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## Isolation of St. Louis Encephalitis Virus from a Naturally-Infected Gray Fox *Urocyon cinereoargenteus.** (32115)

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St. Louis encephalitis (SLE) virus has been repeatedly isolated from wild and domestic birds and from mosquitoes which feed on them; hence, both birds and mosquitoes are implicated in its natural maintenance. There is serologic evidence that feral and domestic mammals are infected (1-10), but recovery of this virus from naturally-infected mammals other than man had not been described prior to 1966 when Sulkin and co-workers reported the isolation of SLE virus from bats(11). This paper reports the isolation of SLE virus from the brain of a California gray fox, *Urocyon cinereoargenteus,* which was shot July 27, 1957 in Amador City, Amador County, California. The animal was obviously ill, but did not attack or run; it was suspected to have rabies and the head was sent to this laboratory for examination.

*Materials and methods.* A 10% suspension of the fox brain tissue in 0.75% bovine albumin in phosphate-buffered saline (BABS) was prepared by grinding the tissue in a mortar, and the suspension was clarified by centrifugation at 3,000 rpm for 20 minutes. The supernatant fluid was removed and penicillin and streptomycin were added to give a final concentration of 1,000 and 5,000 units per ml; the mixture was allowed to stand at room temperature for 30 minutes and was then inoculated intracerebrally (i.c.) in 0.03 ml amounts into 8 adult (4 week old) Swiss albino mice. The suspension was bacteriologically sterile in glucose and thioglycollate broth cultures. For subsequent passages, the brains of sick or dead mice were harvested, made into 10% suspensions as above and inoculated i.c. into groups of 8 adult mice or i.c. and intraperitoneally (i.p.) in 0.01 and 0.03 ml amounts, respectively, into litters of 6 suckling (1- to 4-day-old) mice. Mice were observed for 28 days before being discarded as negative. All brain suspensions were bacteriologically sterile.

Slip smears of brain tissue from the fox and from sick or dead passage mice were stained by Seller's method and examined for Negri bodies. Portions of the brains or brain suspensions were stored at —70°C in flame-sealed glass ampules.

Virus was identified by neutralization tests in suckling mice. Serial 10-fold dilutions of infected mouse brain in BABS were mixed with equal volumes of specific immune or normal rabbit or hamster serum. All sera were inactivated at 56°C for 30 minutes. Serum-virus mixtures were incubated 1 hour at 37°C and then inoculated into mice i.p. in 0.03 ml volumes, using 1 mouse litter (6 mice) per dilution. Mice were observed for 14 days, deaths were recorded and the $LD_{50}$

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titers were calculated by the method of Reed and Muench(12). The difference in titer between virus suspensions mixed with normal serum and those mixed with immune serum represented the neutralization index ($\log_{10}$ N.I.).

Immune sera were prepared by i.p. inoculation of rabbits or hamsters with infected mouse brain suspensions of the following virus strains: (1) fox brain isolate in the third mouse passage; (2) SLE, Ruis strain recovered from human brain, sixth mouse passage; (3) Turlock, MP 781-19, isolated from a pool of *Culex tarsalis* mosquitoes, third mouse passage; (4) Rio Bravo, M 64, type strain from the salivary glands of a naturally-infected *Tadarida brasiliensis* bat (13) third mouse passage; (5) Powassan, type strain isolated from human brain, sixth mouse passage; (6) Modoc, M 544, type strain from a *Peromyscus maniculatus* mouse, fifth mouse passage; (7) Japanese encephalitis (JE), Nakayama, isolated from human brain, forty-sixth mouse passage. Western encephalitis (WE) immune horse serum was obtained from a commercial source.

Cross immunity tests were performed as follows. Weanling or adult mice (3-6 weeks old) were inoculated subcutaneously with a single dose (0.1 ml) containing approximately 10,000 i.c. mouse $LD_{50}$ of the immunizing virus. Immunized mice were held for 1 month, along with uninoculated control mice of the same age. Immunized mice and control mice (6 mice per group) were then inoculated intracerebrally with 1,000 i.c. $LD_{50}$ of the challenge virus and were observed for 21 days. Challenge with homologous virus was done in each instance to demonstrate effectiveness of the immunization, and challenge with heterologous virus demonstrated the presence or absence of cross-protection to the other viruses.

*Results.* Two of 8 adult mice inoculated July 29, 1957, with the fox brain suspension were found dead (no previously recorded symptoms) on the tenth post-inoculation day. The other 6 mice remained well and were discarded after 28 days. Brains of the 2 dead animals were passed to 8 mice, and all were ill or found dead on the 5th or 6th post-inoculation day. In 2 neutralization tests, the titer of the brain suspension from these mice was $10^{-5.6}$ or greater in the presence of normal rabbit serum (NRS); the SLE immune serum reduced the titer to $10^{-3.0}$ ($\log_{10}$ N.I. 2.6 or greater) and $10^{-2.4}$ ($\log_{10}$ N.I. 3.2 or greater), while the titer was unaffected by Turlock virus and WE virus immune sera.

Reisolation of the virus from the fox brain was attempted August 27, 1957 in adult mice. One mouse was found dead on the 7th day, and 1 mouse was found dead and 1 sick on the 9th day after inoculation. The other 5 mice remained well for 28 days and were discarded. On subpassage, all mice were dead or moribund on the 6th day. Titer of the brain suspension from these mice was $10^{-7.6}$ in the presence of NRS and $10^{-1.8}$ or less in the presence of SLE immune serum ($\log_{10}$ N. I. 5.8 or greater).

A third isolation was undertaken November 24, 1965, using 2 litters (12 animals) of 1-day-old mice inoculated i.c. and i.p. with 20% suspension of the fox brain in BABS. One mouse was sick on each of post-inoculation days 7, 9, 13 and 14, while 8 mice remained well for 3 weeks and were discarded. On subpassage into additional suckling mice, all animals were sick or dead on the 4th or 5th day. The titer of the brain suspension from these mice was $10^{-6.7}$ in the presence of NRS, and $10^{-3.3}$ in the presence of SLE immune serum ($\log_{10}$ N.I. 3.4).

Table I shows the results of cross-neutralization tests with various group B arboviruses and the fox virus isolate. The unknown virus was neutralized by SLE immune serum and by the homologous immune serum, but was not significantly neutralized by Rio Bravo, Powassan, Modoc, or JE immune sera. Immune serum prepared with the unknown virus neutralized both test strains of SLE virus, but not Rio Bravo, Powassan, or Modoc viruses.

Table II shows results of the cross immunity tests with the unknown virus and strains of SLE and Rio Bravo viruses. Immunization with the unknown virus resulted in complete resistance to challenge with the

TABLE I. Results of Cross Neutralization Tests with the Fox Brain Isolate and Strains of Group B Arboviruses.

| | Virus Strains | | | | | |
|---|---|---|---|---|---|---|
| | Fox brain isolate | SLE, Ruis strain | SLE, MP 1578 strain | Rio Bravo | Powassan | Modoc |
| *Antiserum* | | | | | | |
| Fox brain isolate | 6.1* | 2.6 | 5.0 | 0.8 | 0.0 | —0.1 |
| SLE, Ruis | 3.4 | 4.0 | 3.2 | 0.4 | ND† | ND |
| Rio Bravo | 0.4 | 1.0 | ND | 5.6 | ND | ND |
| Powassan | 0.7 | ND | ND | ND | 7.0 | ND |
| Modoc | 1.5 | ND | ND | ND | ND | 5.1 |
| Japanese encephalitis | 1.2 | ND | ND | ND | ND | ND |

* $\log_{10}$ neutralization index.
† not done.

TABLE II. Results of Cross-Immunity Tests with the Fox Brain Isolate and with Strains of St. Louis Encephalitis and Rio Bravo Viruses.

| | Challenge virus | | | |
|---|---|---|---|---|
| | Fox brain isolate | SLE, Ruis strain | SLE, MP 1578 strain | Rio Bravo |
| *Immunizing virus* | | | | |
| Fox brain isolate | 6/6* | 6/6 | ND† | 5/6‡ |
| SLE, MP 1578 | 6/6 | ND | 6/6 | 4/6‡ |
| Rio Bravo | 0/6 | ND | 0/6 | 6/6 |

* Ratio of mice surviving intracerebral challenge with 1,000 i.c. $LD_{50}$ virus.
† Not done.
‡ Surviving mice showed transient signs of illness.

homologous virus and SLE virus, and partial resistance to Rio Bravo virus. Immunization with SLE virus resulted in complete resistance to challenge with homologous virus and the unknown virus, and partial resistance to Rio Bravo virus. Immunization with Rio Bravo virus resulted in complete resistance to challenge with homologous virus but not SLE virus or the unknown virus. In all instances the corresponding unimmunized control mice (not shown in Table II) succumbed to the challenge virus, indicating effectiveness of the challenge dose.

*Discussion.* The close serologic relationship between group B arboviruses, particularly between SLE and Rio Bravo viruses, has been noted by a number of workers(11-17). Burns and coworkers(17) found that SLE hyperimmune serum neutralized Rio Bravo virus but that Rio Bravo hyperimmune serum did not neutralize SLE virus. Our studies, and those of Johnson(13), have shown only slight or no cross-neutralization between these two viruses. These disparate findings may perhaps be due to differences between immunization schedules, viral strains, potency of immune sera, or the test procedures employed.

In the cross immunity tests, there was partial protection by both the fox brain isolate and SLE viruses to challenge with Rio Bravo virus. While most of the challenged mice survived, they exhibited ruffled fur, weakness and lethargy, or transient signs of central nervous system involvement. Mice immunized with Rio Bravo virus did not resist challenge with the fox brain isolate or SLE virus. The high brain-passage Ruis strain of SLE virus was found in preliminary tests to be a poor immunizing agent by peripheral inoculation and therefore the MP 1578 strain of SLE virus was used in the cross-protection tests.

The results of the neutralization tests and cross-immunity tests clearly indicated that the unknown isolate was a strain of SLE virus. Partial cross-protection by the isolate and by a standard SLE virus strain

to challenge with Rio Bravo virus was demonstrated.

This recovery of SLE virus from the brain of a fox in 1957 represents, to our knowledge, the first isolation (although not the first reported—see(11)) of this virus from a naturally-infected mammal other than man. Sulkin and coworkers have recently reported isolation of SLE virus from *Tadarida brasiliensis* bats collected in Texas during 1964 (11). Serologic evidence of natural SLE infection has been reported for many feral and domestic mammalian species(1-10). Few members of the *Canidae* have been tested serologically, but SLE antibody has been detected in dogs(1), foxes(8), and a coyote (J. L. Hardy, personal communication). In addition to domestic and laboratory animals, some feral mammalian species have been reported susceptible to experimental infection, including wild mice(14,15) and bats (16). These infections sometimes resulted in illness and death.

Amador County is in the foothills above the central valley of California, a known endemic area for SLE virus. Virus isolations have been made in this laboratory from *C. tarsalis* mosquitoes collected within 50 miles of Amador City. *Culex tarsalis,* the common vector for SLE virus, feeds predominantly on birds, but occasionally takes mammalian blood meals. Tempelis *et al*(18) identified 7 of 8,888 *C. tarsalis* blood meals in Kern County, California, as dog blood, but stated that they could have been from coyotes or foxes. Transmission of SLE virus to hamsters and to wild or laboratory mice by ingestion of infected animal tissues has been reported(15,19-21).

It thus seems possible that the fox forming the subject of this report could have been infected by mosquito bite or by eating an infected mammal or bird. Although this species does not have the population size and reproductive rate necessary to the continuous enzootic maintenance of an arbovirus, it appears to be susceptible to infection and disease with SLE virus.

*Summary.* The virus of St. Louis encephalitis was isolated from the brain of an abnormally-behaving California gray fox, *Urocyon cinereoargenteus,* shot July 27, 1957 in Amador County, California. This represents one of the first known isolations of this virus from a naturally-infected mammal other than man.

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## Effect of Hydrocortisone on Growth and Detachment of Human Heteroploid Cells in Maintenance Media.* (32116)

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The sustaining, or preserving, effect of hydrocortisone, in physiological concentrations, on heteroploid cell monolayers has been noted(1,2), and has been observed independently in this laboratory. The mechanism of this effect is not understood. In the present study, enumeration of cell nuclei and other quantitative studies were performed in order to evaluate the relative contributions of increased cell proliferation and decreased cell detachment and death to the sustaining effect of hydrocortisone.

*Materials and methods. Cells.* Two sublines of a cloned substrain (clone 22) of the Wong-Kilbourne influenza virus-susceptible variant of Chang's human conjunctival cells (3) were used. Mycoplasma were isolated by the method of Pollock *et al*(4) in an atmosphere of 95% nitrogen and 5% $CO_2$ from the subline used in Experiments I and II in 1962, but not from the one used in Experiments III and IV in 1964. *Glassware.* The cells were propagated in 200 ml milk-dilution bottles. Rubber-stoppered, 16 × 125 ml stationary test tubes were used to grow cells for experiments unless stated otherwise. All non-disposable glassware was pyrex and was washed in 7X.§ Disposable DeMuth glass test tubes‖ were used in experiment III. *Media.* Growth medium was mixture 199 (5) with 10% calf serum. Maintenance medium was mixture 199 with 2% horse serum. All sera had been heated for 30 minutes at 56°C. *Propagation and maintenance of cells.* The temperature for growth and maintenance was 37°C. For transfer, cells were dispersed with 0.25% trypsin¶ in phosphate-buffered saline (PBS) in the 1962 experiments or 0.04% versene in 1964. Bottles were seeded with $10^6$ cells in 10 ml medium. Medium was changed every 2-3 days. A continuous monolayer was reached in 7 days in 1962 and in 4 days in the 1964 experiments. Test tubes were seeded with $10^5$ cells. pH was readjusted to 7.2 with 0.5% sodium bicarbonate as needed. *Hydrocortisone.* Pfizer hydrocortisone diethylaminoacetate hydrochloride (aqueous solubility 28 mg/ml) was added to the maintenance medium. *Enumerations of cell nuclei* were performed with Rappaport's technique(6). *Cell viability* was assessed by the trypan blue exclusion test of McLimans *et al*(7). *Stained preparations.* Cells were grown and maintained on cover slips in Leighton tubes, fixed in Bouin's solution and stained with hematoxylin and eosin.

*Results.* The effects of hydrocortisone on

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¶ Trypsin 300, Difco, Detroit, Mich.