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Received December 23, 1957. P.S.E.B.M., 1958, v97.

## Chronic Latent Infections of Birds with Western Equine Encephalomyelitis Virus.* (23862)

W. C. REEVES, G. A. HUTSON, R. E. BELLAMY AND R. P. SCRIVANI
(Introduced by K. F. Meyer)
*School of Public Health, Univ. of California, Berkeley, and Communicable Disease Center, Bureau of State Services, P.H.S., U. S. Dept. of Health, Bakersfield, Calif.*

The endemicity of arthropod-borne viruses such as Western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) in temperate areas indicates the probable existence of a long-term arthropod or vertebrate reservoir of infection. Past studies have indicated that both avian hosts and mosquito vectors are essential in the summer transmission of many of these viruses. This paper reports the finding that WEE virus may persist as a chronic latent infection in experimentally infected birds and the epidemiological significance of this observation.

*Materials and methods.* Wild birds of several species were trapped in the fall and winter of 1953 to 1956, leg banded with identifying numbers, and held in a mosquito-proof outdoor aviary for the duration of the experiments. A pre-inoculation blood sample for antibody determination was obtained by jugular puncture, and within a few days each bird was inoculated subcutaneously with 0.1 or 0.2 ml of 100 to 15,800 mouse $LD_{50}$ concentrations of BFS 1034, BFS 1703 or F 199 strains of WEE virus. The strains were originally isolated from naturally infected mosquitoes or mites and had been through 3 or 4 serial mouse brain passages. One or more post-inoculation blood samples were taken at intervals of 1 to 5 months, and if possible a final blood sample was collected at autopsy. All blood samples were drawn by jugular puncture into a syringe containing heparin diluted in sterile phosphate buffer saline (pH 7.4). The concentration of heparin was 20 U.S.P. units per ml, and for each ml of blood 0.2 ml of heparin solution was used. Blood plasma was tested for neutralizing antibodies to WEE virus, and as a control for SLE neutralizing antibodies when sample size permitted. The neutralization tests were made in 21-day-old mice with a level of 50 $MLD_{50}$ of virus, and when sufficient plasma was available a second level of 500 $MLD_{50}$ of virus. Neutralization indices above 50 were considered positive, 10 to 49 equivocal and below 10 negative. Birds were subjected to autopsy when found dead in the aviary or when sacrificed by bleeding out. When birds remained in good health, they were held for a minimum of 6 months after infection and a portion was then sacrificed for sampling at intervals extending to 10 months post-inoculation. At autopsy separate sterile instruments were used for each bird but not for each organ sample. From each bird the following tissue samples were usually taken: brain, heart, kidney, spleen, lung, liver, lymph and whole heparinized blood. Occasionally fewer or additional sam-

* This investigation was supported in part by research grant from Nat. Inst. of Allergy and Infectious Diseases, N.I.H., U.S.P.H.S.

ples were taken. Each tissue sample was placed in a separate sterile tube which was sealed in a gas flame and stored in dry ice until tested for virus.

Virus tests were made by inoculating 1:2 to 1:5 dilutions of blood samples and approximately 10% emulsions of organs into 21-day-old mice, 10-day-old embryonated chicken eggs, or chick embryo tissue culture. Organ emulsions were prepared by grinding the organs in a mortar with a diluent of 10% rabbit serum in beef heart broth, pH 7.4, containing 625 units of penicillin and 500 $\mu$g of streptomycin/ml. Suspensions were centrifuged at room temperature for 15 to 20 minutes at 2,500 rpm and the supernatant fluid used for inoculation. Each mouse was inoculated intracerebrally with 0.03 ml and intraperitoneally with 0.09 ml of a sample. "Chick embryos" were inoculated with 0.1 ml by the amniotic route. In the last year of study, tissue culture tests similar to those described by Welsh *et al.*(1) replaced the embryonated egg test. Trypsinized cell suspensions of 8 to 11-day-old embryonated eggs were cultured in slanted stationary tubes. The test samples were inoculated in 0.1 ml amounts into the tubes immediately after the addition of the cell suspension. After incubation for 5 days at 37°C, cultures were sub-passed for further establishment of the isolation as well as for confirmation of the absence of a cytopathogenic virus. Any mouse exhibiting signs of encephalitis and sluggish or dead embryos were sacrificed and material was passed for establishment of isolations. The viruses isolated were identified by mouse or tissue culture neutralization tests with specific immune sera. When quantities of the original organ suspensions of positive samples were sufficient, repeat isolations were attempted.

*Results.* From November 1953 through August 1956, 284 birds survived to be autopsied and tested for virus one month or more after WEE inoculations. The 284 birds were distributed among 9 species as follows: 98 tricolored blackbirds (*Agelaius tricolor*), 18 red-winged blackbirds (*Agelaius phoeniceus*), 24 Brewer's blackbirds (*Euphagus cyanocephalus*), 8 yellow-headed blackbirds (*Xanthocephalus xanthocephalus*), 69 cowbirds (*Molothrus ater obscurus* and *M. ater ater*), 2 Chinese spotted doves (*Streptopelia c. chinensis*), 41 house finches (*Carpodacus mexicanus frontalis*) and 24 English sparrows (*Passer d. domesticus*).

Data from isolations from 1 or more organs of 8 birds at intervals of 1 to 10 months postinoculation are summarized in Table I. Little significance can be given to the predominance of findings in male birds, as the majority, 192 of 284 studied, were males. A wide range of organs was tested in the anticipation that 1 or 2 might be found to be the primary sites of latent infection. There is no evidence of such localization, as 6 different tissues were positive in the 8 birds. The finding of positive blood with other autopsy samples negative may be a result of rather complete exsanguination by the jugular vein bleeding technic or failure to sample the organ of primary localization. The limited quantity of test material precluded satisfactory repeat isolation tests on most of the positive samples; however, reisolations were successful in 3 instances. Review of laboratory records of isolations revealed no circumstances that would have favored accidental laboratory contamination of samples. There was no correlation between the dates of introduction of newly infected birds into the aviary and the dates of autopsy of positive birds. The minimum interval was 2 months and the maximum interval was 10 months between dates of autopsy and prior introduction of newly inoculated birds.

A total of 61 tricolored and red-winged blackbirds which were uninoculated, inoculated with normal mouse brain, or inoculated with SLE virus were introduced into the aviary in the course of these studies. The SLE inoculated birds were introduced 3 months following a series of WEE birds. The birds not inoculated with virus were introduced concurrently with a WEE inoculated series. Of these 61 "control" birds, 5 converted from a negative to positive WEE neutralization index during their 4 to 7 months' residence in the aviary. These were all captured wild birds of unknown history, but previous infection with WEE virus was contraindicated by one or more serum samples which

TABLE I. History of Western Equine Encephalomyelitis Isolations from Experimentally Inoculated Birds, 1953 to 1956.

| Species and date of inoculation | Sex | Days elapsed, inoc. to autopsy | WEE serum neutralization titers* | | | Virus isolation history† | | |
|---|---|---|---|---|---|---|---|---|
| | | | Pre-inoc. | Post-inoc. | Autopsy | Tissue | Isolated in | Repeat isolations |
| Brewer's blackbird, 11/10/53 | ♂ | 150 | 0 | 47 | Dead | Gall bladder | Chick embryo | 0 |
| Cowbird 11/10/53 | ♂ | 178 | 0 | 0 | 50 | Lung | *Idem* | 0 |
| *Idem* | ♂ | 198 | 0 | 70 | 32 | Blood, lung | " | 0 |
| Tricolored blackbird, 2/9/54 | ♂ | 133 | 0 | 0 | 24 | Liver | " | |
| *Idem*, 11/3/54 | ♂ | 55 | 0 | 552 | 352 | Brain | Mouse & chick embryo | + |
| " , 11/29/54 | ♂ | 306 | 0 | 260 | 139 | Spleen | *Idem* | |
| House finch, 11/14/55 | ♀ | 245 | | | 490 | Brain | Tissue culture | + |
| English sparrow, 11/14/55 | ♂ | 234 | | | 0 | Blood | *Idem* | + |

* Serum neutralization titers against SLE were all negative.
† Other tissues routinely taken as described in the methods section were negative.

were negative on the WEE neutralization test before the final positive sample. The only blood-feeding arthropods observed in the aviary were nasal mites and the bird mite *Ornithonyssus sylviarum*, neither of which are believed to be vectors of WEE virus.

*Discussion.* This study demonstrates that at least one of the arthropod-borne viruses may persist in avian hosts for as long as 10 months and that virus may recirculate in the blood stream subsequent to the period of active viremia. The obvious implication of this observation is the potentiality of avian hosts with chronic latent infections to serve as long-term reservoirs and sources of virus infection for arthropod vectors. Additional studies are being directed at the evaluation of this possibility. The pattern of virus isolations from naturally infected mosquito vectors under field conditions can be interpreted as supporting the hypothesis that there is an avian source of WEE virus in the winter months(2).

Remarkably little attention has been given to the persistence of any of the arthropod-borne viruses in animal hosts beyond the period of acute viremia. The general assumption that the presence of neutralizing antibodies leads to what might be termed autosterilization of the host and particularly of the blood stream must be re-examined in the light of these findings. The recent reports of Downs(3) and Downs *et al.*(4) of the concurrent circulation of yellow fever virus and neutralizing antibodies is of interest in this regard. The current observation by Chamberlain *et al.*(5) that SLE virus persisted in the gizzard of an experimental cowbird for 38 days further supports the possible importance of birds as reservoirs as well as short-term hosts of these viruses. The earlier report of Webster and Clow(6) of SLE virus persistence for 4 to 6 weeks in the brain and spleen of resistant mice and the study of Slavin(7) in which SLE virus persisted for 162 days in the brains of passively immunized mice may be pertinent to the relationship of virus infection in resistant avian hosts.

The possible development of WEE antibodies by 5 "control" birds is the only evidence we have of bird to bird transmission in the aviary. These apparent conversions may represent errors of the neutralization test as there was insufficient serum to permit confirmatory tests.

A more detailed report of WEE and SLE latent infections in experimentally and naturally infected birds and evaluation of their ability to serve as sources of vector infection will follow completion of current studies.

*Summary.* Western equine encephalomyelitis virus was isolated from tissues of 8 of 284 birds at intervals of 1 to 10 months after experimental infection. Isolations were from Brewer's blackbird, cowbird, tricolored blackbird, house finch and English sparrow. Positive tissues were blood, spleen, liver, lung, brain and gall bladder. The potential importance of birds as long-term reservoirs of virus and sources of vector infection should be determined.

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Received January 16, 1958. P.S.E.B.M., 1958, v97.

## Acquired Tolerance to Skin Homografts in Mice of Different Strains.* (23863)

CARLOS MARTINEZ, JUNE M. SMITH, J. BRADLEY AUST† AND ROBERT A. GOOD‡
*Departments of Physiology, Surgery and Pediatrics, University of Minnesota Medical School, Minneapolis*

Actively acquired tolerance for homologous skin transplantation in mice and chickens has been achieved by exposure of these animals during fetal life to living cells from adult animals of the homologous strain(1,2). These findings were confirmed in rats(3,4). However, since inbred strains of rats were not available, tolerance to homologous skin in this species was obtained when the skin transplanted came from the donor of the spleen used to induce tolerance.

Recently Billingham and Brent(5) reported that approximately 80% of A mice injected intravenously with spleen cells no later than 24 hours after birth from an adult CBA mouse became tolerant to skin homografts taken from mice of the same stock. In contradistinction only one-half of similarly treated CBA mice became tolerant to A skin when spleen cells from the latter were used to induce this phenomenon. Moreover, when the CBA → AU donor host combination was used, not a single AU animal tolerated the CBA skin. These results would indicate the presence of strain differences in susceptibility to the induction of acquired tolerance.

It is the purpose of this report: 1. To document our confirmation of the observation of the British workers that tolerance to subsequent homotransplantation may be produced in mice by intravenous injection of spleen cells in the neonatal period. 2. To present experimental observations which indicate the existence of strain differences in capacity of the donor cells to induce tolerance as well as capacity to develop tolerance as indicated by the British workers.

*Method.* Highly inbred mice of the A, Ce, Z(C3H), C57 Bl(Subline 1), CBA, BALB/C and ZCe $F_1$ and ZBC§ hybrids were used. Acquired tolerance was induced by the method recommended by Billingham and Brent(6,7) consisting of the intravenous injection of viable spleen cells into newborn animals no later than 24 hours after birth. Cells to be in-

* Aided by grants from the U.S.P.H.S. and the Minn. Division Amer. Cancer Soc.

† Am. Cancer Soc. Scholar.

‡ Am. Legion Memorial Heart Research Professor in Pediatrics.

§ ZBC hybrid mice are obtained in the following way:

A ♀ x Z(C3H) ♂
↓
AZ $F_1$ ♀ x Z(C3H) ♂ = ZBC