An Orbivirus of Mosquitoes Which Induces CO₂ Sensitivity in Mosquitoes and Is Lethal for Rabbits

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An orbivirus, JKT-7400, isolated from *Culex* mosquitoes in Indonesia, replicated to a high titer and induced cytopathic effects in *Aedes albopictus* cell cultures. The virus produced lethal sensitivity to carbon dioxide in *Culex* and *Aedes* mosquitoes as well as in *Drosophila melanogaster* fruit flies but was not the agent of the hereditary sensitivity to carbon dioxide previously described for *Culex quinquefasciatus*. When injected intravenously in high doses, JKT-7400 virus was lethal for rabbits, apparently without replicating to a significant extent. It was not pathogenic for adult mice inoculated intravenously or for adult or suckling mice inoculated intracerebrally and intraperitoneally. Unlike an orbivirus isolated from *Culex* mosquitoes in China, JKT-7400 did not interfere with the replication of Japanese encephalitis virus in mosquitoes.

In the course of attempts to identify the infectious agent (Matsu) responsible for hereditary sensitivity to CO₂ in *Culex quinquefasciatus* mosquitoes (7, 8), investigations were undertaken with an orbivirus, isolated from *Culex* mosquitoes in Indonesia, which, like Matsu, produced cytopathic effects in the C6/36 clone of *Aedes albopictus* cells. It was found that although the Indonesian orbivirus did induce CO₂ sensitivity when inoculated into mosquitoes, it was not the cause of hereditary CO₂ sensitivity in *C. quinquefasciatus*. The Indonesian virus was not pathogenic for newborn or weanling mice but was lethal for rabbits at high dosages, though it did not appear to replicate to a significant extent in the latter species. In view of the relatively few viruses known to be toxic for vertebrates in the absence of viral replication and the suggestion that an orbivirus isolated from *Culex* mosquitoes might be of value for the control of Japanese encephalitis (JE) and dengue viruses (1), we record here our observations on the Indonesian virus.

MATERIALS AND METHODS

Viruses. Unless otherwise noted, all experiments were carried out with a virus designated JKT-7400. This agent was originally isolated by the late James Converse in insect cells from a pool of 50 female *Culex* mosquitoes collected in Batutunga, South Kalimantan, Indonesia, on 26 February 1981. The virus was detected by the appearance of cytopathic effects in the C6/36 clone (2) of *A. albopictus* cells. The cell culture was then sent to the Yale Arbovirus Research Unit for further study, where it was found that the isolate contained at least one virus which consisted of 10 segments of double-stranded RNA, presumably an orbivirus (D. L. Knudson, Yale Arbovirus Research Unit, personal communication). We received the virus after it had undergone an unknown number of passages in C6/36 cells. The strain of JE virus we used was isolated from *Culex annulus* mosquitoes in Taiwan in 1972 and had been passaged only in mosquitoes since that time.

Cell cultures. The C6/36 clone of *A. albopictus* cells was cultivated at 28°C with either Eagle minimum essential medium or Dulbecco modified Eagle medium supplemented

with nonessential amino acids as described by Igarashi (2). The antibiotics used were penicillin (100 to 125 U/ml) and streptomycin (100 to 125 µg/ml). Heated (56°C for 30 min) fetal calf serum was used at a concentration of 10% for growth of cultures and, unless otherwise noted, at a concentration of 2% for maintenance of cultures.

Mosquitoes and *Drosophila melanogaster*. The mosquitoes employed were the Oahu strain of *A. albopictus*, which originated from specimens collected on Oahu, Hawaii, in 1971, and the Hokama strain of *C. quinquefasciatus*, which originated from eggs collected on Oahu, Hawaii, in 1980. Both colonies were maintained at 25 to 28°C, with a photoperiod of 16 h. The *D. melanogaster* used was the OM strain, obtained from the Laboratoire de Génétique des Virus, Gif-sur-Yvette, France. This strain was maintained at 25°C, with the ambient photoperiod. Both flies and mosquitoes were maintained at 25°C during all experiments.

Viral assay in mosquitoes and *D. melanogaster*. To assay viral samples for their ability to induce sensitivity to CO₂ in mosquitoes or *D. melanogaster*, serial 10-fold dilutions of test material were inoculated intrathoracically (6) in 0.00017-ml amounts into groups of approximately 25 female mosquitoes or male or female *D. melanogaster* flies. The mosquitoes and flies were kept for 21 days at 25°C, and the surviving specimens were tested for sensitivity to CO₂. The test was carried out by chilling the insects to 1°C and then exposing them to pure CO₂ for 15 min. They were then transferred to room temperature and allowed to recover. Specimens obviously paralyzed 1 h afterwards were considered sensitive. The amount of virus which induced sensitivity in 50% of the insects was considered 1 infectious unit (i.u.) and was estimated by the method of Reed and Muench (4). In some instances, the infection of sensitive insects was confirmed by the head squash technique and immunofluorescence (3).

Viral assay in cell cultures. Viral assay by immunofluorescence in cell cultures was carried out exactly as described previously for the assay of sigma virus (10). In brief, titrations were done in 96-well plastic, flat-bottomed cell culture plates by seeding them with a mixture of *A. albopictus* cells and serial 10-fold dilutions of test material. Four wells were used for each virus dilution, and the titers were calculated by the method of Reed and Muench (4).

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Electron microscopy. A JEOL JEM 100X CM II electron microscope set at 80 kV was used to monitor the viral purification procedures. Viral samples were examined unfixed and stained with 3% phosphotungstic acid.

Viral purification. Purified virus was prepared in the following manner. Fully formed monolayers of *A. albopictus* cells, which had been rinsed to remove fetal calf serum present in the growth medium, were seeded with virus at as high a multiplicity of infection as possible. The inoculum was contained in a volume of 4.0 ml for a bottle with a surface area of 200 cm². After the virus had adsorbed at 25°C for 1 h, 80 ml of maintenance medium, devoid of fetal calf serum, was added to each bottle. Supernatant fluid was harvested from the bottles after they had incubated for 48 h at 25°C and was clarified by centrifugation at approximately 1,200 × g for 20 min in a Beckman JA-14 rotor. The viral material was then concentrated about 10-fold in an Amicon hollow fiber apparatus by using a H1P 100-20 cartridge, which retains particles with a molecular weight of 100,000 or greater. The concentrated viral material was centrifuged at approximately 76,000 × g for 90 min in a Beckman SW27 rotor through a layer of either 40% sucrose or 10% tartrate in Tris hydrochloride buffer (0.002 M, pH 8.8) containing 0.002 M EDTA. Each centrifuge tube contained 10 ml of sucrose or tartrate solution and 28 ml of concentrated virus. The sediment from each tube was collected in 0.2 ml of the same buffer and allowed to stand overnight at 4°C. The sediments were then pooled and centrifuged at approximately 2,400 × g for 30 min to remove any remaining viral aggregates. Supernatant fluid from this centrifugation was placed at the top of either a continuous 30 to 60% sucrose gradient or a continuous 10 to 50% tartrate gradient. Both gradients were prepared in the Tris hydrochloride buffer containing EDTA described above. The sucrose gradient was centrifuged at approximately 90,000 × g for 60 min in a Beckman SW41 rotor, and the tartrate gradient was centrifuged at approximately 60,000 × g for the same length of time in the same type of rotor. Purified virus was recovered from the bottom of the tube with the sucrose gradient and from a band about one-third of the distance from the bottom of the tube with the tartrate gradient.

Preparation of immune sera. Immune sera were prepared in female New Zealand White rabbits weighing approximately 3.5 kg each. The animals received either three or four intravenous injections, the first three at weekly intervals and the last, if given, 4 weeks after injection 3. Rabbits immunized with virus purified in a sucrose gradient received approximately 10^{9.0} i.u. in each injection, and those immunized with virus purified in a tartrate gradient received approximately 10^{7.7} i.u. in each injection. The immune sera were titrated by immunofluorescence in *A. albopictus* cell cultures as described for sigma virus (11).

Neutralization tests in mosquitoes. Neutralization tests in mosquitoes were carried out by mixing equal parts of each test serum, diluted 1:10, and a dilution of virus containing approximately 10^{6.0} i.u. of virus per ml and allowing the mixture to incubate for 1 h at 25°C. At the end of the incubation period, the serum mixture and a titration of the test virus dilution were inoculated into groups of *A. albopictus* mosquitoes as described above.

Histologic procedures. All tissues obtained at autopsy were fixed in Duboscq fluid except for brain tissue, which was fixed in 10% Formalin. All tissues were stained with hematoxylin and eosin.

RESULTS

Observations on infected cell cultures. As assayed by the induction of CO₂ sensitivity in *A. albopictus* mosquitoes, JKT-7400 virus replicated to a titer of approximately 10^{8.5} i.u./ml in *A. albopictus* cell cultures. Such titers were observed as early as 48 h after inoculation of the cultures in the absence of cytopathic effects, which did not appear until about 3 days later. Also, it was found that the virus titer did not increase significantly after 48 h and that the supernatant fluid of the cultures contained more than 10 times as much virus as did washed cells which had been frozen, thawed, and suspended in the same quantity of fresh medium.

Viral purification and preparation of antisera. After a series of preliminary experiments, JKT-7400 virus was purified and rabbits were immunized as described in Materials and Methods. The purification was facilitated by the observation that cultures maintained on media without fetal calf serum yielded as much virus as did those maintained on media containing 2% serum.

When sera were titrated by immunofluorescence with infected and uninfected cell cultures, it was found that sera obtained from two rabbits immunized with virus purified in a sucrose gradient had specific titers of 1:400 after injection 3. Sera collected from the same animals after injection 4 were unsatisfactory because titers were as high against uninfected cells as against infected cells. One of the two serum samples obtained after inoculation 3 was tested for neutralization of the virus in mosquitoes. None of the mosquitoes which received a mixture containing a 1:10 dilution of that serum became CO₂ sensitive, whereas all mosquitoes which received a mixture containing a comparable dilution of preimmunization serum from the same rabbit did become sensitive to CO₂. The simultaneous titration of the virus pool revealed that it had contained 10^{5.6} i.u./ml.

Of two rabbits immunized with virus purified in a tartrate gradient, one had a specific titer of 1:200 after injection 3 and was exsanguinated at that time. The other had a titer too low to be useful after injection 3 but had a specific titer of 1:400 after injection 4.

Physical and chemical properties of JKT-7400 virus. JKT-7400 virus was morphologically similar to an orbivirus (Fig. 1). Note that virus purified in sucrose exhibited distinct capsomeres and was approximately 60 nm in diameter, whereas virus purified in tartrate had an amorphous surface and was approximately 75 nm in diameter.

The size of the virion as seen by electron microscopy was confirmed by filtration experiments with membrane filters (Millipore Corp.). A virus pool with a titer of 10^{8.5} i.u./ml before filtration had titers of 10^{8.0}, 10^{8.0}, and 10^{5.3} i.u./ml after passage through filters with pores of 450, 220, and 100 nm, respectively.

JKT-7400 virus was insensitive to the action of ethyl ether. A pool had a titer of 10^{8.3} i.u./ml after overnight exposure to 20% ether, whereas a control sample exposed for an equal length of time in culture medium without ether had a titer of 10^{8.0} i.u./ml.

CO₂ sensitivity in mosquitoes and flies. JKT-7400 virus induced CO₂ sensitivity in both *C. quinquefasciatus* and *A. albopictus* mosquitoes as well as in *D. melanogaster* flies. CO₂ sensitivity titers appeared to be somewhat higher in *A. albopictus* than in *C. quinquefasciatus*, since one pool of virus had a titer of 10^{7.6} i.u./ml in the latter species and a titer of greater than or equal to 10^{7.8} i.u./ml in the former. The difference in titers was more clearly demonstrated in another experiment in which a pool of another viral isolate (JKT-

FIG. 1. JKT-7400 virus purified in a sucrose (A) or a tartrate (B) gradient. Magnification, $\times 200,000$.

7377), which was said to be identical to JKT-7400 on the basis of the electrophoretic migration of the components of its segmented genome (Knudson, personal communication), was titrated. In that experiment, a titer of $10^{6.6}$ i.u./ml was observed in the *Culex* species, and a titer of greater than or equal to $10^{7.8}$ i.u./ml was observed in the *Aedes* species. In another experiment, a pool of JKT-7400 virus with a titer of $10^{6.8}$ i.u./ml in *A. albopictus* had a titer of only $10^{5.5}$ i.u./ml in *D. melanogaster*. Paralysis following the anesthetization of infected mosquitoes appeared to be specific to CO_2 , since it was not noted after otherwise comparable anesthetization with pure nitrogen.

The specificity of CO_2 sensitivity as an indicator of viral infection was demonstrated by immunofluorescence on head squash samples of *A. albopictus* mosquitoes. Each sample from a mosquito judged sensitive in a viral titration, including those at the endpoint, was found to fluoresce specifically, whereas all samples from insensitive mosquitoes showed no fluorescence. Furthermore, comparative titrations of one viral pool in both cell cultures and by CO_2 sensitivity in *A. albopictus* mosquitoes yielded identical titers of $10^{8.5}$ and $10^{8.6}$ i.u./ml, respectively.

Attempt to demonstrate interference between JKT-7400 and JE virus in mosquitoes. In view of the report (1) that an orbivirus isolated from *Culex* mosquitoes in China interfered with the replication of JE virus in mosquitoes, the following experiment was carried out with JKT-7400 virus. Male *A. albopictus* mosquitoes were inoculated with approximately 400 i.u. each of JKT-7400 virus. One week later, the same mosquitoes were inoculated with approximately 50 i.u. each of JE virus. One group of control mosquitoes received only JKT-7400 virus, another received only JE virus, and group 3 was not inoculated at all. Nine days after inoculation with JE virus, all mosquitoes were killed and examined by the head squash technique. Among mosquitoes which had received both JKT-7400 and JE viruses, the degree of JE fluorescence was as intense as it was in mosquitoes which had received JE virus alone. In other words, there was no evidence of JE viral inhibition. Mosquitoes which had received both viruses also had specific fluorescence for JKT-7400 virus, as did the mosquitoes which had received that virus alone.

Pathogenicity of JKT-7400 virus for rabbits and mice. The pathogenicity of JKT-7400 virus for rabbits was first noted when an attempt was made to immunize the animals intravenously with concentrated virus which had been purified in a sucrose gradient. The first two animals, inoculated with approximately $10^{10.0}$ i.u. each, died between 36 and 48 h after injection 1, but the deaths were not suspected to have been caused by the virus and the animals were not autopsied. When the next two inoculated rabbits died under similar circumstances, a gross autopsy was performed on the one that was not too decomposed when found dead. The most striking finding of this autopsy was the presence of extensive subcutaneous hemorrhages. Subsequently, when the amount of virus inoculated was reduced 10-fold, no further difficulties were encountered in immunizing rabbits.

To obtain further information on the cause of mortality, a female rabbit weighing 4 kg was inoculated intravenously with $10^{10.0}$ i.u. of virus and observed at frequent intervals, so that an autopsy and collection of specimens for histologic and virologic examination could be carried out immediately after the animal died. At the time of death, 39 h after inoculation, the only gross pathology observed was a marbling of the liver and congestion of the kidneys. No hemorrhagic lesions were noted, nor was there an obvious cause of death. A blood sample was collected from the heart at the time of death, and samples of brain, liver, spleen, kidney, lung, heart, and mediastinal lymph node tissues were obtained for histologic and virologic examination.

The only abnormalities noted when the tissues were examined histologically were focal necroses in the liver and, especially, in the spleen. The tissues were triturated in approximately five times their volume of diluent and then assayed for virus in *A. albopictus* cells. This test was not completely satisfactory because some of the triturates (especially those of the liver) were toxic for *A. albopictus* cells at low dilutions. The titers (expressed as infectious units per milliliter of triturate) were as follows: spleen, $10^{4.7}$; liver, $<10^{3.5}$; lung, $10^{2.7}$; lymph node, $10^{2.5}$; heart, $<10^{2.5}$; kidney, $10^{1.7}$; and brain, $<10^{1.5}$. Plasma from the blood sample obtained at the time of death contained approximately $10^{4.0}$ i.u. of virus per ml.

Since it was not clear from the preceding experiment whether or not JKT-7400 virus had replicated in the rabbit, an attempt was made to address that question directly. Two female rabbits weighing 3.5 and 4.2 kg were inoculated intravenously with $10^{8.0}$ and $10^{5.0}$ i.u. of JKT-7400 virus, respectively. Such doses of virus were nonlethal, of course,

and blood samples were obtained from the animals every 12 h for the first 2 days after their inoculation and then at 24-h intervals for the next 2 days. Surprisingly, each of these samples contained approximately the same amount of virus, namely between $10^{3.5}$ and $10^{4.0}$ i.u./ml of serum when assayed in *A. albopictus* cells. The same sera were also assayed in intact mosquitoes and found to have a titer of less than $10^{3.8}$ i.u./ml, the least amount of virus detectable by that assay method. One month after being inoculated with virus, the rabbit which had received the larger dose of virus had a serum antibody titer of 1:200 by immunofluorescence, whereas the rabbit which had received the smaller dose of virus had no detectable antibody in serum diluted 1:80, the lowest dilution that it was possible to assay in C6/36 cells. These two rabbit serum samples were also tested for their ability to neutralize virus in mosquitoes. None of the mosquitoes which were injected with a mixture containing a 1:10 dilution of the postinoculation serum from the rabbit which had received the larger dose of virus became CO_2 sensitive, whereas all mosquitoes injected with a mixture containing a 1:10 dilution of the comparable serum from the other rabbit did become CO_2 sensitive. The simultaneous titration of the virus pool revealed that it had contained $10^{5.6}$ i.u./ml.

The same pool of virus which had proved lethal for rabbits was then tested for pathogenicity in white Swiss mice. Eight adult mice, weighing approximately 15 g each, were inoculated intravenously with approximately $10^{9.8}$ i.u. of virus per animal. Another two groups with five adult mice per group received approximately $10^{9.0}$ i.u. of virus intracerebrally or $10^{8.7}$ i.u. intraperitoneally per mouse. Finally, two litters with eight newborn mice per litter were inoculated with $10^{8.0}$ i.u. of virus per mouse intracerebrally, and two other litters with the same number of mice were inoculated with $10^{8.0}$ i.u. per mouse intraperitoneally. None of the mice showed any sign of illness during an observation period of 12 days.

DISCUSSION

It was originally suspected that JKT-7400 virus might be the infectious agent (Matsu) responsible for hereditary sensitivity to CO_2 in *C. quinquefasciatus* mosquitoes because it was isolated from a pool of *Culex* mosquitoes in nature, produced cytopathic effects similar to that produced by Matsu in C6/36 cells, and induced sensitivity to CO_2 when inoculated into *Culex* mosquitoes. After JKT-7400 virus was purified and an antiserum was prepared, however, it became clear that the virus was not the Matsu agent. Thus, although JKT-7400 virus could be detected easily by immunofluorescence in infected cell cultures or in head squash samples of infected mosquitoes, no fluorescence was seen when the same antiserum was used to react with cell cultures known to contain the Matsu agent or with CO_2 -sensitive mosquitoes which had been injected with the Matsu agent or were from the Matsu colony.

Furthermore, JKT-7400 virus was ether resistant, whereas Matsu was ether sensitive. Finally, the two agents differed greatly in size; JKT-7400 virus passed through a 220-nm filter with no loss of titer, whereas Matsu, propagated in the same type of cell cultures, was completely retained by such a filter.

The demonstration that JKT-7400, an orbivirus, can induce CO_2 sensitivity in mosquitoes adds a third virus family to the group of agents known to produce such a phenomenon

in mosquitoes, the others being rhabdoviruses (5) and bunyaviruses (9). As yet, the mechanism of CO_2 sensitivity is unknown but is suspected to involve changes in the plasma membrane of neurons. It is of interest that, unlike rhabdoviruses and bunyaviruses, orbiviruses do not have an envelope (hence their ether resistance).

It appears that JKT-7400 virus did not replicate to a significant degree in rabbits after intravenous inoculation, or at least not to the extent that would explain the death of an animal in 39 h. If the death had been the result of viral replication, a higher titer of virus in one or more of the various organs sampled would have been expected. It is difficult, however, to explain the persistent viremia observed in the rabbit which received an intravenous injection of only $10^{5.0}$ i.u. unless some replication did occur. The absence of detectable antibody in this animal a month after inoculation argues against extensive replication. If JKT-7400 virus was indeed lethal for rabbits in the absence of replication, it is one of the few viruses known to have a toxic effect *in vivo*. It is of interest that the only other known viruses with similar properties (the iridovirus, frog virus 3, and iridoviruses of insects) also were originally recovered from hosts with normally low temperatures and do not replicate at 37°C .

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