Relationship Between Epidemic Keratoconjunctivitis and St. Louis Encephalitis Viruses. (18698)

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It was recently decided to investigate the relationships of epidemic keratoconjunctivitis virus to certain other viruses. With this plan in mind a strain of virus was secured from Dr. F. S. Cheever in Boston the usual intracerebral titers of which were reported to be approximately 10^{-6} in mice(1-3). Titers obtained in our laboratory were close to 10^{-9}. The virus was neutralized by known St. Louis encephalitis sera and to a lesser degree by Japanese B and West Nile encephalitis sera. These results were fully confirmed by Dr. Cheever(4). Subsequently a strain of keratoconjunctivitis virus obtained from Dr. Alson E. Braley in New York gave the same high titer in mice. This strain was sent to Dr. Cheever who found that it was also neutralized by St. Louis encephalitis serum. Since laboratory contamination was ruled out by these results, tests were set up which established a close immunological relationship between the two strains of epidemic keratoconjunctivitis virus and the virus of St. Louis encephalitis.

Materials and methods. Epidemic keratoconjunctivitis (EK) viruses. Two strains of the virus were employed in these tests. The one (labeled EK-TC17) obtained in 1950 from Dr. Cheever then at Boston was designated the *Cheever* strain. It had been frozen since 1947 and had originally come from Dr. Braley in New York. The other (labeled EK103-p.61) was obtained directly from Dr. Braley in 1950. It had been apparently through more than 100 mouse passages and had been stored at various times either in a mechanical deep freeze or in a solid carbon dioxide cabinet. It was designated the *Braley* strain. Both strains of virus were, in all probability, derived from the same pool of material frozen in 1942 by Dr. Murray Sanders in New York.

Other viruses. Two strains of St. Louis encephalitis virus (SLE) were used. The one, designated *Webster,* had been kept in the lyophilized state since 1943. It was obtained from Dr. A. B. Sabin in Cincinnati and was presumably the strain isolated in 1933 by the late Dr. L. T. Webster of the Rockefeller Institute, New York. The other designated *Hammon* was obtained in 1950 from Dr. W. McD. Hammon in Pittsburgh. Both viruses had been through numerous mouse passages and were supposedly derived from the *Webster Number 3* strain. The Nakayama strain of Japanese B encephalitis virus (Jap B) had been lyophilized in 1943 and was obtained from Dr. Sabin. The West Nile encephalitis virus lyophilized in 1946 was supplied in 1943 by Dr. Joel Warren, Washington, D. C.

Virus suspensions and titrations. Several infected mouse brains were ground in a mortar with alundum and enough undiluted normal rabbit serum to make a 20% suspension. After light centrifugation the supernatant fluid was either used the same day or stored until needed in solid carbon dioxide in approximately one cc amounts in rubber stoppered vials. Titrations of each suspension were performed by making serial tenfold dilutions in saline solution containing 10% normal rabbit serum (rabbit serum-saline) and inoculating them intracerebrally into groups of mice. The normal rabbit serum had been heated at 56°C for one half hour. LD_{50} titers were determined according to the method of Reed and Muench(5).

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

2. Braley, A. E.. *Trans. Am. Acad. Ophth. and Otolaryng.,* 1944, Jan.-Feb.

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

4. Cheever, F. S., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 125.

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

Table I. Neutralization Tests with Epidemic Keratoconjunctivitis Virus (Braley Strain). Protocol

Serum in test	Dilutions of virus in mixture*									LD_{50} titer	Neutralization index
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
Normal rabbit	—	—	—	—	4/4†	4/4	3/4	0/4	0/4	8.3	—
SLE hyperimm. rabbit	4/4	1/4	0/4	0/3	—	—	—	—	—	2.7	400000
Jap B " "	—	—	4/4	2/4	1/4	0/4	—	—	—	5.2	1200
Jap B human‡	—	—	—	4/4	4/4	3/4	1/4	—	—	7.5	6

* Serum-virus mixtures incubated 2 hr in water bath at 37°C and then .03 cc inoculated intracer. into groups of mice.

† Numerator shows No. of mice died; denominator, No. inoculated.

‡ Presumable inapparent laboratory infection.

Neutralization tests. In the neutralization test usually employed, decimal dilutions of virus were added to equal amounts (0.15 cc) of the undiluted serum to be tested. The mixtures were incubated in a water bath at 37°C for 2 hours and then inoculated intracerebrally (0.03 cc) into groups of mice. Deaths were recorded daily and the resultant neutralization indexes were calculated according to the method outlined in Diagnostic Procedures for Virus and Rickettsial Diseases (6). Heated normal rabbit serum was used as a control. A neutralization test was also used in which dilutions of serum were mixed with a constant quantity of virus. Serial four-fold dilutions of the test serum were made in normal rabbit serum and distributed to tubes. Equal amounts (0.15 cc) of the virus dilution selected were added to each tube and after incubation at 37°C for 2 hours the mixtures were inoculated into groups of mice. Normal rabbit serum was used as a control. After the two-hour incubation period the control mixture was serially diluted in rabbit serum-saline and inoculated intracerebrally into mice in order to determine the number of LD_{50} doses present in the mixture.

Sera. Most of the sera were kept in 'lusteroid' tubes or rubber stoppered vials at –30°C in a mechanical deep freeze. Some sera which had been prepared in the past were lyophilized or were stored in the liquid state in the refrigerator.

Animals. The mice were albinos of either sex obtained from a local dealer but bred in our own laboratory for several years. They were between 18 and 24 days old when used and weighed approximately 8 to 11 g. All other animals were obtained from local dealers. The guinea pigs were of both sexes and weighed between 300 and 700 g. Rabbits also were of either sex and weighed between 2 and 3 kg. Albino rats were young male adults weighing approximately 150 g.

Results of neutralization tests. The various neutralization tests were carried out in which undiluted serum and serial dilutions of virus were mixed. The protocol of one test is given in Table I. The neutralization indexes of this and other experiments are summarized in Table II. It is apparent from the data that when the neutralization test is used, serologic distinction of the EK viruses from the SLE viruses is difficult, if not impossible. Most of the sera from EK convalescent animals neutralized the EK and SLE viruses to an equal degree. However there were two exceptions. In one case the pool of serum from the guinea pigs neutralized the homologous virus but not the heterologous virus and in another case the reverse occurred. The serum of Rabbit 1, convalescent from the first intracerebral inoculation neutralized the heterologous SLE virus but not the homologous EK virus. No tests were performed with EK sera against the Jap B and West Nile viruses. The SLE sera obtained from convalescent or hyperimmune animals also demonstrated equal neutralizing ability against both EK and SLE viruses. In addition it was found that the SLE hyperimmune sera neutralized to a lesser but significant degree the related Jap B and West Nile viruses. SLE convalescent

6. *Diagnostic Procedures for Virus and Rickettsial Diseases*, New York, Am. Public Health Assn. 1948, p200.

TABLE II. Results of Cross-Neutralization Tests.
Summary

Sera in test	Virus used in neutralization test: EK Cheever	EK Braley	SLE Webster #3	SLE Hammon	Jap B	West Nile
EK (Braley)						
Rabbit 1 i.cer. conval. (1st)	—	4	320	—	—	—
" 1 " " (2nd)	—	600	1000	—	—	—
" 9 i.perit. "	—	6000	10000	—	—	—
" 10 " "	—	<100	40	—	—	—
Rat pool i.cer. "	—	200	2000	—	—	—
G. pig pool i.cer. "	—	100	3	—	—	—
SLE (Webster #3)						
Rabbit pool hyperimmune	100000	400000	>100000	160000	40000	10000
" " " *	20000	—	12000	10000	320	1000
" " i.cer. conval.	—	6000	4000	—	—	—
" " " " †	—	40000	—	—	—	—
G. pig pool " "	—	3000	2000	—	—	—
Jap B						
Rabbit pool hyperimmune	2500	1200	150	1600	100000	1000
Monkey i.cer. conval.‡	1600	—	<15	500	63000§	—
Human‖	2	6	2	3	12000	60
West Nile						
Rabbit pool hyperimmune*	3000	400	3000	1600	<20	15000
Herpes simplex—						
rabbit—hyperimmune	—	<6	—	—	—	—
Mengo encephalomyelitis—rabbit*	1	<6	—	—	—	—
Western equine encephalitis—rabbit	1	<10	—	—	—	—
Eastern " " "	1	<10	—	—	—	—

* Kindly provided by Dr. Joel Warren, Washington, D.C.
† Hammon strain of St. Louis encephalitis virus.
‡ Kindly provided by Dr. Albert B. Sabin, Cincinnati.
§ Index furnished by Dr. Albert B. Sabin.
‖ Presumable inapparent laboratory infection.

sera were not tested against these last two viruses.

Jap B sera also yielded interesting results. The hyperimmune rabbit serum showed a high index against its own Jap B virus but with it there occurred considerable cross-neutralization against the EK, SLE and West Nile viruses. The results with a Jap B monkey convalescent serum were practically the same. In each case however the homologous index was considerably higher than the heterologous indexes. The human Jap B serum, which neutralized Jap B virus well, showed no significant neutralization of the EK and SLE viruses but did show some neutralization against the West Nile virus. The West Nile serum neutralized the West Nile, EK and SLE viruses but again the index was highest against the homologous West Nile virus. There was little neutralization of the Jap B virus. Sera prepared against the viruses of Herpes simplex, Mengo encephalomyelitis and equine encephalomyelitis (Eastern and Western strains) failed to neutralize both strains of the EK virus.

Neutralization of rabbit passage EK virus. Early in the course of the investigation it was noted that intracerebral inoculation of EK virus into rabbits caused a fatal encephalitis. After 3 intracerebral passages in rabbits followed by three intracerebral passages through mice the virus was used in a neutralization test. After preliminary titration had revealed the same high titers in mice, the dilutions for the test were selected as before. The results are shown in Table III. The SLE hyperimmune serum neutralized the EK virus with an index of 200,000 while the Jap B and West Nile hyperimmune sera yielded indexes of 600. There was no EK hyperimmune serum available but a rabbit convalescent serum gave an index of 3000. These indexes and those obtained with the sera from the same animals in the test against the

TABLE III.
Neutralization of Epidemic Keratoconjunctivitis (Braley Strain) Rabbit Passage Virus.

Rabbit serum in test	Dilutions of virus in mixture								LD_{50} titer	Neutralization index
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
Normal	—	—	—	—	—	4/4	2/4	0/4	8.0	—
SLE hyperimmune	4/4	1/4	0/4	0/4	—	—	—	—	2.7	200000
EK (Braley)—i.cer. conval.	—	4/4	4/4	1/4	0/4	—	—	—	4.7	3000
Jap B hyperimmune	—	—	3/4	3/4	0/4	0/4	—	—	5.2	600
West Nile "	—	4/4	3/4	3/4	0/4	—	—	—	5.2	600

TABLE IV. Cross-Neutralization Tests with Epidemic Keratoconjunctivitis and St. Louis Encephalitis Viruses Using Dilutions of Serum and Constant Quantity of Virus.

Virus in mixture	Serum used	Dilution of serum used						50% protective end-point of diluted serum
		Undil.	1:4	1:16	1:64	1:256	1:1024	
EK	EK rabbit conval.	0/5	4/5	4/5	5/5	5/5	5/5	1:3
160 LD_{50} doses	SLE " "	0/5	0/5	1/5	1/5	5/5	4/5	1:105
	Normal rabbit control	5/5	—	—	—	—	—	—
SLE	EK rabbit conval.	0/5	5/5	5/5	5/5	4/5	5/5	1:2
200 LD_{50} doses	SLE " "	0/5	1/5	5/5	5/5	5/5	5/5	1:7
	Normal rabbit control	5/5	—	—	—	—	—	—

The EK rabbit serum showed neutralization index of 600 against EK virus and 1000 against SLE virus; the SLE rabbit serum showed index of 6000 against EK virus and 4000 against SLE virus.

mouse passage virus were practically identical. The inferences that can be drawn from this experiment are (1) the presence of another virus was not revealed and (2) rabbit passage did not disclose any change in the virus.

Neutralization tests employing dilutions of serum and a constant amount of virus. After it became apparent that the conventional neutralization tests were inadequate to differentiate EK from SLE virus, it seemed desirable to explore the test in which the virus was kept constant but the serum was diluted. The sera from convalescent rabbits with neutralization indexes that were approximately equal against both EK and SLE viruses were used. The serum dilutions were tested against 160 LD_{50} doses of EK virus and 200 LD_{50} doses of SLE virus and the results are given in Table IV. The EK serum gave a 50% protective end-point of 1:3 against the homologous EK virus and a 1:2 end-point against the heterologous SLE virus whereas the 50% end-point of the SLE serum was 1:105 against the heterologous EK virus and 1:7 against the homologous SLE virus. In both tests the SLE serum was more potent regardless of the virus used thus indicating that this method of doing the neutralization test did not serve to differentiate the two viruses. Further tests are in progress.

Infectivity tests in animals. The tests revealed the only marked differences between the EK and laboratory adapted strains of SLE virus. Most of the experiments were done with the *Braley* strain of EK virus but essentially the same results were obtained on a more limited scale with the *Cheever* strain. Sanders(1) had originally reported that EK virus caused a fatal encephalitis in intracerebrally inoculated mice and rabbits but that rats and guinea pigs were refractory. In 3 of these instances the findings were easily confirmed and in addition the discovery was made that guinea pigs were now also susceptible. The results of intracerebral inoculation follow. Rabbits inoculated with EK virus (0.1 cc) developed fever, and central nervous system (CNS) signs. Death ensued between the seventh and fourteenth days. Rabbits inoculated with two laboratory adapted strains of SLE virus remained well. Mice, following inoculation with EK virus (0.03 cc) began as early as 24 hours to show CNS signs which included rotating along the horizontal axis.

TABLE V. Comparison Between Epidemic Keratoconjunctivitis and St. Louis Encephalitis Viruses in Intracerebrally Inoculated Animals.

Animal	Epidemic Keratoconjunctivitis	St. Louis Encephalitis
Mice		
Titer	Ca 10^{-8}	Ca 10^{-8}
Incubation (10% susp.)	72 hr	72 hr
CNS signs first appear	24-48 hr	72 hr
Type of CNS signs	Usual and rotating	Usual
Rats		
Young adult	Refractory	Refractory
G. pigs		
Pyrexia	Present	Present
CNS signs	"	None
Mortality	60-70%	"
Rabbits	Fever, CNS, death	No effect

At 48 hours practically 100% of the mice were showing marked symptoms and at 72 hours the vast majority were moribund. Following inoculation with SLE the mice remained well for the first 48 hours but at 72 hours they were all showing marked CNS signs. Guinea pigs injected with EK (0.1 cc) developed fever and weight loss. About 60 to 70% of the animals developed CNS signs and died between the seventh and twelfth days. Guinea pigs inoculated with SLE virus developed fever and some weight loss but all survived the infection. Albino rats were refractory to both EK and SLE viruses. Thus the apparent pathogenicity of the EK virus for rabbits and to a lesser degree for guinea pigs is at present the only method whereby EK virus can be distinguished from SLE. A comparison between results obtained with the two viruses is given in Table V.

Discussion. As a result of the studies reported here it soon became apparent that it was impossible to differentiate the EK and SLE viruses by means of the neutralization test. Nevertheless by other means certain differences were found. The EK virus caused a fatal encephalitis in rabbits and in a majority of the guinea pigs whereas the SLE viruses were ineffective in rabbits and only caused a febrile response in guinea pigs. In mice, intracerebral inoculation of EK virus induced marked CNS signs, which included rotating along the horizontal axis, as early as 24 hours. These signs continued until death occurred at about 72 hours. Intracerebral inoculation of SLE produced signs at about 72 hours but there was no 'rotating syndrome'. The marked similarities included the crossing over in the neutralization tests and practically identical LD_{50} intracerebral titers in mice. Preliminary tests already indicate that cross-immunity tests in animals do not serve to distinguish the two viruses too well although some differences have been noted. Much of this work has been confirmed by Cheever(4). Further work is in progress and it will be reported later.

What remains unanswered at the present time is the question of whether this is the EK virus or a strain of SLE which can cause disease in rabbits and guinea pigs in addition to the susceptible mouse. Unfortunately no specific immune sera were available to help. If the virus is EK then it is immunologically related to SLE virus and it has now acquired the ability to cause disease in guinea pigs and results in much higher titers in mice than was originally reported. If it is SLE virus it is then unique in two respects. First, SLE does not cause encephalitis in rabbits and second, except for the report by Sulkin, *et al.* (7) in which they found it impossible to repeat their own results, presumably because of different strains of guinea pigs, it is generally agreed that SLE does not cause encephalitis in guinea pigs. Further work along this line is indicated.

Previous reports have tended to show that there is significant crossing over in the in-

7. Sulkin, S. E., Harford, C. G., and Bronfenbrenner, J., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 329.

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.

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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)

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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.