tracerebral neutralization tests with the sera from animals immune to SLE, Jap B and West Nile viruses(8-10). These results were readily confirmed here but it was surprising to find the high neutralization indexes obtained with the heterologous viruses especially when hyperimmune sera were used. Indexes of one thousand or more were quite frequent and several were rcorded of ten thousand or better. Evidently repeated inoculations of animals with living virus serve not only to raise the level of antibody against the homologous virus but also to bring the level of antibody against the heterologous viruses to a significantly high level. Thus as suggested by Lennette and Koprowski(11) it may be of importance in the future to use sera from convalescent animals rather than hyperimmune animals for identification purposes.

Summary. It is impossible at present to distinguish epidemic keratoconjunctivitis virus from St. Louis encephalitis virus by means of the intracerebral neutralization test in mice. The sera from convalescent epidemic keratoconjunctivitis animals and from convalescent or hyperimmune St. Louis encephalitis animals neutralized epidemic keratoconjunctivitis and St. Louis encephalitis viruses to an equal extent. No differences could be elicited by performing neutralization tests with the keratoconjunctivitis virus that had been through 3 successive rabbit passages. Neutralization tests in which serum dilutions were used against a constant amount of virus also failed to show any differences between the 2 viruses. Considerable cross-neutralization was obtained with the related Japanese B and West Nile viruses. Infectivity tests in animals revealed that rabbits and guinea pigs were susceptible to intracerebral inoculation with keratoconjunctivitis virus but not to St. Louis virus.

Received April 17, 1951. P.S.E.B.M., 1951, v77.

8. Smith, M. G. and Reames, H. R., PROC. SOC. EXP. BIOL. AND MED., 1940, v45, 838.

9. Smithburn, K. C. and Jacobs, H. R., *J. Immunol.*, 1942, v44, 9.

10. Casals, J., *J. Exp. Med.*, 1944, v79, 341.

11. Lennette, E. H. and Koprowski, H., *J. Immunol.*, 1946, v52, 235.

A Possible Relationship Between Viruses of St. Louis Encephalitis and Epidemic Keratoconjunctivitis. (18699)

F. S. CHEEVER.

From the Graduate School of Public Health, University of Pittsburgh.

The virus of epidemic keratoconjunctivitis first isolated by Sanders(1,2) has recently been shown by Ruchman to be closely related to the virus of St. Louis encephalitis(3). The present communication confirms this fact and, in addition, presents certain data bearing on the relationship of this agent or agents to the clinical disease of epidemic keratoconjunctivitis.

Materials and methods. Two strains of epidemic keratoconjunctivitis virus were examined. The first, EK-TC 17, was obtained from Dr. A. E. Braley in 1947. This was the strain originally isolated by Sanders(1,2) and carried through an undetermined number of mouse and egg passages. During the Second World War it was sent to the Upjohn Co. in Kalamazoo, Mich., where it was carried through another series of mouse and egg passages(4). At the close of hostilities it was returned to Dr. Braley, who confirmed its specific identification by serological and filtration studies(5). During this time the titer

1. Sanders, M., *Arch. Ophthalmol.*, 1942, v28, 581.

2. Sanders, M., and Alexander, R. C., *J. Exp. Med.*, 1943, v77, 71.

3. Ruchman, I., in press.

4. Calkins, H. E., and Bond, G. C., PROC. SOC. EXP. BIOL. AND MED., 1944, v56, 46.

5. Braley, A. E., personal communication.

of the virus varied from 10^{-5} to 10^{-7}. This strain was subsequently used in protection tests employing gamma globulin *in vitro*(6) and *in vivo*(7); the titer of the virus at this time varied from 10^{-5} to 10^{-6}. The second strain, EK 103-P61, was obtained from Dr. Isaac Ruchman, in July 1950, who, in turn, had received it from Dr. Braley a month previously. While details of the early history of this strain were not available, it had apparently undergone more than 100 mouse passages and was derived from the same pool of material made up by Dr. Murray Sanders in New York City in 1942 as was strain EK-TC 17(3). Attempts to obtain other strains of the virus of epidemic keratoconjunctivitis failed. We were particularly anxious to secure the strain isolated by Maumenee, Hayes, and Hartman(8) which was reported to be antigenically related to *Herpes febrilis* virus. Apparently this strain is no longer available(9). Two strains of St. Louis encephalitis virus, Webster(10) and Hubbard(11), were secured from Dr. W. McD. Hammon. Pools of each of these viral agents were made up in the form of 20% suspensions of infected mouse brain, using 1% bovine albumen solution in broth as the suspending fluid. These were stored in sealed 1 ml ampoules in the CO_2 cabinet until used. All virus preparations were cultured aerobically and anaerobically to insure bacteriological sterility. Rabbits and guinea pigs of both sexes were obtained from a local dealer. Three-four-week-old mice of the Carworth Farm CFW strain were employed in the neutralization and cross immunity tests as well as for the preparation and titration of virus pools. Fertile White Leghorn eggs were used for chick embryo passage experiments.

Titrations of individual virus suspensions were carried out by making serial 10-fold dilutions in 0.2% bovine albumen solution in broth. Five or 6 mice were inoculated with each dilution. The method of Reed and Muench(12) was used in the calculation of LD_{50} titers. Neutralization tests were carried out and the resultant neutralization indices calculated by the methods outlined by Hammon(13), employing undiluted serum and varying amounts of virus. In these tests the virus suspensions were prepared employing 0.4% bovine albumen as diluent, so that when equal amounts of virus suspension and undiluted serum were mixed the final concentration of bovine albumen was 0.2%. These mixtures were placed in a 37°C waterbath for 2 hours; 0.03 ml of each mixture was inoculated intracerebrally into each of 5 or 6 mice. Inactivated normal horse serum was used as a control.

Results. The EK-TC 17 strain has been carried through 4 passages and the EK 103-P61 strain through 2 passages. The LD_{50} titers of these passages have varied from $10^{-7.3}$ to $10^{-8.2}$.

Neutralization tests were carried out with both virus strains employing immune rabbit serum prepared against each of the following encephalitis viruses: St. Louis, Japanese B, and Western Equine. These sera were furnished by Dr. W. McD. Hammon. As shown in Tables I and II, the St. Louis immune serum showed significant neutralization indices against both strains. Slight but not significant protection against one strain was afforded by the Japanese B immune serum, while the Western Equine encephalomyelitis immune serum exerted no demonstrable protective effect. Since these results presented presumptive evidence that the two strains of epidemic keratoconjunctivitis virus were identical, further work was limited to one strain—EK-TC 17.

Host range. Three guinea pigs inoculated intracerebrally with EK-TC 17 developed fever of 3-4 days duration commencing on the

6. Heyl, J. T., Allen, H. F., and Cheever, F. S., *J. Immunol.*, 1948, v60, 37.

7. Cheever, F. S., and Daikos, G., *J. Immunol.*, 1950, v65, 135.

8. Maumenee, A. E., Hayes, G. S., and Hartman, T. L., *Am. J. Ophth.*, 1945, v28, 823.

9. Maumenee, A. E., personal communication.

10. España, C., and Hammon, W. McD., *J. Immunol.*, 1948, v59, 1.

11. American Type Culture Collection: *Virus and Rickettsial Registry*, 1950, 6.

12. Reed, J. L., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

13. Hammon, W. McD. in *Diagnostic Procedures for Virus and Rickettsial Diseases*, New York, American Public Health Association, 1948, 200.

TABLE I. Epidemic Keratoconjunctivitis Virus Neutralization Tests.

Serum	Strain EK-TC 17: Final virus dilutions 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	LD_{50}	NI
SLE* immune	5/5†	4/5	1/5	0/5	0/5	—	—	$10^{-4.5}$	500
Jap B‡ "	5/5	5/5	5/5	2/5	0/5	—	—	$10^{-5.8}$	25
WEE§ "	—	—	5/5	5/5	5/5	2/5	0/5	$10^{-7.8}$	<10
Normal horse	—	—	5/5	5/5	2/5	2/5	0/5	$10^{-7.2}$	—

* St. Louis encephalitis.
† Numerator: No. of deaths.
Denominator: " " mice inoculated.
‡ Japanese B encephalitis.
§ Western equine "

TABLE II.

Serum	Strain EK 103-P61: Final virus dilutions 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	LD_{50}	NI
SLE immune	5/5	4/5	0/5	0/5	0/5	0/5	0/5	$10^{-4.4}$	1000
Jap B "	5/5	5/5	5/5	4/5	1/5	0/5	0/5	$10^{-6.5}$	<10
WEE "	—	5/5	5/5	5/5	5/5	0/5	0/5	$10^{-7.5}$	<10
Normal horse	—	5/5	5/5	4/5	4/5	1/5	0/5	$10^{-7.4}$	—

fourth day after inoculation. One pig died on the 11th post-inoculation day with no definite neurological signs. The brain was bacteriologically sterile and attempts to isolate the virus by mouse inoculation were successful. Five guinea pigs inoculated with the 2 strains of St. Louis encephalitis virus showed a similar but less well marked febrile response and all recovered. Reinoculation experiments demonstrated cross immunity between the 2 viruses —*i.e.*, St. Louis encephalitis-recovered guinea pigs showed no febrile response after inoculation with epidemic keratoconjunctivitis virus 4 weeks later, and vice versa.

Of 2 rabbits inoculated intracerebrally with EK-TC 17 one remained apparently well throughout the period of observation. The second developed signs suggesting CNS involvement on the 11th post-inoculation day and was sacrificed when moribund on the 14th day. A 20% suspension of brain tissue was prepared and cultured under aerobic and anaerobic conditions; no growth occurred. A few of the mice injected intracerebrally with this material died 96 hours after inoculation, but attempts to pass the agent further failed.

Three groups of 3-4-week-old mice were inoculated intraperitoneally with epidemic keratoconjunctivitis virus (EK-TC 17 strain) and the 2 strains (Webster and Hubbard) of St. Louis encephalitis virus respectively. The intracerebral titers of the 3 virus pools were approximately equal. Each mouse received 0.1 ml of a 10% suspension of virus. The mortality ratios varied considerably; the actual ratios were SLE Hubbard: 4/15 (27%), SLE Webster: 12/15 (80%), EK-TC 17: 15/16 (94%).

No difficulty was experienced in propagating the virus of epidemic keratoconjunctivitis (strain EK-TC 17) in 8-day embryonated hens' eggs. The yolk sac route of inoculation was employed. As a general rule, the embryos began to die in significant numbers between 48 and 72 hours after inoculation. When sacrificed at this time the embryos usually appeared hemorrhagic. After removal of the eyes, beak and feet the remainder of the embryo was ground up in 1% bovine albumen solution so as to give a 10 or 20% suspension, and this was used as inoculum for the next passage. The LD_{50} titers of the first 10 passages varied from $10^{-4.5}$ to $10^{-7.7}$; the titers obtained with the 4th and subsequent passage materials were somewhat higher than has been the result when St. Louis encephalitis virus has been inoculated by the same route.

Mice immunized against St. Louis encephalitis virus (Hubbard strain) by a series of 3 graded intraperitoneal injections at weekly intervals showed marked immunity to intracerebral challenge with the homologous virus 10 days after the last immunizing injection as compared to control animals of the same age.

TABLE III. Cross Immunity Tests. Mice Immunized Against SLE Hubbard.

Mice	Challenge virus	Virus dilutions 10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9	LD_{50}	NI
SLE immune	SLE	0/7	1/8	1/7	0/7	1/7	0/8	1/8	0/5	—	$<10^{-1}$	>2000000
Controls	SLE	—	—	—	8/8	9/9	8/8	6/9	1/8	0/8	$10^{-7.3}$	—
SLE immune	EK	9/10	9/9	9/9	10/10	10/10	6/9	2/10	1/10	—	$10^{-6.5}$	20
Controls	EK	—	—	—	7/7	7/7	7/7	6/7	3/7	0/7	$10^{-7.8}$	—

TABLE IV. Cross Immunity Tests. Mice Immunized Against EK-TC 17.*

Mice	Challenge virus	Virus dilutions 10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9	LD_{50}	NI
EK immune	EK	10/10	10/11	11/11	9/9	8/9	6/10	2/7	0/10	—	$10^{-6.2}$	16
Controls	EK	—	—	—	9/9	8/8	8/8	5/7	1/8	0/7	$10^{-7.4}$	—
EK immune	SLE	1/10	2/8	2/9	3/9	3/9	3/9	0/8	0/7	—	$10^{-1.7}$	790000
Controls	SLE	—	—	—	8/8	8/8	8/8	7/8	2/8	0/7	$10^{-7.6}$	—

* Exp. 3.

When challenged with epidemic keratoconjunctivitis virus only a slight degree of increased immunity was demonstrated (Table III). Mice were immunized against epidemic keratoconjunctivitis virus (strain EK-TC 17) in similar fashion and subsequently challenged with the homologous and the heterologous viruses. In 3 different experiments only a minimal amount of acquired immunity against the homologous virus (EK) was demonstrated. In all 3 experiments the degree of acquired immunity against the heterologous virus (St. Louis Hubbard) was greater, although the actual neutralization indices showed considerable variation. The explanation of these findings is not clear (Table IV).

Attempts were made to secure specific immune sera against epidemic keratoconjunctivitis virus from outside sources. Through the kindness of Dr. Murray Sanders, serum drawn from a patient 8 years after an experimental infection with EK virus was made available to us. During the course of his experimental but clinically typical infection, this patient (M.S.) developed neutralizing antibodies against the specific virus(2). To his knowledge he had never had any contact with St. Louis encephalitis virus. Two convalescent serum specimens from the Schenectady outbreak of 1943(14) were furnished us through the kindness of Dr. R. F. Korns. One was a sample from a pool of convalescent serum obtained in 1943 from patients, all of whom had suffered clinically typical attacks of epidemic keratoconjunctivitis. An undetermined number of these individuals had been hyperimmunized with epidemic keratoconjunctivitis virus vaccine during convalescence (but prior to bleeding) as well. The second specimen was drawn in 1943 from an individual patient (Crouch) who during convalescence from a typical attack of the disease had received several injections of the same vaccine prior to being bled as a source of hyperimmune serum. Neutralization tests were set up with these specimens against the viruses of St. Louis encephalitis and epidemic keratoconjunctivitis.

As seen in Tables V and VI, patient MS possessed moderate levels of protective antibody against both viruses. Both patient Crouch and the pool of convalescent sera from the Schenectady outbreak showed significant levels of protective antibody against St. Louis encephalitis virus (Table V). Neither specimen showed any protective effect against the epidemic keratoconjunctivitis virus (Table VI).

Discussion. The findings reported here confirm Ruchman's observations(3) that a close serological relationship exists between the viruses of St. Louis encephalitis and epidemic keratoconjunctivitis. Further and more extensive tests must be carried out before it can be determined if the differences noted are more than one would expect to exist between recog-

14. Korns, R. F., Sanders, M., and Alexander, R. C., *Am. J. Public Health*, 1944, v34, 567.

TABLE V. St. Louis Encephalitis Virus. Neutralization tests.

Serum	SLE Hubbard strain: Final virus dilutions 10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9	LD_{50}	NI
SLE immune	—	5/5	1/5	0/4	0/5	0/5	0/5	—	$10^{-3.6}$	7940
Patient M.S.	—	5/5	5/5	2/5	1/5	1/5	0/5	0/5	$10^{-5.2}$	200
Normal horse	—	—	5/5	5/5	5/5	4/5	1/5	0/5	$10^{-7.5}$	—
SLE immune	6/6	5/5	2/6	0/6	0/6	0/6	—	—	$10^{-3.7}$	5010
Schen. Conv. pool	6/6	6/6	5/6	0/6	0/5	0/5	0/6	0/6	$10^{-4.4}$	1000
Patient Crouch	6/6	6/6	6/6	4/6	1/6	0/5	0/6	0/6	$10^{-5.4}$	100
Normal horse	—	6/6	6/6	6/6	5/6	5/6	1/6	0/6	$10^{-7.4}$	—

TABLE VI. Epidemic Keratoconjunctivitis Virus. Neutralization tests.

Serum	Strain EK-TC 17: Final virus dilutions 10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9	LD_{50}	NI
SLE immune	5/5	4/5	3/5	1/5	0/5	0/4	—	—	$10^{-4.2}$	3160
Jap B	—	5/5	5/5	5/5	2/5	0/5	0/5	—	$10^{-5.8}$	79
Patient M.S.	—	5/5	5/5	3/5	0/5	0/5	—	—	$10^{-5.2}$	316
Normal horse	—	—	5/5	5/5	5/5	4/5	2/5	0/5	$10^{-7.7}$	—
SLE immune	6/6	4/6	2/6	1/6	0/6	0/6	—	—	$10^{-3.5}$	1590
Schen. Conv. pool	6/6	5/5	6/6	6/6	3/5	2/6	0/5	0/6	$10^{-6.5}$	<10
Patient Crouch	6/6	6/6	6/6	6/6	5/6	4/6	0/6	0/6	$10^{-7.1}$	<10
Normal horse	—	6/6	6/6	6/6	6/6	2/6	0/6	0/6	$10^{-6.7}$	—

nized strains of St. Louis encephalitis virus (*e.g.* Webster and Hubbard). The serological results presented suggest that patient MS did suffer from an infection of an agent closely related to the virus of St. Louis encephalitis, if it can be assumed that no other unrecognized exposure to the latter agent had occurred. The results obtained from the Schenectady outbreak are more difficult to interpret since artificial immunization as well as natural disease presumably played a part in the stimulation of antibody production, but it seems probable that an agent closely related to St. Louis encephalitis virus was the causative agent of the outbreak. The failure to demonstrate neutralizing antibodies against the virus of epidemic keratoconjunctivitis is difficult to explain in the face of the strong presumptive evidence presented by Korns, Sanders, and Alexander(14) that unvaccinated individuals involved in the same outbreak developed specific antibodies against this agent in the course of typical attacks of the disease. It is obvious that outbreaks of epidemic keratoconjunctivitis as they occur today should be carefully studied and that in addition to carrying out the conventional antibody studies every effort should be made to isolate and identify the etiological agent. From the epidemiological point of view it is interesting that a good proportion of the reported outbreaks of epidemic keratoconjunctivitis have occurred under conditions which did not favor arthropod transmission of the agent.

As first pointed out by Ruchman(3) both strains of epidemic keratoconjunctivitis virus have shown a marked increase in virulence as measured by intracerebral titrations in mice. The explanation for this finding is not clear. This agent (strain EK-TC 17) was titrated simultaneously in Carworth Farm CFW mice and in the Tumblebrook strain of Swiss white mice. This latter strain had been employed in the studies involving the protective effect of gamma globulin(6,7). The LD_{50} titer of the virus pool was identical in both titrations. Similar results were obtained when the Hubbard strain of St. Louis encephalitis virus was titrated simultaneously in the two groups of animals. It seems unlikely, therefore, that the strain of mouse employed was responsible for this finding.

Summary. Evidence has been presented which confirms Ruchman's observations that the viruses of St. Louis encephalitis and epidemic keratoconjunctivitis are closely related. The importance of careful study of current outbreaks of epidemic keratoconjunctivitis as a means of assessing the significance of these findings is stressed.

Received April 16, 1951. P.S.E.B.M., 1951, v77.