# Protective Studies in Mice and Monkeys with an Inactivated Japanese Encephalitis Virus Vaccine Grown in Hamster Diploid Cell Culture.* (32210)

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Recent studies of Japanese encephalitis virus (JEV) in tissue culture have demonstrated that this virus propagates satisfactorily in most cell cultures of Syrian hamster origin (1,2,3). We have been attempting to employ these cell cultures as sources of inactivated vaccines. Preliminary data suggested that a continuous strain of hamster diploid embryonic cells was the most promising for this purpose(3). The present report deals with potency of this cell culture vaccine tested in laboratory animals.

*Material and methods. JEV virus strains.* JEO strain isolated from Okinawa mosquitoes (4) was used for the vaccine and HV1 isolated from the brain of a human case in Taiwan(5) and Nakayama strains were employed to challenge immunized animals. Both HV1 and JEO strains were used after 3 passages in suckling mouse brain (wild virus). The Nakayama strain was obtained from the U.S. Army 406 General Medical Research Laboratory, Japan. It is a high mouse brain passage strain (adapted virus), but the exact passage history is unknown.

*Preparation of the cell culture vaccine.* A continuous strain of hamster embryonic skin and muscle cells (HSS) established in 1962 (3) was used to grow virus. The karyotype of the HSS strain was diploid when tested at the 18th passage level. Passage levels used for vaccine in this study were between the 12th and 20th. Thirty two-ounce bottles of HSS cell culture were incubated with approximately $10^7$ $LD_{50}$ of JEO in 2 ml of minimal essential medium (MEM) with 10% calf serum after discarding the maintenance medium. The virus was allowed to absorb for 2 hours while the bottles were rocked in an incubator at 36°C. Then the bottles were washed twice in Hanks' balanced salt solution (BSS) at pH 7.3, fed with 100 ml of MEM with 5% calf serum and incubated at 36°C for 2 days. When early but definite signs of cytopathic effect appeared, about 48 hours after virus inoculation, the bottles were washed 3 times with BSS, and the medium was replaced by 100 ml of MEM containing 0.4% bovine albumin (or human albumin) at pH 7.5 and incubation continued at 36°C. The pH of the media was maintained during incubation by addition of 5.6% Bicarbonate. The culture fluid was harvested about 20 hours later when more than 50% of the cells showed cytopathic change and the hemagglutinin (HA) titer of the supernate was 1:192 or greater. The fluid was centrifuged twice at 2000 rpm for 30 minutes. Formalin was added to the virus suspension to a final concentration of 1:2,000 and stored at 4°C for 3 weeks. Infectivity usually was lost after one week as shown by inoculation into suckling mouse brain. For longer storage the vaccines were frozen at −65°C.

*Mouse brain vaccine.* In 1965 the Japanese government provided the Taiwan Provincial Health Department with a large amount of commercially manufactured mouse brain JEV vaccine for a field study in the Taipei-Hsinchu area. An aliquot of commercial vaccine and of reference vaccine (Lot No. 64-1) of the Japanese National Institutes of Health was made available for laboratory test at NAMRU-2 by Dr. M. Kitaoka. Both of these

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TABLE I. Evaluation of JEV Tissue Culture Vaccines by Mouse Protection Test.

| Vaccine lot | Virus titer before inactivation $Log_{10} LD_{50}$/ml in mice | HA/0.5 ml | Days inactivation | $ED_{50}$* | Challenge dose ($LD_{50}$) |
|---|---|---|---|---|---|
| V 12 | 9.5 | 24 | 52 | <1 | 33 |
| V 33 | 9.5 | 6 | 27 | <1 | 33 |
| V 18 | >9.5 | >48 | 32 | 4.0 | 15 |
| V 32 | >9.5 | 384 | 29 | 3.2 | 33 |
| V 35 | >9.5 | 384 | 254 | 7.1 | 13 |
| V 37 | 8.4 | 384 | 242 | 10.7 | 13 |
| V 47 | 8.6 | 192 | 205 | 5.5 | 13 |

* Maximal vaccine dilution protecting 50% of mice from intracerebral challenge of 10-50 $LD_{50}$ of HV1 virus.

vaccines were made with the mouse adapted Nakayama strain.

*Mouse protection test.* The method of the Japanese National Institutes of Health was used with slight modification(6). Serial 4-fold dilutions of vaccine were made. Groups of 7-10 four-week-old mice (white Swiss NIH strain) were inoculated intraperitoneally every other day, four times, with 0.5 ml of dilutions of the vaccine. These mice were then challenged intracerebrally with 10-50 $LD_{50}$ of the challenge strain 14 days after the first immunization. The maximal dilution of the vaccine which protected 50% of the mice from death was calculated by the method of Reed and Muench and expressed as $ED_{50}$(7).

*Monkey protection test.* Adult Taiwan monkeys, *Macaca cyclopis,* weighing 5-6 kg were used. Groups of 5 or 6 monkeys were inoculated 4 times with 1 ml of undiluted vaccine, once a week for 3 doses followed by a booster dose 2 weeks later. Other groups received 2 doses one month apart of vaccine emulsified with an equal portion of mineral oil adjuvant (85% Bayol F and 15% Arlacel A). Each dose consisted of 1 ml divided into 4 portions given at one time in different I.M. sites. Two weeks after the last immunization, monkeys were challenged intranasally (I.N.) under light ether anesthesia with 0.5 ml of $10^{-3}$ suspension of suckling mouse brain HV1 virus. This dilution was chosen after titration of the challenge inoculum in 2 monkeys at each 10-fold dilution $10^{-2}$ to $10^{-7}$. One of 2 monkeys given the $10^{-4}$ and $10^{-5}$ suspensions died. All monkeys given more concentrated virus died, while none died after more dilute suspension. In the protection tests, in addition to groups of immunized and non-immunized control monkeys, others that had been previously infected were included. These monkeys that had been injected I.V., I.N., or subcutaneously with HV1 did not show encephalitic symptoms but did have viremia and developed antibody. The monkeys were closely observed for clinical symptoms. The presence of viremia was tested every other day during the first 6 days after challenge by inoculating whole heparinized blood into a litter of 6-11 suckling mice. Each suckling mouse was inoculated with 0.01 ml intracerebrally and 0.02 ml subcutaneously. Mouse encephalitic deaths during a 3-week observation period were recorded. Blood specimens were collected serially during the experiment for serological study.

*Hemagglutination inhibition test (HAI).* The procedure for HAI test described by Buescher *et al*(8) was followed. The lowest serum dilution employed was 1:20.

*Results.* JEO strain propagated to high titer in the HSS cells(3). Table I shows 7 lots of vaccine prepared in hamster cell culture. The first 3 were grown in secondary cultures of whole embryo, while the rest were grown in HSS. All the vaccines had high infectivity titers, but only those prepared in continuous cells had consistently high HA titers. The potency of these vaccines in the mouse protection test was better correlated with HA titer than with infectivity. The mouse potency for the vaccines prepared in the continuous cell line compares favorably with the minimal requirements for JEV mouse brain vaccines prescribed by the Japanese National Institutes of Health(6).

Cell culture vaccine lot V 32 was inoculated into groups of monkeys as fluid vaccine and

TABLE II. HAI Antibody Titers in Monkeys After Immunization.

| Vaccine | MK # | Weeks after first vaccination: 0 | 4 | 6 |
|---|---|---|---|---|
| | | Antibody titer | | |
| V 32 (fluid) | 145 | 0 | 160 | 160 |
| | 146 | 0 | 160 | 160 |
| | 154 | 0 | 40 | 160 |
| | 167 | 0 | 80 | 160 |
| | 195 | 0 | 160 | 320 |
| V 32 (oil adjuvant) | 243 | 0 | 0 | 160 |
| | 244 | 0 | 0 | 40 |
| | 245 | 0 | 0 | 160 |
| | 246 | 0 | 20 | 320 |
| | 247 | 0 | 40 | 640 |
| Control | 249 | 0 | | 0 |
| | 250 | 0 | | 0 |
| | 251 | 0 | | 0 |
| | 252 | 0 | | 0 |
| | 253 | 0 | | 0 |

with mineral oil adjuvant. Table II shows the HAI antibody response to these vaccines. Response to one dose of oil adjuvant vaccine was much poorer than to the initial 3 doses of fluid vaccine. However, after booster inoculations with both types of vaccine, substantial titers of 1:160 or greater were obtained in all but one monkey.

The monkeys were challenged 2 weeks after the last dose of vaccine along with nonimmunized control monkeys and 9 previously infected monkeys. Table III shows the frequency of viremia in these monkeys. Although some viremia was demonstrated in the immunized monkeys, it was much less than in the controls. The previously infected monkeys had occasional low level viremia similar to the immunized monkeys.

The clinical course of the monkeys after challenge is shown in Table IV. With one exception, all control monkeys developed fever and were moribund on the sixth to ninth day after challenge. Paralysis and death occurred in the following few days. Two monkeys given oil adjuvant vaccine developed complete paralysis and one died 21 days after challenge. This animal (MK244) had the poorest HAI antibody response to vaccination. None of the monkeys immunized with fluid vaccine or previously infected developed paralysis or died.

Animal protection tests on another lot of vaccine (V-37) were conducted to compare the potency of cell culture with mouse brain vaccine. The results of mouse protection tests using both HV1 and Nakayama strains for challenge are shown in Table V. No direct comparison is possible between the potency as measured against HV1 and Nakayama because of the considerable variation in $LD_{50}$ of the challenge virus. However, the mouse brain vaccines clearly protected best against the mouse adapted Nakayama strain, while the cell culture vaccines protected much better against the recent low passage "wild" strain (HV1).

Groups of monkeys were also immunized with these vaccines. No matter whether the Nakayama or HV1 strain was used as antigen in the HAI test, the monkeys inoculated with cell culture vaccine produced higher antibody titer than those given mouse brain vaccine. Table VI shows the HAI results with the Nakayama strain as antigen. All of the monkeys inoculated with cell culture vaccine developed HAI titers of 1:160, but much poorer

TABLE III. Viremia After Intranasal Challenge of Immunized, Previously Infected and Control Monkeys.

| Vaccine group | MK # | HI titer before challenge | Days after challenge: 2 | 4 | 6 |
|---|---|---|---|---|---|
| V 32 (fluid) | 145 | 160 | 1/6* | 2/9 | 1/7 |
| | 146 | 160 | 0/7 | 0/11 | 0/2 |
| | 154 | 160 | 0/6 | 3/7 | 0/8 |
| | 167 | 160 | 1/6 | 1/7 | 0/8 |
| | 195 | 320 | 0/6 | 1/8 | 0/4 |
| V 32 (oil adjuvant) | 243 | 160 | 1/4 | 0/7 | 0/6 |
| | 244 | 40 | 0/5 | 1/7 | 0/5 |
| | 245 | 160 | 0/7 | 1/11 | 0/4 |
| | 246 | 320 | 0/7 | 0/7 | 0/3 |
| | 247 | 640 | 2/7 | 1/7 | 0/4 |
| Control | 249 | 0 | 7/7 | 5/5 | 0/5 |
| | 250 | 0 | 2/2 | 6/7 | 0/5 |
| | 251 | 0 | 6/6 | 5/7 | 0/7 |
| | 252 | 0 | 6/6 | 1/1 | 0/5 |
| | 253 | 0 | 4/4 | 6/9 | 0/4 |
| Previously infected | 76 | 320 | 0/6 | 1/8 | 1/9 |
| | 218 | 320 | 1/8 | 3/5 | 0/5 |
| | 220 | 640 | 0/7 | 1/7 | 0/8 |
| | 222 | 640 | 0/6 | 0/3 | 0/8 |
| | 223 | 80 | 0/8 | 0/6 | 2/4 |
| | 224 | 40 | 0/7 | 0/4 | 0/4 |
| | 225 | 80 | 0/8 | 1/6 | 0/6 |
| | 226 | 160 | 1/7 | 0/7 | 7/7 |
| | 227 | 80 | 0/7 | 0/8 | 1/7 |

* No. of suckling mice encephalitis deaths/No. of mice tested.

TABLE IV. Results of Intranasal Challenge of Immunized, Previously Infected and Control Monkeys.

| Vaccine lot | No. tested | Died of infection | Paralysis | Tremor or paresis | No neurological sign |
|---|---|---|---|---|---|
| V 32 (fluid) | 5 | 0 | 0 | 2 | 3 |
| V 32 (in oil) | 5 | 1 | 2 | 1 | 2 |
| Control | 5 | 4 | 4 | 0 | 1 |
| Previously infected | 9 | 0 | 0 | 1 | 8 |

TABLE V. Comparative Results in the Mouse Protection Test with Mouse Brain and Tissue Culture Vaccines.

| Type of vaccine (virus strain) | Challenge strain HV1 (13 $LD_{50}$) | Nakayama (44 $LD_{50}$) |
|---|---|---|
| Mouse brain (Nakayama) | $ED_{50}$ | $ED_{50}$ |
| Commercial vaccine | 5.6 | 6.6 |
| Reference vaccine (Lot No. 64-1) | 7.2 | 6.3 |
| Tissue culture (JEO) | | |
| V-37 | 10.7 | 2.6 |

response followed mouse brain vaccine. A neutralization test was performed in HSS cell culture with sera obtained from control monkeys prior to challenge and immunized monkeys prior to the first dose of vaccine. No neutralizing antibody was found in any of the monkeys.

TABLE VI. HAI Antibody Response in Monkeys to Mouse Brain and Tissue Culture Vaccines.

| Type of vaccine | MK # | Weeks after the first immunization 0 | 4 | 6 |
|---|---|---|---|---|
| | | Antibody titer | | |
| Control, no immunization | 340 | | | 0 |
| | 341 | | | 0 |
| | 342 | | | 0 |
| | 313 | | | 0 |
| | 311 | | | 0 |
| | 310 | | | 0 |
| Mouse brain (fluid) | 356 | 0 | 20 | 20 |
| | 357 | 0 | 0 | 0 |
| | 360 | 0 | 0 | 0 |
| | 362 | 0 | 40 | 40 |
| | 361 | 0 | 40 | 40 |
| | 355 | 0 | 80 | 160 |
| Tissue culture (fluid) | 204 | 0 | 160 | 160 |
| | 323 | 0 | 40 | 160 |
| | 325 | 0 | 40 | 160 |
| | 329 | 0 | 160 | 160 |
| | 331 | 0 | 80 | 160 |
| | 337 | 0 | 40 | 160 |
| Previous infection, no immunization | 154 | | | 40 |
| | 167 | | | 20 |
| | 227 | | | 160 |
| | 230 | | | 160 |
| | 307 | | | 80 |
| | 225 | | | 80 |

The clinical disease observed in these monkeys after I.N. challenge with HV1 is presented in Table VII. Five of 6 controls and 4 of 6 given mouse brain vaccine died. All of the monkeys immunized with cell culture vaccine or previously infected survived the challenge. Although many showed signs of encephalitis, recovery was complete. A more detailed description of Japanese encephalitis virus infection of Taiwan monkeys, including these protection tests, will be reported(9).

TABLE VII. Comparative Results in Monkey Protection with Mouse Brain and Tissue Culture Vaccines.

| Vaccine group (virus strain) | No. tested | No. sick | No. died |
|---|---|---|---|
| Control | 6 | 6 | 5 |
| Mouse brain (Nakayama) | 6 | 5 | 4 |
| Tissue culture (JEO) | 6 | 5 | 0 |
| Previous infection | 6 | 2 | 0 |

*Discussion.* There have been two types of inactivated JEV vaccines available in the past, one grown in mouse brain, the other in chick embryo. The former was initiated by Japanese workers in the 1930's and studied by Sabin (10) and others, while the latter was developed separately by Warren and Hough(11) and Koprowsky and Cox(12). Field studies in Japan suggested that these types of vaccine gave some protection based on serological conversion and lowered incidence of the disease among the vaccinated(13-19). Nevertheless, no completely satisfactory controlled field trial has been available and the true efficacy and amount and severity of side reactions to these vaccines remain controversial. The American military services after some initial interest have ceased using JEV vaccine. Mouse brain vaccine has been used extensively in

Japan for decades. The possibility of inducing demyelinating encephalitis by mouse brain material provides some risk to its use.

With the simplification of tissue culture technique, there has been renewed interest in preparation of JEV vaccines. A tissue culture source is simpler and less expensive than mouse brain for vaccine production. Potent vaccine may be prepared from wild strains of JEV so that best protection can be expected under natural conditions of challenge. In contrast, potent mouse brain vaccine has been prepared only with a strain well adapted to mice and protects animals poorly from challenge with wild strains of JEV(10,19).

Many problems remain to be solved before attenuated virus strains can be used as live vaccines(20,21). Otani has used chick embryo cell culture to produce a vaccine with the Nakayama strain(22). Hammon and his associates have recently switched from attempts to develop a live attenuated JEV vaccine to a formalin inactivated vaccine. However, for the inactivated vaccine they have employed an attenuated JEV strain grown in primary hamster kidney cell culture. In a series of careful studies with this system, Darwish and Hammon have characterized the optional conditions for growth and inactivation of the virus(23,24). They have produced an inactivated vaccine with potency in the mouse intraperitoneal challenge test equal to that required by the United States government for previously approved chick embryo and mouse brain JEV vaccine. In addition, this vaccine has been shown to produce neutralizing antibodies in monkeys and guinea pigs.

The advantages of human diploid cell strains over primary cell cultures as sources of virus vaccines have been discussed by Hayflick(25). The use of human diploid cells or continuous cell strains of normal animal tissue sources in the preparation of virus vaccine has been considered by a Committee on Tissue Culture Viruses and Vaccines(26). There is increasing concern over the potential dangers of adventitious agents in primary monkey kidney cell cultures used for vaccine. The same may hold true for the less well studied primary hamster kidney cultures. Recently, Pagano has reported the use of attenuated poliovirus vaccine grown in human diploid cells immediately after birth in humans and argued that such preparations are safer than monkey kidney grown vaccines(27). A diploid cell strain can be studied extensively for the presence of extraneous virus and for oncogenic capacity prior to the preparation of vaccine. The remote chance that continuous cell cultures may carry unidentified agents which may be infectious and pathogenic to a vaccinee under particular conditions will be extremely difficult to disprove. Well characterized diploid cells of hamster origin should be even safer than similarly studied cells of human origin for preparation of vaccine since hypothetical carcinogenic agents not revealed by methods now known would be less likely to effect heterologous species.

*Summary.* Formalin inactivated Japanese encephalitis virus vaccine prepared with a recently isolated strain (JEO) in tissue culture of a continuously propagated hamster embryo skin and muscle diploid cell has been tested for potency in mice and monkeys. Several lots of vaccine had a protective potency in the mouse test with intracerebral challenge similar to that required by the Japanese government. In 2 separate experiments in monkeys the vaccine produced substantital titers of HAI antibdy, reduced the amount of viremia after intranasal challenge and protected them from death. The potency of cell culture vaccine was compared to vaccine produced from mouse brain. In the mouse the cell culture vaccine gave better protection against challenge with a low passage strain, while the mouse brain vaccine protected better against the homologous high mouse passage Nakayama strain. In the monkey protection test, cell culture vaccine produced higher HAI antibody and much better protection against intranasal challenge with the low passage strain.

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## A Relationship of Direct Coombs Test Pattern to Autoantibody Specificity in Acquired Hemolytic Anemia. (32211)

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In the past 15 years evidence has accumulated(1,2) that "warm-type" autoantibodies in acquired hemolytic anemia are frequently reactive with components of the Rh complex on the human red blood cell (RBC). Independently, it has been learned that these "warm-type" autoantibodies are always or virtually always γG (IgG) globulins (3,4). The presence of such autoantibodies on the surface of a patient's RBC is readily demonstrated *in vitro* by producing agglutination with an anti-γG Coombs serum. In some cases the patient's RBC can also be agglutinated by specific antiserum to human complement (C′) components; in other cases anti-C′ serum gives no reaction(1,4,5). Thus, human RBC autosensitization by "warm-type" γG autoantibodies may or may not be accompanied by bound C′ components. From these observations it seemed reasonable to assume that some γG autoantibodies were C′-fixing while others were not(cf. 4). This difference might, in turn, depend on differences in specificity of the autoantibodies(6), by analogy to observations with blood group isoantibodies. Since it is well known that human Rh *iso*antibodies seldom are C′-fixing (7), a survey was begun in 1965 to evaluate the possibility that, among γG autoantibodies, Rh-related specificity might be found more often in patients whose RBC gave positive direct antiglobulin (Coombs) reactions with anti-γG alone. Conversely, autoantibodies from patients whose RBC were autosensitized with both γG globulin and C′ might, by this reasoning, be less likely to have Rh specificity.