Chikungunya Virus Vaccine Prepared by Tween-Ether Extraction

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Chikungunya virus vaccines prepared by Tween 80 and ether inactivation of virus grown in green monkey kidney cell cultures were shown to be as immunogenic as comparable Formalin-inactivated vaccines. Both types of vaccine stimulated hemagglutination-inhibiting, complement-fixing, and neutralizing antibody and afforded protection to mice against a live virus challenge. It was shown after Tween-ether treatment of chikungunya virus that the infectivity of the virus was lost and the hemagglutinin titer was increased. By characterization of the resultant hemagglutinin by sucrose and cesium chloride density gradient centrifugation, it was found that the extracted particle was smaller in size and greater in density than the parent virus particle. Removal of lipid may account for the alterations in the physical characteristics of the infectious virus particle. Conditions for treatment of chikungunya virus with Tween and ether were found that preserved high titers of hemagglutinin as well as the immunogenicity of the virus preparations.

Chikungunya (CHIK) virus is a group A arbovirus which produces a febrile illness in man and has been isolated from human cases during widespread epidemics occurring in Africa and Asia (5, 8, 9, 13). Although CHIK infection is rarely fatal, it may incapacitate a person for several days, making a protective vaccine desirable for control of this disease.

A Formalin-inactivated vaccine for CHIK virus has been prepared in green monkey kidney cell culture and has been shown to protect mice against an intracerebral challenge and also to stimulate the production of hemagglutination-inhibiting (HI), complement-fixing (CF), and neutralizing antibodies (6).

Extraction of several lipid-containing viruses with the surfactant Tween 80 and ether results in a product which is immunogenic but which lacks infectivity. Experimental influenza and measles vaccine have been prepared by this method (3, 11). Tween-ether extraction has also been used in the preparation of hemagglutinins for use in arbovirus HI antibody tests (15).

This work is concerned with attempts to apply the Tween-ether extraction procedure for preparation of a vaccine against CHIK virus and to compare immunogenic potencies of Formalin vaccines with those of vaccines prepared by the Tween-ether method.

MATERIALS AND METHODS

Cell cultures. Flask cultures of green monkey kidney cells were obtained from Microbiological Associates, Bethesda, Md.

Nine-day-old embryonated chick eggs were obtained from Duckworth Hatcheries, Hanover, Md. Chick embryo cell cultures were prepared by methods described elsewhere (12).

Viruses. African CHIK strain 168, the virus used in the preparation of vaccines, was subjected to 12 suckling mouse brain passages in this laboratory prior to use. Other viruses used were the Asian CHIK strain BAH-306, Indian CHIK strain C-266, Mayaro virus strain B, and Semliki Forest virus, Kumba strain. These viruses were kindly supplied by E. L. Buescher of this Institute and K. V. Shah, Department of Pathobiology, Johns Hopkins University, Baltimore, Md.

Virus titrations. Infectious virus was titrated by using the plaque technique described by Porterfield (12) on chick embryo cell culture dishes.

Concentration of virus by pelleting. Virus was inoculated into green monkey kidney cell culture flasks at a multiplicity of infection of approximately one plaque-forming unit per 350 cells. Virus was harvested at 48 hr postinoculation, and cellular debris was removed by centrifugation at 1,250 × g for 20 min. Supernatant fluids were centrifuged in a Spinco 40 rotor at 82,000 × g for 2 hr. After sedimentation of
the virus into a pellet, the supernatant fluids were removed from the centrifuge tube and the pellet was resuspended to 5% of its original volume in phosphate-buffered saline containing 0.5% bovine plasma albumin.

**Twee-ether extraction of virus.** The procedure of Muggay and Rott (10) for extraction of Sindbis virus with Tween and ether was followed closely for CHIK virus. Clarified virus harvest was mixed with Tween 80 at a concentration of 5 mg of the surfactant per ml of virus harvest and was shaken for 15 min at room temperature. An equal volume of diethyl ether was added, and the mixture was shaken for 15 min at room temperature. Ether was separated from extracted virus by centrifugation at 1,200 × g for 20 min. Residual ether was removed from the aqueous phase by aeration with nitrogen gas. This procedure was found to give the highest yield of hemagglutinin (HA) from live-virus harvests, resulting in a four- to eightfold increase in HA titer.

**Density gradient centrifugation.** Rate-zonal centrifugations of pelleted CHIK virus or Tween-ether-extracted virus were performed on gradients of 5 to 30% sucrose in phosphate-buffered saline. Layers of sucrose were allowed to diffuse overnight at 4 C to obtain a smoother gradient. A 0.5-ml sample of pelleted virus or Tween-ether-extracted virus was layered onto a gradient and centrifuged at 40,000 × g for 1 hr in a Spinco SW-50L rotor. The rotor was allowed to come to rest with the brake disengaged, and fractions were removed from the centrifuge tubes by piercing the bottom of the tube and allowing the fractions to drip out. The refractive index of each fraction was measured on an Abbé 3-L refractometer, and hemagglutination tests were immediately done on each fraction. A sample of each fraction, diluted in phosphate-buffered saline containing 0.5% bovine plasma albumin, was frozen for later infectivity titrations.

Equilibrium density gradient centrifugations were performed by mixing 0.5 ml of pelleted CHIK virus or Tween-ether-extracted virus with 4.5 ml of CsCl in phosphate-buffered saline to give a final density of 1.23 g/ml. Centrifugation was carried out in the SW-50L rotor at 133,000 × g for 18 hr. The rotor was allowed to come to rest with the brake disengaged, and fractions were collected by the drip-out method. The refractive index of each fraction was measured on a refractometer, and densities were computed by the equation of Ifft et al. (7). Hemagglutination tests were done immediately on all fractions, and samples of fractions for infectivity titrations were stored at −60 C in 0.5% bovine plasma albumin-buffered saline diluent.

**Serological tests.** For hemagglutination and HI antibody tests, the methods of Clarke and Casals (2) were used employing goose erythrocytes. The CF antibody test was described by Buescher et al. (1). The volumes in these tests were modified so that a microtechnique could be employed. Sera, or fluids containing HA, were serially diluted in "W"- or "U"-bottom plates by using micro diluting loops, and reagents were added by the use of 0.025- or 0.05-ml micropipet droppers. All microtitration equipment used was manufactured by the Cooke Engineering Co., Alexandria, Va. Antigens for HI and CF antibody tests were prepared from virus-infected suckling mouse brain by sucrose-acetone extraction.

Neutralizing antibody tests were performed by the plaque-inhibition technique described by Porterfield (12).

Sera for CF and neutralizing antibody tests were inactivated at 60 C for 30 min, whereas those for HI antibody tests were kaolin-extracted and adsorbed with goose erythrocytes to remove natural agglutinins.

**Vaccine preparation and potency testing.** The procedure of Harrison, Binn, and Randall (6) was followed for the preparation of Formalin-inactivated vaccine for CHIK virus. The Tween-ether extraction procedure previously described was also used for preparation of Tween-ether vaccine. After removal of residual ether by aeration with nitrogen gas, the fluids were filtered through 0.45-μm Nalgene filters; bacterial sterility tests were performed and the fluids were stored at 4 C. Tests for residual virus after inactivation were performed in suckling mice for both the Formalin and Tween-ether vaccines by intracerebral inoculation of serial dilutions of inactivated virus fluids.

Potency assays of the vaccines were performed in male, Swiss Bagg strain mice, 3 to 4 weeks old, which were obtained from the Department of Laboratory Animals, Walter Reed Army Institute of Research. Vaccine was serially diluted and groups of mice were inoculated intraperitoneally with a 0.5-ml dose of vaccine. All groups were challenged with 100 to 1,000 mouse lethal doses (LD₅₀) of the challenge virus 14 days after vaccination. Effective dose (ED₅₀) values were calculated by the method of Reed and Muench (14).

Antibody assays were performed in groups of adult mice by giving two 0.25-ml doses of vaccine intraperitoneally, spaced 7 days apart. All mice were bled by cardiac puncture 14 days after their second dose of vaccine. Bleedings were pooled for each group of mice, and the serum was separated from the clot and stored at −20 C.

**RESULTS**

**Growth curve of CHIK virus in green monkey kidney cell cultures.** Growth characteristics of CHIK virus in green monkey kidney cell cultures were studied so that harvests could be made that would result in the greatest yield of infectious virus or HA. Cell culture flasks were inoculated with 5 ml of CHIK virus diluted in medium 199 to give multiplicities of infection of 1:3, 1:50, 1:350, and 1:7,000 plaque-forming units per cell. At selected time intervals, samples from each input multiplicity were removed and tested for viral and HA content. Also, observations were made for the presence of cytopathic effect (CPE) at the various multiplicities. From the results summarized in Fig. 1, it may be seen that an input multiplicity of 1:350 gives a maximum infectious virus titer at 48 hr and a maximum HA titer at 96 hr. At all multiplicities, HA production and CPE appeared to increase after infectious virus had reached peak titer. A multiplicity of 1:350
it was found that the HA particle was indistinguishable from the infectious particle of untreated CHIK virus preparations after rate-zonal sedimentation in sucrose gradients. After CsCl equilibrium density gradient centrifugation, most HA activity was associated with the infectious particle, although at times a second small peak or shoulder of HA of lower density was evident in the gradient. The possibility of a degradative effect by CsCl on the CHIK virion has not been ruled out.

Vaccine preparation and assay. By using techniques already described, CHIK virus was Tween-ether-treated, with the object being to use the

was used in all subsequent experiments for the preparation of stock CHIK virus.

Rate-zonal and equilibrium density gradient centrifugation. As previous experiments indicated, the HA titer of CHIK virus increased after Tween-ether treatment. The physical alterations of the virus particle after treatment were studied by analysis of sedimentation rates in sucrose gradients and also by buoyant density determinations in CsCl gradients. After centrifuging the untreated infectious particle and the Tween-ether-extracted HA in similar gradients, the sedimentation rate of the HA particle was found to be less than the sedimentation rate of the infectious particle (Fig. 2). Treatment of virus with Tween and ether also results in a homogeneous yield of HA particles, as indicated by the well-defined peak of HA activity.

To supplement physical characterization of the infectious and HA particles, the buoyant density of the particles was determined by equilibrium density gradient centrifugation. From Fig. 3, it can be seen that the whole infectious virus particle equilibrates at a density of approximately 1.23 g/ml, whereas the HA particle derived by Tween-ether treatment equilibrates at the slightly higher density of 1.26 g/ml.

From previous experiments (unpublished data),
TABLE 1. Comparison of chikungunya virus harvests and Formalin and Tween-ether vaccines

| Harvest or vaccine          | Infectivity (log_{10} plaque-forming units/ml) | Hemagglutinin (log_{10} units/ml) | ED_{50}³ |
|----------------------------|-----------------------------------------------|-----------------------------------|--------|
| 48-hr Virus harvest        | 8.5                                           | 3.1                               | 0.172  |
| 48-hr Formalin vaccine     | <1.0                                          | <0.3                              | 0.166  |
| 48-hr Tween-ether vaccine  | 1.0                                           | 3.7                               | 0.243  |
| 96-hr Virus harvest        | 7.3                                           | 3.4                               |        |
| 96-hr Formalin vaccine     | <1.0                                          | <0.3                              | 0.166  |
| 96-hr Tween-ether vaccine  | 1.0                                           | 3.7                               |        |

³ ED_{50} is the volume of vaccine per dose protecting 50% of vaccinated mice against a lethal challenge. Groups of mice were vaccinated on day zero with serial dilutions of vaccine and were challenged 14 days later with 300 mouse LD_{50}.

TABLE 2. Antibody response in mice after vaccination with Formalin and Tween-ether chikungunya vaccines

| Vaccine             | Hemagglutination inhibition test CHIK 168³ | Complement fixation test CHIK 168³ | Bead neutralization tests⁴ |
|---------------------|-------------------------------------------|-----------------------------------|---------------------------|
|                     | CHIK 168                                  | C-266                             | BAH-306                   | Mayaro       | SFV⁴        |
| 48-hr Formalin      | 20                                        | 8                                 | 11                        | 8            | 9           | 0           | 0           |
| 48-hr Tween-ether   | 20                                        | 4                                 | 14                        | 8            | 11          | 0           | 0           |
| 96-hr Formalin      | 10                                        | 4                                 | 10                        | 0            | 9           | 0           | 0           |
| 96-hr Tween-ether   | 20                                        | 16                                | 14                        | 9            | 11          | 0           | 0           |

³ Vaccine given in two doses (0.25 ml intraperitoneally) 7 days apart.
⁴ HI titer represents the reciprocal of the highest dilution of serum exhibiting complete inhibition of eight units of hemagglutinin.
CF titer is expressed as the reciprocal of the highest dilution of serum reacting with four units of viral antigen and resulting in fixation of two full units of complement.
Neutralization was measured as the diameter (in millimeters) of the zone of plaque inhibition.
Semliki Forest virus.

product as a vaccine. Both Formalin and Tween-ether vaccines were prepared simultaneously for reference purposes. Stock CHIK virus harvested from infected green monkey kidney cell cultures at 48 and 96 hr was used in the preparation of the vaccines. The levels of infectious virus and HA vary between 48 and 96 hr postinoculation (Fig. 1). It was of interest to uncover any differences between vaccines prepared from a harvest containing mostly infectious virus and one in which HA was at its highest level with infectivity on the decline. Experiments were also designed to compare the immunogenicities of vaccines prepared by the Tween-ether and Formalin methods.

Infectious virus and HA levels before and after inactivation by Tween-ether or Formalin are summarized in Table 1. Residual virus in the vaccines was tested for by intracerebral inoculation of suckling mice and by plaque titration on chick embryo cell culture dishes. Formalin was neutralized in Formalin-inactivated vaccines by the addition of sodium bisulfite before safety testing. No live virus was detected in either test system. From Table 1, it can be seen that Formalin destroys HA activity of CHIK virus and that Tween-ether extraction raises the HA level.

One-dose potency assays of the vaccines were performed by inoculating mice with various dilutions of vaccine and then challenging the mice 2 weeks later with a constant amount of CHIK virus. The challenge dose of virus after titration was found to be approximately 300 mouse LD_{50} with an intracerebral challenge in the range of 100 to 1,000 mouse LD_{50} considered to be adequate in CHIK vaccine potency assays. The ED_{50} value for each vaccine is listed in Table 1. The ED_{50} is the volume of vaccine per dose protecting 50% of vaccinated mice against a lethal challenge.

Immunological response in mice to the Tween-ether and Formalin vaccines was determined by neutralizing HI and CF antibody tests. Two-dose assays were performed in mice for each vaccine (Table 2). For the HI and CF antibody tests, postvaccine mouse sera were tested against the African CHIK 168 strain virus which was used in the preparation of vaccines. Results (Table 2) show that all four vaccines stimulated CF and HI antibodies when postvaccine sera were tested against the homologous virus. However, to determine the degree of specificity of antibodies produced in response to the vaccines, several different strains of CHIK virus were used in the
neutralizing antibody tests. Mayaro and Semliki Forest viruses were also included since they belong to the same subgroup as CHIK virus within the group A arboviruses. Testing of postvaccine mouse sera listed in Table 2 showed stimulation of neutralizing antibody against several strains of CHIK virus but not against antigenically related Mayaro and Semliki Forest viruses.

**DISCUSSION**

After characterization of the Tween-ether-derived CHIK virus particle by rate-zonal sedimentation and buoyant density determinations, it was found to be smaller in size and greater in density than the mature CHIK virion. Other investigators have shown similar results with Tween-ether extraction of Sindbis virus (10) and measles virus (4).

After treatment of CHIK and other arboviruses with ether, a marked diminution is observed in HA and infectious virus activity, suggesting that ether disrupts the integrity of the virus by attacking the lipid-containing envelope. When Tween 80, a nonionic surfactant, was used in conjunction with ether, infectivity of CHIK virus was greatly reduced but HA activity increased. The combined action of Tween and ether appears to result in a mild delipidation of the envelope of CHIK virus, resulting in increased HA activity. Removal of viral envelope lipid by Tween-ether extraction was shown to be a possible explanation for the increased density and smaller size of the extracted hemagglutinin when it was characterized by density gradient centrifugation.

Chikungunya virus vaccines prepared by the Tween-ether extraction method have been shown to be as immunogenic as Formalin-inactivated vaccines prepared from the same virus stock. Potency assay ED50 values for the vaccines showed no significant variation when 95% confidence limits were applied in the chi square test. Vaccines did not differ in potency when 48- or 96-hr virus harvests were used for the preparation of either Formalin or Tween-ether vaccines. Although the ratio of HA to infectious virus varied between the 48- and 96-hr harvests, immunizing antigen concentration, as reflected in vaccine potency, remained constant.

After two doses of either Tween-ether or Formalin vaccine, mice showed a stimulation of neutralizing, HI, and CF antibodies to the homologous virus strain and neutralizing antibodies to antigenically similar strains of CHIK virus. The Tween-ether vaccines were not broadened antigenically, and no neutralizing antibody after vaccination of mice with two doses of vaccine could be detected for Mayaro and Semliki Forest viruses.

Although a direct relationship between HA and the immunizing antigen was not demonstrated, the graded protective response in mice to various amounts of HA was observed in the potency assays for the Tween-ether vaccines. The Formalin vaccines, lacking demonstrable HA activity, also show a graded response in potency assays. Further studies are required to elucidate the nature of the immunizing antigen with respect to the infectious CHIK virus particle.

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