RELATIONSHIP OF THE VIRUS OF LOUPING ILL IN SHEEP AND THE VIRUS OF RUSSIAN SPRING-SUMMER ENCEPHALITIS IN MAN*

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During the past 5 years Russian investigators have described a new clinical type of encephalitis occurring during the months of May and June in the thickly forested parts of Soviet Russia (1). Furthermore, they report having obtained from the brain tissue of fatal cases a filterable virus similar to but not identical with the Japanese B encephalitis virus. The Russian virus was recovered from pasture ticks (*Ixodes persulcatus*) in certain isolated endemic regions and from wild rodents, and in turn was passed by the tick under experimental conditions from infected to healthy animals, thus bringing about the disease.

In June, 1941, the Russian virus was successfully transported in ticks from the laboratory of Dr. Chumakov in Moscow to that of Dr. Parker in Hamilton, Montana (2). It was sent to us for study about 16 months later, through the courtesy of the United States Public Health Service and the agency of the Commission on Neurotropic Virus Diseases of the United States Army. We have now compared this virus with several others of the encephalitis group and have found it unrelated to all except that of louping ill which it resembles closely.

Louping ill, a disease of sheep in Northern England and Scotland, has been known for more than 100 years. It is most common from mid-March to mid-May. From the brains of fatal cases virus was secured (1931) which proved capable of infecting sheep. Moreover, the virus was obtained from ticks (*Ixodes ricinus*) which had been fed on sheep, and was transferred by the ticks to healthy sheep which contracted the disease.

The results of comparative studies on these two viruses have already (3) been summarized briefly; they are now presented in detail.

Materials and Methods

Viruses: Russian Spring-Summer Encephalitis.—Three strains of Russian spring-summer encephalitis virus have been studied in this laboratory. The first one, desig-

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nated by us as Russian No. 1, was obtained from the National Institute of Health through the cooperation of Dr. Parker, as indicated above. It was made available to us in the form of a mouse brain specimen preserved in 50 per cent glycerine; from this material, successive mouse-to-mouse passages by the intracerebral route were carried out. Most of the present work has been done with this strain. The second specimen, presumably of the same strain, of Russian spring-summer encephalitis virus, also sent to us by the National Institute of Health, at a later date, and designated in our laboratory as Russian No. 2, has yielded results identical with those of the Russian No. 1 strain. Finally, the third specimen, B-4, was brought by hand to The Rockefeller Institute directly from Russia. This material reached our laboratory in the form of a lyophilized preparation which, on intracerebral inoculation in mice, proved to have retained full virulence. Again, the results obtained with this virus coincide with those elicited by the Russian No. 1 strain.

Louping Ill.—Our strain of louping ill virus has been maintained in this laboratory for a period of 10 years. Originally it was obtained from Dr. Rivers, shortly after he had received it from Scotland.

These two viruses have been maintained in our laboratory in the following manner: W-Swiss mice, 20 to 25 days of age, were infected intracerebrally with a 10^{-2} or 10^{-3} suspension of virus; when prostrate, their brains were removed, made into a 10 per cent suspension in buffered distilled water, frozen quickly, and stored in dry ice at $-76°C$. The number of mouse passages was thus reduced to a minimum since when the virus was needed we used the original suspension. The Russian virus employed in these experiments has had only two or three mouse passages in this laboratory. The louping ill virus had had many more passages prior to 1941, during which period it was maintained by alternating mouse inoculation with storage in 50 per cent glycerine. Since then, however, it has been preserved in dry ice and has had no more than two or three passages.

Experimental Animals.—A few rabbits, guinea pigs, and hamsters were used for the purpose of furnishing immune sera, as well as determining their susceptibility to the two viruses. With this exception, all animals employed in these experiments were W-Swiss mice (4) which were either raised in our own laboratory or purchased from a reliable outside dealer. For virus passages, either for infection or for the preparation of complement-fixing antigens, very young mice were used, that is, between 20 and 25 days of age. For vaccination to obtain immune sera, somewhat older mice were employed, those from 50 to 60 days old at the beginning of the test. In all cases the age and weight of the mice were known and emphasis was placed on their uniformity.

Serological Tests: Immune Sera.—For both complement fixation and neutralization tests immune sera were obtained by inoculation of mice with the corresponding virus. Several different batches of mice were immunized and tested on separate occasions. In general the immunization was carried out by injecting the mice with two doses of avirulent, formolized, 10 per cent infected mouse brain emulsion, then following these with from three to six injections of virulent mouse brain material in dilutions varying from 10^{-4} to 10^{-2}, given at intervals of about 6 days. The emulsions were made up in distilled water to which citric acid-sodium diphosphate buffer at pH 7.2 had been added. A detailed description of the course of immunization is given later under Experimental. For collection of serum, mice were anesthetized, and approximately

0.6 cc. of blood was withdrawn from the heart of each animal. The bloods were then pooled, centrifuged, and the serum was stored in lusteroid tubes at −76°C. after it had been frozen quickly in a dry ice-alcohol mixture.

Guinea pigs were immunized against Russian and louping ill viruses by injection of mouse infected brain; hamsters, which proved to be susceptible to Russian virus, by injection of hamster infected brain. The process of immunization of these animals is given in detail under Experimental.

Complement Fixation Tests.—The technique used for the complement fixation test has been described in detail elsewhere (5). Mouse brain antigens were prepared from each virus by freezing and thawing a 10 per cent brain emulsion which was then centrifuged in an angle head centrifuge at 5,000 R.P.M. for 1 hour. The supernatant, to which merthiolate in a concentration of 1:10,000 was added, constituted the antigen. Amounts of 0.25 cc. of antigen were used in the test. The complement was composed of fresh guinea pig serum, of which two units in a volume of 0.5 cc. were employed for the test. The mouse sera were inactivated at 60°C. for 20 minutes and tested in serial twofold dilutions, commencing with either undiluted serum or serum in dilution of 1:2, the volume of serum dilutions being 0.25 cc. The first phase of the reaction, namely, the mixture of antigen, complement, and serum, was incubated in the ice box at 4°C. for 18 hours and then left at room temperature for an additional half hour. Following this the hemolytic system was added, which consisted of rabbit anti-sheep hemolysin, 3 M.H.D. in 0.25 cc. volume plus 3 per cent packed sheep cells also in a volume of 0.25 cc. Then the tubes were incubated in a water bath at 37°C. for ½ hour and the reaction was read. The highest dilution of a serum giving a 2 plus or better fixation indicated its titer. The necessary controls were included in each test.

Neutralization Tests.—Mouse, hamster, and guinea pig hyperimmune sera and human convalescent sera have been tested for neutralizing antibodies. As source of virus for the tests, fresh virus was employed each time, consisting of the brains of three or four young mice infected intracerebrally with either Russian No. 1 or louping ill virus. When these animals had become prostrate owing to the infection, their brains were removed, ground in a mortar, and emulsified in the proper amount of diluent to give a 10 per cent brain tissue emulsion. The diluent was prepared with physiological saline and normal rabbit serum in proportions of 9 to 1 respectively. Before use the diluent was filtered through a Seitz pad. The original 10 per cent infected brain emulsion was centrifuged in a horizontal centrifuge at 2,000 R.P.M. for 10 minutes and the supernatant fluid diluted in serial tenfold dilutions, starting with 1:50; next, 0.2 cc. amounts of the serum to be tested for neutralizing antibodies were placed in small, sterile test tubes; to each tube, 0.2 cc. of the proper virus dilution was added, giving mixtures in equal volume of constant amount of serum and decreasing concentrations of virus—from 10^{-2} to 10^{-10}. These serum-virus mixtures were incubated in a water bath at 37°C. for 2 hours and then immediately injected intracerebrally into mice from 25 to 30 days of age, four mice being employed per dilution of virus. The animals were kept under observation for a period of 21 days and then discarded. Surviving mice were regarded as protected. The protection given by the sera was estimated according to the method of Reed and Muench (6).

Cross-Resistance Tests.—Mice were vaccinated to determine the degree of cross-

resistance existing between the Russian No. 1 and louping ill viruses. The challenge test was a subcutaneous or intraperitoneal inoculation of virus. The vaccine employed was a formolized mouse brain virus suspension prepared as follows: Batches of about thirty young mice were infected intracerebrally with the corresponding virus. When prostrate, their brains were removed, weighed, and then emulsified in physiological saline to a concentration of 10 per cent brain tissue. A mechanical blendor was used for this purpose. This 10 per cent suspension was centrifuged in a horizontal centrifuge at 2,500 R.P.M. for 10 minutes; the supernatant was pipetted off, put in a flask, sufficient formaldehyde added to give a concentration of 0.5 per cent formaldehyde, and the flask was closed with a glass stopper and kept in the refrigerator. These emulsions were tested for virulence at intervals by mouse inoculation and when found avirulent they were used for vaccination. The particular batches of vaccine employed in the tests to be described had been kept in the refrigerator for about 60 days and were avirulent.

Human Sera.—Human sera from two individuals were tested for both complement-fixing and neutralizing antibodies. For the complement fixation tests the sera were inactivated at 60°C. for 20 minutes; for neutralization tests they were used unheated.

EXPERIMENTAL

Pathogenicity of the Russian Spring-Summer Encephalitis Virus and Louping Ill Virus.—Rabbits, guinea pigs, and mice were used in attempts to bring to light any similarities in the pathogenicity of the two viruses. Intracerebral inoculations of either Russian No. 1 or louping ill virus in amounts of 0.3 cc. and 0.2 cc. respectively in 10^{-1} and 10^{-2} dilutions of infected mouse brain did not kill rabbits or guinea pigs, nor did animals so inoculated show any signs of illness.

The infectivity of both the Russian No. 1 and louping ill virus was studied in detail in mice, especially with reference to the titer of virulence and the route of inoculation. Also, in the case of the Russian virus, mice of different ages were tested on account of the striking susceptibility to peripheral inoculation exhibited by this species.

Experiment 1.—A series of tenfold dilutions of Russian No. 1 virus obtained from two mice prostrate following intracerebral infection was prepared, using as diluent 10 per cent rabbit serum in saline. A similar series of dilutions was prepared in an identical manner with the louping ill virus.

Russian No. 1 Virus.—The virulence of this virus for mice was tested by intracerebral, intranasal, subcutaneous, and intraperitoneal routes in animals 30, 60, and 100 days of age and weighing respectively 13, 22, and 25 gm. The amounts given were 0.03 cc., 0.03 cc., 0.5 cc., and 0.5 cc. respectively. The result of the test is shown in Table I.

The high virulence of the Russian virus for mice following peripheral inoculation by both subcutaneous and intraperitoneal routes was very striking;

TABLE I

Virulence of Russian Spring-Summer Encephalitis Virus for W-Swiss Mice, Given by Different Routes

Route of inoculation and amount injected	Age of mice	Fate of mice following injection of virus in dilution:								
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
	days									
Intracerebral	30					4/4*	4/4	4/4	3/4	0/4
0.03 cc.	60					4/4	4/4	4/4	1/4	0/4
	100					3/3	4/4	4/4	1/4	
Intranasal	30	4/4	3/4	3/4	3/4	3/4				
0.03 cc.	60	4/4	4/4	4/4	3/4					
	100	3/3	3/3	4/4	4/4					
Subcutaneous	30	4/4	4/4	4/4	4/4	4/4				
0.5 cc.	60	4/4	4/4	4/4	4/4					
	100	4/4	4/4	4/4	4/4					
Intraperitoneal	30	4/4	4/4	4/4	4/4	4/4				
0.5 cc.	60	4/4	4/4	4/4	4/4					
	100	4/4	4/4	4/4	3/4					

* 4/4 = four mice out of four died.

TABLE II

Virulence of Louping Ill Virus for W-Swiss Mice, Given by Different Routes

Route of inoculation and amount injected	Age of mice	Fate of mice following injection of virus in dilution:								
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
	days									
Intracerebral	30			4/4	4/4	3/4	1/4	0/4	0/4	0/4
0.03 cc.										
Subcutaneous		1/4	1/4	1/4	1/4					
0.5 cc.										
Intraperitoneal		4/4	4/4	3/4	3/4					
0.5 cc.										

Footnote as in Table I.

titers of at least 10^{-6} were obtained even in very old mice. In numerous tests we have encountered still higher titers—from 10^{-7} to 10^{-9}—on subcutaneous and intraperitoneal infection of mice between 60 and 90 days old.

Louping Ill Virus.—The virulence of this virus was tested only in mice 30 days of age and weighing approximately 13 gm.; intracerebral, subcutaneous, and intraperitoneal routes were employed with results shown in Table II.

Considerable difference in susceptibility of mice of the same age to the two viruses was observed. 30-day-old mice were highly susceptible to intraperitoneal inoculation of both viruses, but on subcutaneous inoculation they were far more susceptible to Russian than to louping ill virus. In older mice (up to 100 days), the difference in susceptibility to the two viruses became greater; it decreased for the louping ill, whereas it remained unchanged for the Russian virus.

Serological Tests: Complement Fixation Tests.—The first indication of the existence of a close relationship between the Russian spring-summer encephalitis and louping ill viruses resulted from our use of the complement fixation test. Extensive investigations have been carried out since in attempts to establish not only the degree of crossing at a given time but also to follow the development of both complement-fixing and neutralizing antibodies in batches of mice, some immunized with Russian No. 1 virus and others with louping ill virus.

Experiment 2.—An experiment was undertaken to determine serological relationships among six central nervous system viruses, namely, St. Louis encephalitis, Japanese B encephalitis, West Nile encephalitis, louping ill, Russian spring-summer encephalitis, and Western equine encephalomyelitis (W.E.E.). The virus of W.E.E. was included in this experiment to provide a control since prior experience has shown this virus to be entirely unrelated to the other five. Only results with the Russian No. 1 and louping ill viruses are given here; those obtained with the other viruses are included only in so far as they control the experiment.

Two hundred and eighty mice, from 60 to 70 days of age, were divided in seven groups of forty each. One group was immunized against St. Louis encephalitis virus, a second against Japanese B encephalitis virus, a third against West Nile encephalitis virus, a fourth against louping ill virus, a fifth against Russian No. 1 virus, a sixth against W.E.E., and the last group was left untreated as controls. Each group of mice was vaccinated according to the following schedule:—

1st day—formolized 10 per cent mouse brain vaccine, 0.25 cc. intraperitoneally; *3rd day*—formolized 10 per cent mouse brain vaccine, 0.25 cc. intraperitoneally; *10th day*—virulent 10^{-4} emulsion of infected mouse brain in distilled water, 0.5 cc. intraperitoneally; *15th day*—virulent 10^{-2} emulsion of infected mouse brain in distilled water, 0.5 cc. intraperitoneally; *20th day*—virulent 10^{-2} emulsion of infected mouse brain in distilled water, 0.5 cc. intraperitoneally.

After this course of immunization the mice were bled on the following days, counting from the time of the last injection: 10th, 25th, 50th, and 140th. The sera were pooled in each group and stored in dry ice at −76°C.

The complement fixation tests were performed in such a manner that all the sera of a given date were tested simultaneously against all antigens. Thus far four such tests have been carried out, namely, on the 10th, 25th, 50th, and 140th days. The results obtained with the Russian and louping ill viruses are given in Table III; this table includes also results with another virus, the Japanese B encephalitis, which was used as a control.

Table III shows that crossing between the Russian No. 1 and louping ill viruses was complete on all four occasions; in each case the crossing was to half the titer obtained with the homologous antigen. Neither the Russian nor the louping ill immune sera reacted with the Japanese B antigen, on the other hand, not even in a dilution of 1:2, nor did the Japanese B immune serum react with the Russian or louping ill antigens. The findings with the Japanese B virus were the same as those presented in the case of the three other central

TABLE III

Complement Fixation Tests with Mouse Hyperimmune Sera. Russian Spring-Summer Encephalitis, Louping Ill, and Japanese B Encephalitis Viruses

Days elapsed between end of vaccination and bleeding for test	Antigens	Mouse hyperimmune sera																							
		Russian spring-summer encephalitis								Louping ill								Japanese B encephalitis							
		Dilution of serum								Dilution of serum								Dilution of serum							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
10	Russian	4	4	4	4	4	4	0	0	4	4	4	4	4	1	0	0	0	0	0	0	0	0	0	0
	Louping ill	4	4	4	4	2	0	0	0	4	4	4	4	4	3	0	0	0	0	0	0	0	0	0	0
	Japanese B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4	4	4	0
25	Russian	4	4	4	4	2	0	0	0	4	4	4	4	2	0	0	0	0	0	0	0	0	0	0	0
	Louping ill	4	4	4	4	±	0	0	0	4	4	4	4	4	2	0	0	0	0	0	0	0	0	0	0
	Japanese B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4	4	0	0
50	Russian	4	4	4	2	0	0	0	0	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0
	Louping ill	4	4	3	0	0	0	0	0	4	4	4	2	0	0	0	0	0	0	0	0	0	0	0	0
	Japanese B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	3	0	0	0	0
140	Russian	4	4	4	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Louping ill	4	4	0	0	0	0	0	0	4	4	3	0	0	0	0	0	0	0	0	0	0	0	0	0
	Japanese B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2	0	0	0	0	0	0

4 = no hemolysis; 0 = complete hemolysis; 3, 2, 1 ± = intermediate degrees of hemolysis.

nervous system viruses studied, none of which reacted at any time with either Russian No. 1 or louping ill virus. This amount of crossing between the Russian and louping ill virus is the most pronounced that we have observed in complement fixation tests carried out with viruses of the central nervous system group.

A high degree of crossing was also found between Russian and louping ill antigens when the titer of the antigens was determined by testing dilutions of antigen with a constant amount of immune serum, as shown in Table IV.

Serial twofold dilutions of Russian No. 1, louping ill, and St. Louis encephalitis antigens were tested against a constant amount of immune sera in dilution of 1:12. The table shows that the titer of the Russian antigen was 1:32 with

the homologous serum and 1:16 with the louping ill serum; it did not react with the St. Louis antiserum. The titer of louping ill antigen was 1:8 with louping ill serum and 1:8 with Russian serum; again there was no reaction with the St. Louis immune serum. On the other hand, the St. Louis antigen had a titer of 1:8 with its immune serum while neither the Russian nor louping ill serum reacted with the St. Louis antigen.

Similar crossing between the Russian and louping ill viruses has been observed consistently in complement fixation tests with sera from other batches of immune mice, as well as with the other strains of Russian virus, the Russian No. 2 and the Russian B-4, obtained by us at a later date.

Furthermore, by means of the complement fixation test it was ascertained that the three strains of Russian spring-summer encephalitis virus were iden-

TABLE IV

Complement Fixation Test. Russian Spring-Summer Encephalitis, Louping Ill, and St. Louis Encephalitis Viruses

Mouse hyperimmune serum (constant amount—dilution 1:12)	Mouse antigens in dilution:																							
	Russian spring-summer encephalitis							Louping ill							St. Louis encephalitis									
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:1	1:2	1:4	1:8	1:16	1:32	1:64			
Russian spring-summer encephalitis	4	4	4	4	4	3	0	4	4	4	3	0	0	0	0	0	0	0	0	0	0			
Louping ill	4	4	4	4	4	±	0	4	4	4	4	±	0	0	0	0	0	0	0	0	0			
St. Louis encephalitis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	4	4	2	0	0	0			

Footnote as in Table III.

tical, and also that each had a close relation to the louping ill virus. The complement fixation test showed, moreover, an absence of any relationship between the strains of Russian virus and our strains of Japanese B encephalitis virus. Evidence on these points is given in the following experiment.

Experiment 3.—Batches of mice were immunized against the following viruses: Russian strain 1, Russian strain 2, Russian strain B-4, louping ill, Japanese B encephalitis (Kobayashi and Nakayama strains), and West Nile encephalitis. Immunization consisted of two intraperitoneal injections of inactivated virus followed by intraperitoneal injections of live virus. At the appropriate time the mice were bled from the heart and their sera tested for complement-fixing antibodies against the corresponding antigens. The result of one such test is given in Table V.

The three strains of Russian spring-summer encephalitis virus showed complete crossing and hence were indistinguishable. The louping ill immune serum reacted with a maximum titer with louping ill antigen (1:128), and it also reacted with all three Russian antigens with a titer of 1:64 in each case. Con-

versely, the three Russian immune sera reacted with louping ill antigen in titers of 1:64 respectively; in each case the titer proved to be about one-half that shown with the homologous antigen. On the other hand, the two Japanese B strains, which reacted with each other very closely, showed no reaction at all with the Russian or louping ill viruses, with the possible exception of the Japanese (Kobayashi) immune serum which gave a weak reaction (1:2) with one of the Russian antigens. The West Nile immune serum did not react with any of the Russian virus antigens. The question of crossing between the Japanese B and West Nile viruses will be discussed in another publication.

TABLE V

Complement Fixation Tests with Three Strains of Russian Spring-Summer Encephalitis Virus, Two Strains of Japanese B Encephalitis Virus, and One Strain of West Nile Encephalitis Virus

Mouse hyperimmune sera—mouse brain antigens.

Antigens	Mouse sera						
	Russian No. 1	Russian No. 2	Russian B-4	Japanese B (Nakayama)	Japanese B (Kobayashi)	Louping ill	West Nile encephalitis
Russian No. 1	1:64*	1:128+	1:128	0	0	1:64	0
Russian No. 2	1:128	1:128+	1:128+	0	0	1:64	0
Russian B-4	1:64	1:128+	1:128	0	1:2	1:64	0
Japanese (Nakayama)	0	0	0	1:128+	1:64	0	1:4
Japanese (Kobayashi)	0	0	0	1:128+	1:128	0	1:8
Louping ill	1:64	1:64	1:64	0	0	1:128	0
West Nile encephalitis	0	0	0	1:8	1:8	0	1:64

* Highest dilution of serum giving a 2+ or better fixation. The first dilution of serum was 1:2.

Neutralization Tests.—Neutralization tests with mouse hyperimmune sera did not yield results as conclusive as those obtained by means of the complement fixation test. In general, mice hyperimmunized with Russian virus have been slow in exhibiting neutralizing antibody, as has already been reported by Silber (7). Mice immunized with louping ill virus, on the other hand, have shown a more prompt response.

Table VI gives the results of neutralization tests on the mouse sera described in Experiment 2.

Each serum was tested for neutralizing antibody against viruses of Russian encephalitis, louping ill, and Japanese B encephalitis. For each serum-virus combination, including the normal serum control, the 50 per cent endpoint titer was figured by the Reed and Muench method (6). The columns in the table under the heading "Titer of virus, etc." give the decimal logarithm, disregarding the minus sign, of the 50 per cent endpoint titer; the next columns under the heading "Neutralization index, etc."

give the ratio between the titer of virus in the presence of immune serum and the titer of virus in the presence of control serum. According to this system, the neutralization index of the control serum is always 1, and the neutralization index of an immune serum indicates the maximum number of M.L.D. protection given by that serum. Neutralization index values of 10 or less are considered as negative, those between 11 and 50 as doubtful, and those above 51 as significant.

The data in Table VI show that at the 10th day bleeding the Russian No. 1 serum showed a slight protection against Russian virus, with a neutralization index of 100, and no protection against either louping ill or Japanese B encephalitis viruses. The louping ill serum showed a moderate protection against louping ill virus but no significant protection against Russian or Japanese viruses. Finally, the Japanese B encephalitis serum showed only doubtful protection against Japanese and Russian viruses and none against louping ill.

On the 25th day bleeding, the Russian serum showed a moderate protection against its homologous virus, with a neutralization index of 650, but still no protection against either louping ill or Japanese viruses. The louping ill serum showed only slight protection against both louping ill and Russian viruses, and none against Japanese B encephalitis. The Japanese serum now showed a good level against Japanese virus (1,000) but did not protect against Russian or louping ill viruses.

On the 50th day bleeding, the protective level of Russian No. 1 serum was good, with an index of 2,200; this serum, which had no effect when tested against Japanese B virus, gave slight though significant protection against louping ill virus, with an index of 100. The louping ill immune serum, which had a neutralization index of 1,000 against the homologous virus, showed moderate protection against Russian virus, with an index of 200, and none against Japanese B virus. Finally, Japanese B serum showed no protection against either Japanese B, Russian, or louping ill virus.

Although a certain degree of crossing was found to exist between the Russian and louping ill viruses in some of the tests described above, it was not very conclusive. Since the protective levels of mouse sera for the homologous viruses were in general low, animals from other species were immunized with the thought that they might react more strongly to the immunization and thus better bring to light relationships among the different viruses.

Guinea pigs were immunized against Russian No. 1 and louping ill viruses by injections of mouse infected brain in dilutions of 10^{-2} and 10^{-3}. Four to six intraperitoneal injections of 4 cc. each were given at intervals of 1 week or 10 days, and the sera were tested for neutralizing antibody 10 weeks after the beginning of immunization. Hamsters were immunized against Russian and Japanese B virus with hamster infected brain. Four injections of 1 cc. of virus in dilutions of 10^{-2} and 10^{-3} were given at intervals of several days and the sera were tested for neutralizing antibody about 10 weeks from the time of the first injection. The results of these tests are presented in Table VII.

TABLE VI

Neutralization Tests with Mouse Hyperimmune Sera. Russian Spring-Summer Encephalitis, Japanese B Encephalitis, and Louping Ill Viruses

Immune mice		Fate of mice following intracerebral inoculation of virus in dilution:																			Titer of virus in presence of serum*			Neutralization index of serum against virus of:‡		
		Russian encephalitis virus							Louping ill virus						Japanese B virus											
Day of bleeding	Serum	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Russian	Louping ill	Japanese B	Russian	Louping ill	Japanese B
10	Russian encephalitis	4/4§	2/4	0/4	1/4	0/4	0/4		4/4	4/4	3/4	3/4	0/4	0/4		4/4	4/4	3/4	1/4	0/4	7.22	6.17	6.50	100	2	1
	Louping ill	4/4	4/4	1/4	0/4	0/4	0/4		3/4	0/4	1/4	1/4	0/4	0/4		4/4	4/4	3/4	0/4	0/4	7.67	3.60	6.34	35	800	1
	Japanese B	4/4	4/4	0/4	0/4	0/4	0/4		4/4	4/4	4/4	4/4	1/4	0/4	2/4	4/4	3/4	0/4	0/4	0/4	7.50	6.67	5.00	50	−1	32
	Unvaccinated controls	4/4	4/4	4/4	2/4	0/4	1/4	0/4		4/4	4/4	3/4	1/4	0/4			4/4	4/4	0/4	0/4	9.22	6.50	6.50	1	1	1
25	Russian encephalitis	4/4	1/4	0/4	0/4	0/4	0/4		4/4	4/4	1/4	0/4	0/4	0/4			4/4	4/4	0/4	0/4	6.67	4.67	6.50	650	21	7
	Louping ill	4/4	4/4	0/4	0/4	0/4	0/4		4/4	2/4	1/4	0/4	0/4	0/4			4/4	4/4	0/4	0/4	7.50	4.23	6.50	90	60	7
	Japanese B	4/4	4/4	3/4	0/4	0/4	0/4		4/4	4/4	3/4	1/4	1/4	0/4	3/4	3/4	1/4	0/4	0/4	0/4	8.34	5.66	4.32	13	2	1000
	Unvaccinated controls	4/4	4/4	4/4	3/4	0/4	1/4	0/4		4/4	4/4	2/4	0/4	0/4			4/4	4/4	3/4	0/4	9.47	6.00	7.34	1	1	1
50	Russian encephalitis	3/4	1/4	0/4	0/4				4/4	4/4	1/4	0/4	0/4			4/4	4/4	3/4	0/4	0/4	6.50	4.67	6.34	2200	100	1
	Louping ill	4/4	3/4	1/4	0/4	0/4	0/4		4/4	1/4	0/4	0/4	0/4			4/4	4/4	3/4	1/4	0/4	7.50	3.67	6.50	200	1000	1
	Japanese B	4/4	4/4	1/4	2/4	2/4	0/4		4/4	4/4	4/4	3/4	1/4	0/4	4/4	4/4	4/4	0/4	1/4	0/4	8.67	6.50	5.63	15	1	7
	Unvaccinated controls	4/4	4/4	4/4	3/4	1/4	2/4	0/4		4/4	4/4	3/4	1/4	1/4			4/4	4/4	0/4	0/4	9.84	6.66	6.50	1	1	1

* = titer of virus is expressed by the exponent of the highest dilution of virus giving a 50 per cent mortality.

‡ = neutralization index is the ratio between the titer of the virus in the presence of an immune serum and the titer of the virus in the presence of the unvaccinated mouse serum.

§ 4/4 = four of four mice injected died.

TABLE VII

Neutralization Tests with Guinea Pig and Hamster Hyperimmune Sera. Russian Spring-Summer Encephalitis, Japanese B Encephalitis, and Louping Ill Viruses

Serum	Fate of mice following intracerebral inoculation of virus in dilution:																								Titer of virus in presence of serum			Neutralization index of serum against virus of:		
	Russian encephalitis virus								Louping ill virus								Japanese B virus													
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Russian	Louping ill	Japanese B	Russian	Louping ill	Japanese B
Russian encephalitis guinea pig	4/4	2/4	3/4	0/4	0/4	0/4			4/4	4/4	4/4	1/4	0/4	0/4	0/4		4/4	4/4	4/4	4/4	4/4			6.00	4.67	7.50	1700	1000	5
Louping ill, guinea pig	4/4	4/4	4/4	2/4	1/4	0/4			4/4	4/4	3/4	0/4	0/4	0/4	0/4		4/4	4/4	4/4	4/4	4/4			7.23	4.34	7.50	100	2100	5
Normal guinea pig			4/4	4/4	4/4	2/4	1/4	0/4				4/4	4/4	4/4	1/4	0/4			4/4	4/4	3/4	3/4	0/4	9.23	7.67	8.17	1	1	1
Russian encephalitis, hamster	4/4	3/4	0/4	0/4	0/4	0/4			4/4	4/4	3/4	0/4	0/4	0/4	0/4		4/4	4/4	4/4	4/4	0/4			5.34	4.34	6.50	2100	2100	10
Japanese B encephalitis hamster	4/4	4/4	4/4	4/4	1/4	2/4	0/4	2/4		4/4	4/4	4/4	4/4	3/4	0/4		4/4	3/4	1/4	0/4	0/4			8.67	7.34	4.50	1	2	1000
Normal hamster			4/4	4/4	4/4	1/4	0/4	0/4				4/4	4/4	4/4	1/4	0/4			4/4	4/4	4/4	0/4	0/4	8.67	7.67	7.50	1	1	1

Footnotes as in Table VI.

The data in the table show that guinea pig Russian No. 1 immune serum gave almost as good protection against louping ill virus as it did against Russian virus, the neutralization index being 1,000 and 1,700 respectively, whereas this serum gave no protection against Japanese B virus. Again, a hamster Russian immune serum showed equally high protection against louping ill virus and against Russian virus, with an index of 2,100 in both cases, while the protection against Japanese B virus was negative. On the other hand, although a guinea pig louping ill immune serum protected well against louping ill virus, with an index of 2,100, the amount of protection shown by this immune serum against Russian virus was less, the index being 100 (about one-tenth of the former), whereas Japanese B virus was not neutralized. Finally, the hamster Japanese B immune serum, although giving good protection against Japanese B virus, with an index of 1,000, had no effect on either Russian or louping ill viruses.

Results similar to those set forth in Table VII with reference to Russian and louping ill viruses have been obtained in repeated tests of animal sera, that is, Russian immune serum protected equally well against louping ill and Russian viruses, whereas louping ill immune serum showed less protection against Russian virus than against louping ill virus.

The results presented in Table VII show clearly that the close relation between Russian and louping ill viruses could be demonstrated by neutralization tests as well as by complement fixation tests, and that neither one of the two viruses is related to the Japanese B encephalitis virus.

Cross-Resistance Test.—In view of the pronounced relationship between the two viruses under investigation, it was found advisable to determine the degree of cross-resistance, if any, existing between the Russian and louping ill viruses. Hence mice were vaccinated with avirulent preparations and then tested intraperitoneally for resistance. The reasons for the intraperitoneal challenge test were as follows. First, it had been found difficult, in general, to immunize mice against an intracerebral injection of most central nervous system viruses by using avirulent suspensions of virus. Second, the high susceptibility of mice to the Russian virus given either subcutaneously or intraperitoneally precludes the use of live virus to immunize against the Russian No. 1 strain, unless the mice have first been immunized with inactivated material. Third, the intraperitoneal route of infection was employed rather than the more natural subcutaneous one because of the low susceptibility of mice to louping ill virus injected subcutaneously, as shown above.

Experiment 4.—240 W-Swiss mice, 24 to 28 days of age and weighing 12 or 13 gm., were divided into four groups. Group I, consisting of 65 mice, was vaccinated with louping ill vaccine; group II, of 65 mice, with Russian No. 1 vaccine; group III, of 50 mice, with W.E.E. vaccine, and finally, group IV, of 60 mice, was left untreated as controls. In each case the course of vaccination consisted of two subcutaneous in-

jections, one on 2 consecutive days, of an avirulent 10 per cent mouse brain vaccine containing 0.5 per cent formaldehyde as described under Materials and Methods. 14 and 15 days later the mice were infected with the Russian No. 1 and louping ill viruses respectively by intraperitoneal injection of 0.5 cc. of a series of tenfold dilutions of virus. The results are shown in Table VIII.

None of the mice died as a consequence of vaccination. Following intraperitoneal infection with Russian virus, the titer of the virus was $10^{-8.50}$ in the control mice; in mice vaccinated with W.E.E., the titer was $10^{-7.50}$ plus; in the mice given louping ill

TABLE VIII

Cross-Resistance Test. Mice Vaccinated with Either Russian Spring-Summer Encephalitis, Louping Ill, or W.E.E. Formolized Mouse Brain Emulsions Tested Intraperitoneally for Immunity against Russian and Louping Ill Viruses

Vaccine	Fate of mice following intraperitoneal inoculation of 0.5 cc. of virus in dilution:																			
	Russian encephalitis virus												Louping ill virus							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer of virus in different groups of mice*	Resistance index‡	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Titer of virus in different groups of mice*	Resistance index‡
Russian encephalitis.	§ 2/5	1/5	1/5	0/5	0/5	0/5				1.20	20,000,000	3/6	0/5	1/5	0/5	0/5	0/5		1.16	100,000
Louping ill.	5/5	4/5	2/5	2/5	0/5	0/5	0/5			3.00	320,000	0/5	0/5	1/5	0/5	0/5	0/4		0.6 or less	370,000 or more
W.E.E.....			5/5	5/5	5/5	5/5	5/5			7.50+	10 or less			3/5	5/5	4/5	0/4		5.14	11
None—controls.....			5/5	5/5	5/5	5/5	5/5	5/5	0/5	8.50			5/5	4/5	5/5	3/5	3/5	3/5	6.17	

* = titer of virus is expressed by the exponent of the highest dilution of virus giving a 50 per cent mortality.

‡ = resistance index is the ratio between the titer of the virus in a vaccinated group and the titer of the virus in the control group.

§ 2/5 = two of five mice injected died.

vaccine, the titer was $10^{-3.00}$, and in mice given Russian vaccine the titer was $10^{-1.20}$. Furthermore, following injection of louping ill virus, the titer was $10^{-6.17}$ in the controls, $10^{-5.14}$ plus in mice vaccinated with W.E.E. vaccine; $10^{-1.16}$ in those given Russian No. 1 vaccine, and less than 10^{-1} in mice given louping ill vaccine.

This experiment shows that under the conditions of the test, formolized, inactivated, Russian spring-summer encephalitis virus protects mice against approximately 20,000,000 intraperitoneal minimal lethal doses of Russian virus and against 320,000 intraperitoneal minimal lethal doses of louping ill virus. On the other hand, formolized, inactivated louping ill vaccine protects mice against a challenge test dose of 100,000 intraperitoneal minimal lethal doses of Russian virus given 15 days later, and against 370,000 plus minimal lethal doses

of louping ill virus given intraperitoneally. That this cross-resistance is not due to some non-specific factor is indicated by the fact that another formolized, inactivated vaccine prepared identically from another virus (W.E.E.), does not give the slightest protection against either the Russian No. 1 or louping ill virus. This marked cross-resistance is therefore further proof of the close relationship between these two viruses.

Human Convalescent Sera

Further confirmation of the similarity between the two viruses under study was obtained in tests with two human convalescent sera.[1]

The first serum, W, was derived from an individual who, in 1933, while engaged in work on louping ill virus, came down with an acute infection which later, on the basis of neutralization tests (8), was diagnosed as louping ill. This diagnosis was confirmed by complement fixation tests carried out in 1941 (5). This individual was not in contact with the Russian virus until the summer of 1942; yet, a sample of serum drawn in 1936 and kept lyophilized in the refrigerator, when tested for complement-fixing antibodies against Russian No. 1 and louping ill viruses, gave a positive reaction while it failed to react with other central nervous system viruses. Moreover, a specimen of serum drawn in 1942 proved to be positive for both complement-fixing and neuralizing antibodies against Russian No. 1 and louping ill viruses, while it was negative when tested against other central nervous system viruses.

The serum of a second person, K, was tested for antibodies against these viruses. This individual became ill in September, 1942, with an acute disease clinically diagnosed as an encephalitis-like infection. His serum was tested for complement-fixing antibodies against a number of viruses with which he had been working and showed a strong reaction against louping ill and Russian No. 1 antigens. Neutralizing antibodies were also present against these two viruses, while no antibodies of any kind were found against other viruses tested. A sample of serum taken in 1936 and kept lyophilized in the refrigerator was entirely negative. Unfortunately it was not possible to obtain an early sample of blood from this patient in the first days of illness; the two specimens taken on the 15th and 30th days of illness respectively were both positive. Although there is the possibility that this individual might have had a simultaneous infection with the Russian and louping ill viruses, this is rather remote. It is far more probable that he had but one infection, either Russian encephalitis or louping ill, with the result that his blood acquired antibodies against the two viruses. The sample of serum W-1936 proves beyond doubt that a louping ill convalescent serum reacts with the virus of Russian spring-summer encephalitis.

Table IX shows the results of complement fixation tests. W-1936 serum reacted in a dilution of 1:8 with both Russian and louping ill antigens, while it

[1] In a previous publication (3) mention was made of a third convalescent serum from an individual who became ill with louping ill infection in 1933, simultaneously with the patient W. The results obtained with this serum are not reported here because of their incompleteness: the supply of lyophilized serum kept in the refrigerator since 1936 had become exhausted and fresh serum was not available.

failed to react with either Japanese B or W.E.E. antigens. Likewise, serum W-1942 reacted in a dilution of 1:8 with the Russian antigen, in a dilution of 1:8 with the louping ill antigen, and not at all with the other antigens tested. Serum K-1936, a preinfection specimen, was negative in all cases. On the other hand, serum K-10-1942 reacted in a dilution of 1:16 with Russian and louping ill antigens. Serum K-11-1942 reacted in a dilution of 1:32 with Russian and louping ill antigens. None of these sera reacted with the other antigens tested.

Table X presents the results of one of several neutralization tests carried out with the human sera. Serum W-1942 had a neutralization index of 1,400 against louping ill virus, of 6,800 against Russian virus, and of −10 against

TABLE IX

Human Convalescent Sera. Complement Fixation Tests

Serum	Mouse brain antigens																							
	Russian spring-summer encephalitis							Louping ill							Japanese B encephalitis				W.E.E. virus			
	Dilution of serum							Dilution of serum							Dilution of serum				Dilution of serum			
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
W-1936	4	4	2	0	0	0	0	4	4	2	0	0	0	0	0	0	0	0	0	0	0	0
W-1942	4	4	4	0	0	0	0	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0
K-1936	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K-10-1942	4	4	4	4	0	0	0	4	4	4	3	1	0	0	0	0	0	0	0	0	0	0
K-11-1942	4	4	4	4	4	1	0	4	4	4	4	3	1	0	0	0	0	0	0	0	0	0

Footnote as in Table III.

Japanese B encephalitis virus. Serum K-11-1942 gave a protection of 300 M.L.D. against louping ill virus, of 10,000 M.L.D. against Russian virus; it did not protect against Japanese B encephalitis virus. In every instance the degree of cross-resistance present between Russian and louping ill viruses was highly significant.

DISCUSSION

Complement fixation, neutralization, and cross-resistance tests all indicate that there exists a close relationship between the Russian spring-summer encephalitis and louping ill viruses. There are indications, however, that these viruses may not be identical, for in almost every instance the reaction or protection shown by the homologous serum-antigen or vaccine-virus was greater than that exhibited by heterologous combinations. Moreover, the varying susceptibility of mice, especially old ones, to peripheral inoculation of the two

TABLE X

Human Convalescent Sera. Neutralization Tests

Serum	Fate of mice following intracerebral injection of virus in dilution:																											
	Russian encephalitis virus										Louping ill virus									Japanese B encephalitis virus							
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	Titer of virus in presence of serum	Neutralization index	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer of virus in presence of serum	Neutralization index	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titer of virus in presence of serum	Neutralization index
W-1942	4/4	4/4	1/4	0/4	0/4	0/4	0/4		5.67	6800	4/4	1/4	1/4	0/4	0/4	0/4		3.84	1400	4/4	4/4	4/4	4/4	1/4	0/4	7.67	−10
Guinea pig, normal			4/4	4/4	4/4	4/4	0/4	0/4	9.50																		
Mouse, normal												4/4	4/4	3/4	2/4	1/4	0/4	7.00		4/4	4/4	4/4	1/4	0/4	0/4	6.67	
K-11-1942	4/4	3/4	1/4	0/4	0/4	0/4	0/4		5.50	10,000	3/4	1/4	0/4	0/4	0/4			3.50	300	4/4	4/4	4/4	2/4	1/4	0/4	7.23	−5
Guinea pig, normal			4/4	4/4	4/4	4/4	0/4	0/4	9.50																		
Mouse, normal												4/4	4/4	2/4	0/4	0/4	0/4	6.00		4/4	4/4	4/4	0/4	0/4	0/4	6.50	

Footnotes as in Table VI.

viruses seems to point to a slight difference between them, although it may be added that such difference was not greater than would be expected to exist between two strains of the same virus, nor than that resulting from changes induced in a virus by repeated mouse passage in the laboratory.

On the other hand, not only the serological relationships here reported but other characteristics as well point to a close similarity between Russian spring-summer encephalitis virus and louping ill virus; for example, the scope of animal susceptibility. Both these viruses were found to be infective for mice, sheep, and monkeys, while they were not virulent for guinea pigs, rabbits, or rats. Moreover, the epidemiology of the natural infection is distinctly similar, each being a tick-transmitted disease, one of sheep, the other of man and some wild rodents.

We have not been able to confirm the observations of Smorodintseff (1) that there is cross-neutralization between the Russian spring-summer encephalitis virus and that of Japanese B encephalitis. Furthermore, complement fixation tests as well as cross-resistance tests have likewise failed to reveal any relationship between these two viruses.

The close relationship here described between the Russian spring-summer encephalitis virus and louping ill virus is more marked than any thus far encountered between any two central nervous system viruses. In view of these findings we consider the specimens of Russian spring-summer encephalitis virus and of louping ill virus received in our laboratory as alike.

SUMMARY

An experimental study of three strains of Russian spring-summer encephalitis virus and one of louping ill virus has yielded the following results:—

1. The sera of mice hyperimmunized to the viruses of Russian encephalitis and louping ill respectively have produced complement fixation with both antigens in almost precisely the same titer.

2. In neutralization tests hyperimmune sera against the Russian virus strains protected against louping ill virus to the same extent as against the Russian virus strains. Conversely, hyperimmune sera against the louping ill virus protected against the Russian viruses, although to a less degree than against louping ill virus.

3. In cross-resistance tests in mice, a vaccine consisting of formolized Russian virus gave strong protection against this latter and moderate protection against louping ill virus. Formolized louping ill virus gave moderate protection against infection with louping ill and considerably less against the Russian virus.

4. Serum from an individual recovered from a laboratory infection with louping ill virus contracted in 1933 gave positive complement fixation and neutralization tests with the Russian spring-summer encephalitis, as well as with louping ill virus.

5. Serum from a patient who became infected with either Russian or louping ill virus or both while working with the viruses in the laboratory in the fall of 1942, gave positive reactions on complement fixation and neutralization tests against them both.

6. No such similarities have been found with other central nervous system viruses. Hence it would appear that they are specific.

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