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Immunogenicity of purified, inactivated chikungunya virus in monkeys*

EIICHI NAKAO[1] & SUSUMU HOTTA[2]

Chikungunya (CHIK) virus, harvested from infected BHK-21 cell cultures and highly purified by a method combining zinc acetate precipitation, Sephadex–Sepharose column chromatography, and sucrose density gradient centrifugation, was subjected to ultraviolet (UV) irradiation and treatment with formalin. Inactivation of the virus by UV light was apparently a first-order reaction. The virus treated with 0.05% formalin at 4°C was inactivated completely after 7 weeks, but when treated with 0.025% formalin retained its plaque infectivity at least 17 weeks. Both UV- and formalin-inactivated viruses induced production of anti-CHIK neutralizing antibodies in Japanese monkeys with no preimmune antibodies. Judging by the titres of antibodies produced, the immunogenic effect of the UV-irradiated virus was superior to that of the formalin-treated virus.

In view of the distribution of chikungunya (CHIK) virus in tropical areas of Asia and Africa and the possible danger of laboratory infection, the need for anti-CHIK vaccines is obvious. Harrison et al. (1967), dealing with formalin-inactivated CHIK virus, and Eckels et al. (1970), with Tween–ether extracted virus, reported the immunizing effects of their experimental vaccines on mice and monkeys. A trial of the formalinized vaccine in human volunteers has also been reported (Harrison et al., 1971). In this paper, experiments are described in which highly purified CHIK virus was inactivated by ultraviolet (UV) irradiation or formalin and tested for its immunogenic effect in inducing production of neutralizing antibodies in monkeys.

MATERIALS AND METHODS

Virus

The CHIK virus, African strain, from the 177th suckling mouse brain passage (kindly supplied by Dr A. Igarashi and Dr K. Fukai of Osaka University), was cultivated in BHK-21 monolayer cultures (Macpherson & Stoker, 1962) at 37° C in a medium consisting of Eagle's minimum essential medium, 5% inactivated calf serum, and 0.03% glutamine, supplemented with 6 μg of kanamycin per ml. The infected culture fluids harvested after incubation for 17–20 hours were purified by a method combining zinc acetate precipitation, Sephadex–Sepharose column chromatography, and sucrose density gradient centrifugation. The procedures used for cell culture and virus purification were the same as reported previously (Yoshinaka & Hotta, 1971).

Virus diluent

Phosphate buffer saline (PBS) (Dulbecco & Vogt, 1954) containing 0.2% bovine serum albumin (BSA) (Difco) was used to dilute the viral materials throughout the experiments. When UV irradiation and formalin treatment were applied, the diluent was used without BSA.

UV irradiation

The purified virus suspension was placed in a 66-mm diameter Petri dish to a depth of 3 mm with a stirrer. It was then irradiated by UV lamp at the rate of 4.7 W/m²/s at a distance of 25 cm. The temperature of the fluid was maintained at 4° C.

Formalin treatment

The purified virus was mixed with formalin (neutralized by the addition of magnesium carbonate) to a final concentration of either 0.05% or 0.025%.

* From the Department of Microbiology, Kobe University School of Medicine, Kusunoki-cho, Ikuta-ku, Kobe, Japan 650. Originally presented to the 1971 Conference on the Pathogenesis of Arboviral Infections held under the US-Japan Cooperative Medical Science Program in Hawaii, USA.

[1] Virologist.

[2] Professor and Chairman.

The mixture was sealed in a glass tube and held at 4°C, being shaken by hand every third day.

Virus assay

The infectivity of viral materials was determined by the plaque technique on BHK-21 cell monolayer cultures. The well-grown cells in 80-ml plaque bottles were washed with Hanks' salt solution (pH 7.0) and inoculated with 0.1 ml of the virus suspension per bottle. After the cultures had remained at room temperature for 90 min, with rocking every 7 min, 10 ml of an overlay medium (Eagle's minimum essential medium containing 1.2% methyl cellulose, 5% calf serum, 0.03% glutamine, 6 μg of kanamycin per ml, and 0.12% sodium hydrogencarbonate) was added to each bottle (Hotchin, 1955; Schulze & Schlesinger, 1963). After incubation at 37°C for 4 days, the plaques were counted. The cells were stained with crystal violet.

Immunization of monkeys

The irradiated or formalin-treated virus, after confirmation that it had completely lost its plaque infectivity, was injected subcutaneously into Japanese monkeys *(Macaca fuscata)* procured from the Japan Monkey Centre. No anti-CHIK antibodies were present in the sera before injection. Three shots were given in the flexor side of the upper arm: 1 ml at the first injection; 2 ml 2 weeks later; and 2 ml 9 weeks after the first. No abnormal sign was noted in the inoculated animals. At an appropriate time serum was taken from each monkey and tested for its anti-CHIK neutralizing antibody titre.

Titration of anti-CHIK neutralizing antibody

The monkey sera were inactivated by heating at 56°C for 30 min and then stored at −20°C until tested. One ml of a 2-fold dilution of each serum was mixed with 1 ml of a CHIK virus suspension of about 200 plaque-forming units (PFUs) per 0.1 ml. The mixture was held at 37°C for 60 min; 0.1 ml was then assayed for plaque titres on BHK-21 cells by the method described above. As a control, the same virus suspension was mixed with the PBS–BSA diluent instead of the test sera and assayed for plaque titres in the same manner. A 50% plaque reduction rate was calculated by probit chart (Finney, 1952).

RESULTS

Inactivation of CHIK virus by UV irradiation and formalin

The results obtained by UV irradiation and formalin treatment are illustrated in Fig. 1 and 2 respectively. Inactivation of the purified CHIK virus by UV light was linear, and was complete within 5 min. The virus was completely inactivated when treated in 0.05% formalin kept for 7 weeks at 4°C, but the virus treated with 0.025% formalin still retained its plaque infectivity after 17 weeks.

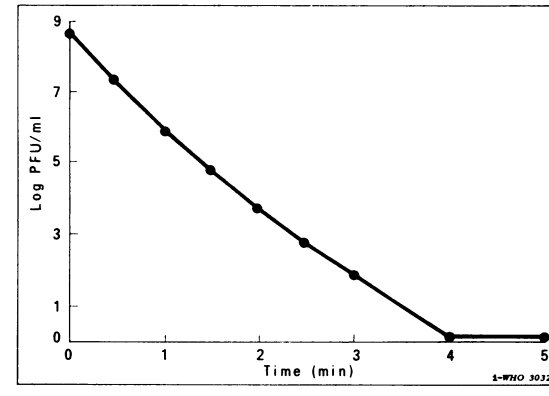

Fig. 1. Inactivation of purified CHIK virus by UV irradiation. Ordinate indicates titre of active virus as expressed in log plaque-forming units (PFU)/ml. Abscissa indicates irradiation time in minutes.

Production of anti-CHIK neutralizing antibodies in vaccinated monkeys

In 2 monkeys inoculated with UV-inactivated CHIK virus, neutralizing antibodies of a significant titre were produced after the second injections; the titres rose promptly after the third injections, showing a maximum level of about 400 (Fig. 3). In 2 other

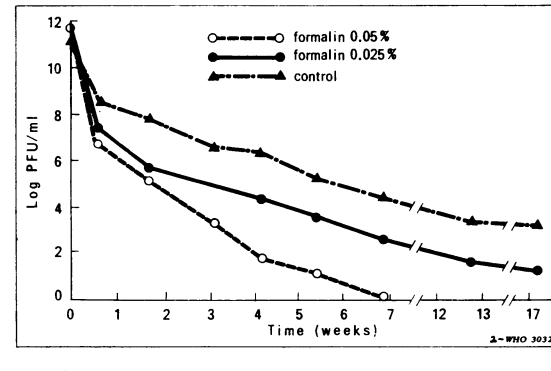

Fig. 2. Inactivation of purified CHIK virus by addition of formalin. Units of ordinate are the same as in Fig. 1. Abscissa shows duration of exposure to formalin, in weeks.

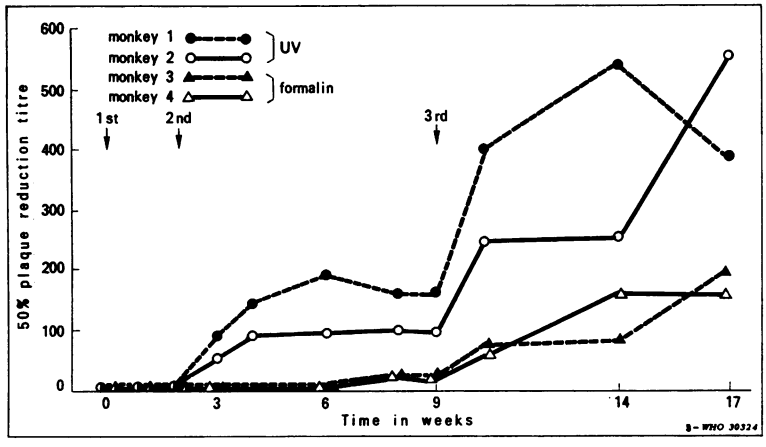

Fig. 3. Production of anti-CHIK neutralizing antibodies in Japanese monkeys (*Macaca fuscata*) after subcutaneous injection of purified CHIK virus inactivated by UV irradiation or formalin treatment. Ordinate indicates titre of antibodies as measured by the 50% plaque reduction method. Abscissa indicates observation period in weeks. Arrows indicate times of the 3 injections.

monkeys inoculated with virus inactivated with 0.05% formalin, production of the specific neutralizing antibodies was comparatively low even after the third injection. A similar tendency was noted in another series of experiments. Our data indicated that the UV-inactivated virus was superior to the formalinized virus in regard to its immunogenicity in monkeys.

DISCUSSION

UV-irradiated CHIK virus was shown to be effective in stimulating monkeys to produce anti-CHIK neutralizing antibodies. Virus inactivation by UV irradiation is apparently a first-order reaction and is complete within several minutes under experimental conditions. Complete inactivation of the virus by 0.05% formalin required about 7 weeks, and this period was prolonged considerably when the concentration of formalin was reduced to 0.025%. Moreover, the antibody-producing capacity of the formalin-treated virus was lower than that of the UV-inactivated virus, at least under the conditions studied. Thus the anti-CHIK UV vaccine appears to be better than the formalin-treated vaccine, although further studies are neccessary before a definite conclusion can be drawn.

In a previous paper (Nakao, 1972) we reported that the African strain of CHIK virus used here was indistinguishable from an Asian strain of virus (BaH306), isolated in Thailand, in kinetic neutralization tests using anti-CHIK mouse sera. The vaccine against the African strain may therefore also give effective protection against CHIK virus of Asian origin.

It should be noted that the virus materials used in the present study were highly purified, containing only very small amounts of contaminants. The properties of the chemical components of the virus should be further explored from the immunological viewpoint. Igarashi et al. (1970) have reported findings bearing on this problem.

A number of practical considerations should be borne in mind. To avoid the hazard of adventitious agents, cellular substrates for virus propagation should be carefully selected. For example, human diploid cells may be used. Attention must also be given to the basic and technical aspects of the viral inactivation mechanism. Gruber (1971) has reported that ionizing radiation by cobalt-60 was effective in retaining a good level of immunogenicity against purified Venezuelan equine encephalitis virus, another group A arbovirus.

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RÉSUMÉ

IMMUNOGÉNICITÉ DU VIRUS CHIKUNGUNYA, PURIFIÉ ET INACTIVÉ, CHEZ LE SINGE

Une souche africaine de virus chikungunya entretenue sur cultures cellulaires BHK-21 a fait l'objet d'une purification poussée combinant la précipitation par l'acétate de zinc, la chromatographie sur colonne Sephadex-Sepharose et la centrifugation en gradient de densité de saccharose. Elle a ensuite été inactivée soit par irradiation aux rayons ultra-violets soit par traitement formolé.

Le virus traité par le formol à 0,05% à 4°C a été complètement inactivé après 7 semaines; traité par le formol à 0,025‰, il a conservé son activité formatrice de plages pendant 17 semaines au moins. Le virus soumis au rayonnement UV a été totalement inactivé après 5 minutes. Injectés à des singes, les virus inactivés par le rayonnement UV ou par le formol à 0,05% ont tous deux suscité la production d'anticorps neutralisants anti-chikungunya, mais à en juger par les titres obtenus le virus irradié a fait preuve d'un pouvoir immunogène supérieur à celui du virus inactivé par le formol.

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