Isolation of Influenza Viruses Types A and B in Ferret Kidney Cell Culture[1] (35025)

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Influenza A virus was isolated and characterized using the ferret as the experimental host (1). The ferret is still the best experimental host for the study of the clinical syndrome produced by influenza A virus. The studies of the susceptibility of ferret kidney cell cultures (FKCC) to influenza virus came about as the result of the isolation of influenza B virus in 1966 in the course of testing skunk kidney cell cultures (SKCC) as a system for isolation of viruses from throat wash specimens, which were negative for virus by the chicken embryo test. Since then it has been found that SKCC and FKCC host systems are suitable for isolation of both influenza A and B viruses and for cultivation of rabiesvirus. In testing the susceptibility of other cell culture systems, it was found that bat lung cell cultures (BLCC) were susceptible to infection with influenza B virus.

Materials and Methods. Animals. The striped skunks, *Mephitis mephitis*, used in this study were wild caught skunks from Alameda County, California. Throat swab specimens were taken at the time of capture and these were tested for virus by ic inoculation into a litter of infant mice, 1 to 2 days old. The skunks were bled prior to removal of the kidneys and lungs for cell culture. Blood serum was saved for serological studies. The brain was tested for rabies by the fluorescent rabies antibody (FRA) test. The control cell cultures were tested for the presence of hemadsorbing viruses by the addition of guinea pig erythrocytes and for viruses pathogenic for infant mice. The ferrets were purchased from Marshall Research Animals, Inc., North Rose, New York. The control cell cultures were tested as noted above. The rhesus monkeys were obtained from Woodard Asiatic Corp., San Francisco. The control rhesus monkey kidney cell cultures were tested for naturally occurring viruses by the hemadsorption test and by observation for cytopathic effects. *Tadarida brasiliensis mexicana* bats were collected in Berkeley, California. The salivary glands of each bat were tested in infant mice. The *Myotis lucifugus* and *Pipistrellus subflavus* bats were collected from Boone cave, near Columbia, Missouri. The control cell cultures from the three species of bats were tested for viruses as noted above.

Specimen collection and preparation. The patients included in this study were from the one author's family and from the family of one of the laboratory staff. Throat wash or nose and throat swab specimens were taken in cases of severe respiratory tract infections in an attempt to obtain a definitive diagnosis. The throat wash specimens were obtained by having the patient gargle with 10 ml of 0.75% bovalbumin fraction V in phosphate saline solution. The material was centrifuged at 2500 rpm for 20 min and a portion of the supernatant fluid was taken for testing and the rest was stored in sealed glass ampoules at a temperature below −60°. Antibiotics prepared as a 10× concentrate were added to the material to be tested to give 2 mg of streptomycin and 1000 units of penicillin/ml. A volume of 0.1 ml of the throat wash specimen was added to each tissue culture tube. The throat and nose swab specimens were put into 2 ml of 0.75% bovalbumin fraction

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TABLE I. Hemagglutination–Inhibition Reactions of JNJ Virus with Other Influenza Type B Viruses.

Viruses	Animal immune sera				
	G. Lakes/54	Maryland/59	Arizona/62	Taiwan/62	JNJ/66
Great Lakes/54	128[a]	16	128	<8	8
Maryland/59	32	*64*	128	<8	16
Arizona/62	64	32	*256*	<8	32
Taiwan/62	<8	<8	16	*128*	8
JNJ/66	8	32	128	8	*32*

[a] Reciprocal of dilution which completely inhibited 4 hemagglutinating units of virus.

V in phosphate saline, and after rinsing the swab in this solution the swab was rolled against the side of the tube and removed. The specimen was processed as noted above.

Tissue culture systems. The skunks, ferrets, and bats were bled under ether anesthesia until dead. The kidneys and lungs were then removed aseptically and minced in Hanks' balanced salt solution. The tissue was trypsinized at room temperature for 2 to 3 hr in Hanks' solution at a pH of about 7.4. The outgrowth medium for the lung and kidney cell cultures was Hanks' balanced salt solution containing 0.5% lactalbumin hydrolysate (LAH), 15% inactivated fetal calf serum (FCS) and antibiotics; streptomycin, penicillin, and neomycin at 250 units/ml, and bacitracin at 2.5 units/ml. A 8.8% sodium bicarbonate solution was added to give a pH of 6.9 to 7.0. For maintenance medium (MM) the FCS was reduced to 2%. The rhesus monkey kidney cell cultures (MKCC) were prepared by the usual method (2). The MM for the MKCC was Leibovitz medium (3). When this was used for the FKCC the cytopathic effect (CPE) was delayed. The controls for each cell culture system were tested for natural virus infections.

Indirect fluorescent antibody (FA) test. The indirect FA method used for identification of influenza viruses was essentially that described by Lennette *et al.* (4).

Hemagglutination-inhibition (HI) test. Guinea pig erythrocytes were used in the HI test and serum end points were determined by the pattern method. The details of the test have been reported elsewhere (5).

The hemadsorption test. To test for hemadsorption, 0.2 ml of a 0.4% suspension of guinea pig red blood cells in phosphate saline was added to the 1.5 ml of medium in the cell culture tube. The tube was held at 4° for 30 to 60 min, with the slant in the same position as for incubation. A control tube of the same cell culture system was treated in like manner.

Results. Virus isolation and identification. The first isolation of influenza B virus in SKCC was obtained from a throat wash specimen of JNJ, a 23-year-old-male, taken on February 6, 1966. He became ill on the 5th when on a tour of Southern California with the University of California Glee Club. Twelve of 45 members of the club became ill on the 5th and a total of 15 developed symptoms of an acute respiratory tract infection before the tour ended on the 7th. When JNJ was seen on the 6th he had a temperature of 102.4 and complained of chills, acute sore throat, and a "head cold." The throat wash specimen was tested first in embryonated eggs. No virus was isolated in this host system. On February 25th the original throat wash specimen was tested in SKCC. A CPE was noted at 72 hr. Hemadsorption tests were positive and the virus was identified as influenza B by the FA test. The identification was confirmed by serological tests. Table I shows the results of hemagglutination–inhibition tests, comparing the JNJ virus with other strains of influenza B virus. The JNJ strain was passed 4 times in SKCC and a CPE was noted by days 3 to 4 at each passage. In February 1967 the virus was reisolated from the original throat wash specimen in both FKCC and MKCC. A CPE was

observed in the FKCC by day 3. The reisolation was identified as influenza B virus by the FA test. The JNJ strain of influenza B virus grew readily in ferret lung cell culture (FLCC). The CPE was less marked and developed later than in the FKCC. The supernate of the FLCC taken on day 7 after inoculation had a HA titer of >1:8.

A strain of influenza A virus was isolated from a throat wash specimen from JNJ, taken on Oct. 11, 1957, when he was 15 years of age. This virus isolation was done using the embryonated egg culture system. The virus was characterized as belonging to the Asian type of influenza virus. The throat wash specimen was taken on the first day of illness at which time he complained of temporal headache, sore throat, dizziness, sensitivity of the eyes to light, and aching in the chest, arms, and legs. His temperature ranged from 99.6 to 102 on the day the specimen was taken and reached a peak of 102.6 on the third day of illness. His 10-year-old brother (MWJ) developed a similar illness on Oct. 13th and influenza A virus was recovered from a throat wash specimen taken on that day. The third embryonated egg (E3) passage of the JNJ-57 virus was tested in SKCC and FKCC and a CPE was observed in both systems. The virus passaged in the FKCC was identified as influenza A by the FA test.

In testing specimens from the children of one of the laboratory assistants in 1968, a strain of influenza A virus was isolated from a throat and nose swab specimen from MW, a 5-year-old female, taken Jan. 19, 1968. The child had a mild upper respiratory infection with sore throat and cough. She made a prompt and uneventful recovery. The specimen was tested in FKCC and a CPE was observed on the fourth day. The virus was identified as influenza A by the FA test.

The Tadarida kidney cell cultures were tested for susceptibility to the JNJ strain of influenza A virus. The virus multiplied in this cell culture system without CPE. The Tadarida, Myotis, and Pipistrellus lung cell cultures were susceptible to infection with the JNJ strain of influenza B virus as shown by the hemadsorption and hemagglutination tests. The supernate of the Tadarida lung cell culture had a HA titer of >1:4. The Myotis and Pipistrellus lung cell cultures were inoculated with the J-305 Asian strain of influenza A virus and these were negative for virus by the hemadsorption and hemagglutination tests.

Discussion. The successful isolation of influenza A and B viruses in FKCC gives us a new host system for use in the routine isolation of these viruses. The SKCC also was suitable for this purpose. It would be of interest to determine whether kidney cell cultures from other mustelids would be good host systems for the isolation of influenza viruses, for example, minks and spotted skunks, which are available from commercial sources for use as experimental animals. Ferrets are much cheaper than monkeys and are easier to house and feed in the laboratory.

The studies of bat kidney cell culture systems is part of our surveillance of bats for latent viruses. We prepare pools of trypsinized lung and kidney cells from several bats of one species. If the cell culture from these animals does not show a CPE after changing from outgrowth medium to maintenance medium, the cell culture fluid is tested for virus by the infant mouse test, and by inoculation into MKCC and human fetal diploid (HFD) cell cultures. The bat cell culture systems have been tested for susceptibility to certain arboviruses. The Rio Bravo, Modoc, Powassan, Myotis meningoencephalitis and St. Louis viruses all multiplied in Tadarida kidney cell cultures without producing CPE. These viruses also multiply in FKCC without CPE. The Kern canyon virus isolated from Myotis yumanensis bats (6) multiplies in Tadarida cell cultures without CPE. Western and St. Louis encephalitis viruses multiply in Tadarida kidney cell cultures without CPE. Both of these viruses multiply in Tadarida lung cell cultures with CPE. Western encephalitis virus multiplies in FKCC with CPE. Nonneuroadapted strains of rabiesvirus have been cultivated in SKCC and FKCC.

As noted previously, cell culture of the lungs and kidneys of wild caught animals can be used as a method of surveillance for

latent virus infections in wildlife. In addition, the susceptibility of the cell culture from a species of animal, gives an indication of the possible role that this animal could play in the maintenance of a virus in nature. For example, if a virus causes a CPE in a lung or kidney cell culture system, one would expect that the host from which the cells were obtained would not be suitable for the maintenance of the virus in nature, because it would be expected to produce disease in this host. The hamster kidney cell culture system can be used as an example, that is, Western encephalitis virus multiplies in this cell culture system with CPE and when this host is inoculated with Western encephalitis virus by the sc route it ordinarily dies of the infection. On the other hand, the ability of a virus to multiply in a cell culture system from an animal and not produce CPE would favor the survival of the virus in this host in nature. The Tadarida bat kidney cell culture system is suitable for cultivating Rio Bravo virus and the virus does not produce CPE in this cell system. The virus can exist in this host as a symptomless infection of the salivary glands which shows that this host is a good one for the maintenance of the virus in nature (7).

The susceptibility of bat cell cultures to influenza viruses warrants consideration of this animal as a possible source of myxoviruses. If fruit and insectivorous bats are infected in nature with myxoviruses that are also infectious for man, there are ample opportunities for man to be infected with aerosols of virus from this animal. Many of the great cave temples in the Orient have bat roosts with large numbers of bats and this is one type of ecological situation that should be studied for myxoviruses. We do know that persons have been infected with rabies from aerosol exposure in bat caves containing large numbers of bats (8). There are several species of bats that tend to colonize in human habitation.

Summary. The isolation of influenza A and B viruses in primary ferret kidney cell cultures is described. A cytopathic effect is produced by these viruses in this cell culture system. The skunk kidney cell culture system is equally satisfactory for isolation of these viruses. Influenza A and B viruses were found to multiply in bat cell culture systems without a cytopathic effect.

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