50}/0.02 ml. The young adult mice inoculated intracerebrally with fresh or desiccated material showed no clinical signs during the 21-day observation period and they were resistant to subsequent intracerebral inoculation with a virulent strain of WEE. (Table 4)

Since the criterion of attenuation of the WEE-clone 15 described by Dr. Johnson was based on the loss of pathogenicity for young adult mice inoculated intracerebrally, it was decided to investigate the behavior of this strain in other laboratory animals known to be susceptible to the WEE virus.

Results of Monkey Inoculation - Four Rhesus monkeys of about 4 to 5 pounds were inoculated intracerebrally; each animal received 0.50 ml of 10% chick embryo suspension containing 2.0 X 10^6 MLD_{50} (suckling mouse I.C. doses). The animals were bled before inoculation, daily for 5 consecutive days after inoculation, and at the end of the 1 month observation period. All the animals had viremia on the 1st and 2nd days post-inoculation; only 2 had viremia on the 3rd day and no virus was recovered on the 4th and 5th days. The titer of the viremia was never higher than 10^2 MLD_{50}/0.02 ml. None of the monkeys showed clinical signs during the one-month observation period (Table 1). Two of the animals were sacrificed for histopathological examination of the central nervous system by Dr. E. Jungherr who stated: "On the whole, the lesions were mild but definitely encephalic in character, as indicated by increased intensity in the hemisphere contralateral to the inoculation." Neutralization tests performed on the pre and post-inoculation blood sera showed that all the animals developed antibodies. The post sera neutralized 10^2 MLD_{50} of homologous virus. The two remaining monkeys were challenged intracerebrally 3 months later with a virulent strain of WEE obtained from the Biological Production Section. The results of the challenge inoculation are summarized in Table 2. The two monkeys previously inoculated with the attenuated strain WEE-clone 15 showed a definite immunity; they had no viremia and developed no clinical signs during the one-month observation period, whereas the 2 normal control monkeys developed encephalitic symptoms, showing paralysis, and became prostrate 5-6 days after inoculation. They were sacrificed for pathological studies. These results are not yet available.

Results of Intracerebral Inoculation of Guinea Pigs - Six 500 gm. guinea pigs were injected intracerebrally; each animal received 0.~~52~~ 25 ml of 10% chick embryo suspension containing 10^6 MLD_{50} (suckling mouse I.C. doses). The animals were bled daily from the 1st to the 4th day after inoculation and the sera titrated by intracerebral inoculation of suckling mice (Table 3). All the animals had circulating virus on the first and second days; on the 3rd day, 2 out of 4 (2 died of traumatism of heart punctures) showed traces of virus. No viremia was demonstrated on the 4th post-inoculation day. None of the animals which survived the traumatism of the daily bleeding demonstrated clinical signs during the one-month observation period.

Potency Test - In Table 4 are summarized the results of the potency tests performed with the attenuated strain WEE-clone 15 in mice and guinea pigs. All the animals previously inoculated with live attenuated virus resisted the intracerebral challenge inoculation. In the group of animals which

were injected with inactivated virus (The 10% chick embryo material was treated with ethylene oxide at the rate of 1.0 ml per each 100.0 ml chick embryo suspension), the mice showed only a partial resistance, but the guinea pigs were fully protected.

Stability of Attenuation of WEE-Clone 15 Strain - This strain did not regain virulence for young adult mice injected intracerebrally after being cultivated serially in eggs for 8 passages and in chick embryo tissue culture for 20 passages (Tables 5 and 6).

Circulating virus recovered from Rhesus monkeys and guinea pigs after intracerebral inoculation with WEE-clone 15 was not pathogenic for young adul. mice inoculated intracerebrally.

Comments - On the basis of the results reported above, the WEE-clone 15 strain appears to be quite attenuated with loss of pathogenicity for young adult mice, guinea pigs and Rhesus monkeys inoculated intracerebrall y with 10^6 MLD_{50} or more virus (suckling mouse I. C. doses). When the behavior of WEE-clone 15 in the Rhesus monkey and white mouse is compared with that observed in the 17D attenuated strain of yellow fever virus, the WEE-clone 15 appears to be milder. None of the monkeys inoculated with WEE-clone 15 showed clinical signs whereas up to 33% of monkeys inoculated intracerebrally with 17D might develop encephalitis manifested by paralysis and other clinical signs, sometimes producing death. Yellow fever strain 17D is virulent for adult mice inoculated intracerebrally with small doses (less than 5 MLD_{50}) whereas young adult mice do not show symptoms when inoculated intracerebrally with more than 10^6 MLD_{50} (suckling mouse IC doses) of WEE-clone 15.

The WEE-clone 15 has retained its antigenicity as demonstrated by the immunity induced in monkeys, white mice and guinea pigs after those animals have been exposed to this strain (Tables 2 and 4).

I concur with Dr. Harald N. Johnson in recommending that the behavior of a live virus vaccine prepared from this strain be investigated in horses; if successful results are obtained, it would be desirable to try the WEE-clone 15 in human beings.

M. Roca-Garcia

M. Roca-Garcia

dg

TABLE I

Results of Intracerebral Inoculation of Rhesus Monkeys with Attenuated WEE Strain 2628-TC. 00218 Clone 15 CETC-1 Led. CE-2(8103B-9) -

Each animal received 0.50 ml of 10% chick embryo suspension (2 X 10^6 MLD_{50})

Animal No.	MLD_{50}* Circulating Virus (Days after Inoculation) 1	2	3	4	5	Symptoms and fate of the animals during a one-month observation period
N-540	$10^{1.5}$	$10^{1.0}$	0	0	0	Remained normal through the observation period - Sacrificed for pathological studies
N-541	$10^{2.0}$	$10^{2.0}$	$<10^{1.0}$	0	0	"
N-542	$10^{1.3}$	$<10^{1.0}$	+	0	0	Remained normal through observation period - Challenged later I.C. with virulent WEE strain (see Table 2)
N-543	$10^{2.0}$	$10^{1.0}$	0	0	0	"

*Titration performed in suckling mice inoculated intracerebrally, MLD_{50}/0.02 ml.

+ One mouse died out of 6 inoculated with undiluted serum.

TABLE 2

Results of Intracerebral Challenge Inoculation of Monkeys #N-542 and N-543 (see Table 1) with WEE Virulent Strain 3 Months after Receiving Intracerebral Inoculation of WEE Attenuated Strain 2628-TC. 00218 Clone 15 CETC-1-Led. CE-2 (8103B-9)

Inoculum: Each animal received 0.50 ml of 4% chick embryo suspension = 10^6 MLD_{50}*

Monkey No.	MLD_{50}* Circulating Virus (Days after Inoculation) 1	2	3	4	Symptoms and fate of the animals during a one-month observation period
N-542	0	0	0	0	No clinical signs during the observation period
N-543	0	0	0	0	"
N-559 (control)	$10^{4.0}$	$10^{1.5}$	$10^{1.0}$	0	4th day after inoculation: tremor, complete paralysis of right arm and partial paralysis of right leg - Next day: prostrate. Sacrificed, brain and cord removed for histopathological examination.
N-560 (control)	$10^{5.0}$	$10^{1.5}$	0	0	4th day after inoculation: tremor, right leg weak. 5th day: paralysis of right leg and arm, partial paralysis of left leg. Next day: prostrate. Sacrificed, brain and cord removed for histopathological examination.

* Suckling mouse intracerebral titration.

TABLE 3

Results of Intracerebral Inoculation of Guinea Pigs with Attenuated WEE Strain 2628-TC. 00218 Clone 15 CETC-1 Led. CE-2 (8103B-9)

Each animal received 0.25 ml. of 10% chick embryo suspension (10^6 MLD_{50})

Animal No.	MLD_{50}* Circulating Virus (Days after Inoculation) 1	2	3	4	Symptoms and fate of the animals during one-month observation period
G.P. #1	$10^{2.0}$	$10^{1.0}$	$10^{1.0}$	0	No clinical signs during the observation period
G.P. #2	$10^{1.5}$	$10^{1.0}$	$10^{1.0}$	0	"
G.P. #3	$10^{2.5}$	D**			Died from traumatism of heart puncture
G.P. #4	$10^{2.5}$	$10^{1.5}$	0	0	No symptoms during the observation period
G.P. #5	$10^{2.5}$	$10^{1.5}$	0	0	"
G.P. #6	$10^{2.5}$	$10^{2.5}$	D		Died from traumatism of heart puncture

* Titration performed in suckling mice inoculated intracerebrally, MLD_{50}/0.02 ml.

** D - Found dead.

TABLE 4

Potency Tests of WEE Attenuated Strain 2628-TC-00218-Clone 15 CETC-1-Led.-CE-2 in White Mice and Guinea Pigs Using Live or Inactivated Virus.

Animal	Inoculated w/live material - 10% chick embryo susp. $10^{7.3}$ MLD_{50}/ml.			I.C. Chall. Inoc. w/ virulent WEE Strain- 10% chick embryo susp. 10^{6} MLD_{50}/ml.		Inoc. w/Inactivated virus-10% chick embryo susp. prepared from live material $10^{7.3}$ MLD_{50}/ml.			I.C. Challenge Inoc. w/ virulent WEE Strain - 10% chick embryo susp. 10^{6} MLD_{50}/ml.	
	Animal Group #	Route of Inoc.	ML.	ML.	Results S/R	Animal Group #	Route of Inoc.	ML.	ML.	Results S/R
White mouse 21-day-old	40432	I.C.	0.03	0.03	20/20	40435	I.P.	0.50	0.03	9/20
"	40433	I.P.	0.50	0.03	20/20	40436	I.P.	0.50*	0.03	8/20
Guinea Pig 500 Gr. Wt.	1	I.C.	0.25	0.25	3/3	3	I.P.	1.0	0.25	3/3
"	2	I.P.	1.0	0.25	3/3	4 (control)	None	None	0.25	0/2
White mouse 21-day-old	40434 (control)	None	None	0.03	0/19					

I.C. - intracerebral; I.P. - intraperitoneal; S/R - survival ratio (numerator indicates number of animals which survived; denominator indicates number of animals inoculated in the test)

* This group received 2 inoculations, one week apart.
Challenge inoculation took place one month after inoculation of attenuated virus.

TABLE 5

Stability of the Attenuated WEE Strain after Serial Passages in the Chick Embryo, Estimated by the Lack of Virulence for Young Adult White Mouse Inoculated Intracerebrally

Inoculum: 10% chick embryo suspension, considered as undiluted material

Virus Dilutions	Chick Embryo-5th Passage Infant mice I. C.	Chick Embryo-5th Passage Young Adult mice I. C.	Chick Embryo-7th Passage Infant mice I. C.	Chick Embryo-7th Passage Young Adult mice I. C.	Chick Embryo - 8th Passage Infant mice I. C.	Chick Embryo - 8th Passage Young Adult mice I. C.
10^{-0}	ND	0/6*	ND	0/6	ND	0/6
10^{-1}	ND	0/6	ND	0/6	ND	0/6
10^{-2}	ND	0/6	ND	0/6	ND	0/6
10^{-3}	ND	0/6	7/7	0/6	7/7	0/6
10^{-4}	7/7	ND	7/7	ND	7/7	0/6
10^{-5}	7/7	ND	0/7	ND	7/7	ND
10^{-6}	7/7	ND	0/7	ND	7/7	ND
10^{-7}	0/7	ND	0/7	ND	7/7	ND

ND - not done

* mortality ratio

TABLE 6

Stability of the Attenuated WEE Strain after Serial Passages in Chick Embryo Tissue Culture (CETC), estimated by the lack of Virulence for Young Adult White Mouse Inoculated Intracerebrally.

Virus Dilutions	CETC - 5th Passage: Infant mice I.C.	CETC - 5th Passage: Young adult mice I.C.	CETC-15th Passage: Infant mice I.C.	CETC-15th Passage: Young adult mice I.C.	CETC - 20th Passage: Infant mice I.C.	CETC - 20th Passage: Young adult mice I.C.
10^{-0}	ND	0/6*	ND	0/6	ND	0/6
10^{-1}	ND	0/6	ND	0/6	ND	0/6
10^{-2}	7/7	0/6	7/7	0/6	7/7	0/6
10^{-3}	7/7	0/6	7/7	ND	7/7	0/6
10^{-4}	7/7	ND	7/7	ND	0/7	0/6
10^{-5}	3/7	ND	0/7	ND	0/7	ND
10^{-6}	0/7	ND	0/7	ND	0/7	ND

ND - not done

* mortality ratio

Virus Research - August 7, 1967

Dr. H. R. Cox

Western EE Vaccine

The following is a summary of our production of bulk WEE vaccine:

Seed: **A vial of the chick embryo vaccine (from the batch prepared by Dr. M. Roca-Garcia and subsequently sent to Dr. H. N. Johnson) was used to initiate our first chick embryo (CE) tissue culture passage (LTC-1). The 48-hour harvest of this passage which represented lactal maintenance medium plus 2% Eastman gelatin #1099 was combined with an equal volume of normal horse serum and dried down to serve as stable seed. Its titer post-drying was 5.7 $TCID_{50}$/0.2 ml.**

Bulk Vaccine Production: **RIF-free chick embryo monolayers, 24 to 48 hours old, in Blake bottles were seeded. Seventy-five ml of the growth medium were removed and 1 ml of a 10^3 dilution of seed virus was added to the remaining 25 ml of growth medium. Virus was allowed to adsorb for 1 hour at room temperature (RT). The supernatant medium was then removed and the monolayer was washed 3 times with 50 ml volumes of PBS. Each Blake bottle then received 100 ml of Eagle's maintenance medium containing 2% human serum albumin. The calculated residual amount of normal horse serum from the growth medium was 0.004 ppm. Cultures were incubated at 34°C for 24 to 36 hours, and then harvested. Fifteen Blake bottles were pooled and samples removed for testing. In each of the 3 lots thus prepared an uninoculated control bottle was similarly harvested for RIF testing. Each of the 3 lots after removal of sampbs, represented approximately 1400 ml. The titers of the 3 harvest pools were 8.2, 8.5 and 9.2 $TCID_{50}$/ml. At no point in the production process was penicillin used.**

Following the satisfactory completion of sterility, safety and RIF tests on each of the 3 harvests, the frozen fluids were thawed, pooled in a common container and then filtered through an 8 micron Millipore filter to remove intact cells. Samples were set aside for the testing department. The fluids were filled in 5.5 ml amounts and lyophilized in #1 glass vials.

All routine tests for sterility, the presence of RIF and intact cells were satisfactory. No intracerebral or other safety tests in monkeys were done on this product. The contents of the final container were tested for identity and sterility and found to be satisfactory.

A portion of one harvest pool was pig-dried and tested for stability. The results (reported in the September 1966 Monthly Summary) ma y be summarized as follows:

Titer immediately post drying was 8.4/ml; this represented a loss of one log of virus in drying.

Weeks Stored	Room Temp.	37°C	4°C
1	7.0*	6.4	--
2	7.2	5.7	--
4	7.2	5.0	7.7

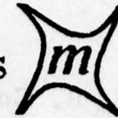
* All titers are expressed as $TCID_{50}$/ml.

Samples for stability tests of the completed batch were held at room temperature and at 37°C for 1, 2, 3, 4, 6 and 8 weeks. These have not yet been titrated owing to the strike and the absence of chick embryo tissue culture tubes.

Floyd S. Markham

APPENDIX 3

A

MICROBIOLOGICAL ASSOCIATES 
Division of DYNASCIENCES Corporation

4733 Bethesda Avenue / Bethesda, Maryland 20014 / (301) 654-3400

DIAGNOSTIC SERVICES TEST REPORT

Ref: Our Code #MVS 2593

TO: Dr. Philip Coleman
Virginia Commonwealth University
Medical College of Virginia
Microbiology Department
MCV Station
Richmond, Virginia 23298

FROM: Dr. Michael Collins

DATE: December 13, 1974

TEST: Mouse antibody production (MAP) test

SPECIMEN: WEE vaccine, strain B628, C1-15, HK4CEC31CE2CEC4

RECEIVED: October 31, 1974

RESULTS:

MA Log	Inoculum	Viruses found as contaminants by the MAP test											
Number	(dilution)	PVM	Reo3	Sendai	GDVII	K	Polyoma	MVM	M.Ad.	MHV	LCM	Ectromelia	LDH
MVS-2593	undiluted	-	-	-	-	-	-	-	-	-	-	-	-
	1:10	-	-	-	-	-	-	-	-	-	-	-	-

BRANCH OFFICE / *2330 Centinela Ave., Los Angeles, California 90064 (213) 820-5250*

APPENDIX 4

4733 BETHESDA AVENUE • BETHESDA, MARYLAND 20014
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11841 MISSISSIPPI AVENUE • LOS ANGELES, CALIFORNIA 90025
TELEPHONE: (213) 820-6250

DATE: April 12, 1976

REPORT TO: Dr. Philip H. Coleman, D.V.M., Ph.D.
Medical College of Virginia
Department of Microbiology
Richmond, Va. 23298

TEST: COFAL (Avian Leucosis)

DATE RECEIVED: January 27, 1976

MBA LOG #	SPECIMEN	COFAL ON QUAIL CELLS*	COFAL ON CHICK EMBRYO CELLS*
D-7359	Group O** Final Wee vaccine	<1:2	<1:2
D-7360	Group I media	<1:2	<1:2
D-7361	Group II** WEE unclarified vaccine - passed one time in Chick embryo cells	<1:2	<1:2
D-7362	Group III WEE unclarified vaccine - reacted with anti WEE and than anti-antiserum centrifuged and grown in Chick embryo cells with 2% anti WEE serum	<1:2	<1:2
D-7363	Group IV Chick embryo cell control with anti-WEE serum, no virus	<1:2	<1:2
D-7364	Group V** WEE unclarified vaccine grown in Chick embryo cells in presence of 2% anti-WEE serum	<1:2	<1:2

*Specimens grown in quail and chick embryo cell cultures for 3 weeks (3 passages.)
**Rabbit anti-WEE serum in media.

REPORT FROM: [signature]
John C. Parker, Ph.D.
Director, Diganostic Laboratory

dmr

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