IMMUNOLOGICAL RELATIONSHIPS AMONG CENTRAL NERVOUS SYSTEM VIRUSES

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In the past few years there has been increased interest in the study of the immunological relationships that exist among viruses that, either in a natural host or in experimental animals, affect chiefly the central nervous system.

Webster (1) and several Japanese workers (2) undertook to compare the characteristics of the St. Louis and Japanese B viruses by means of neutralization tests, using either human convalescent sera or animal hyperimmune sera. The results of these investigations indicated that no definite relationship exists between these two viruses.

In 1940, Smithburn and coworkers (3), studying the properties of a newly discovered virus called by them the West Nile virus, compared it with the Japanese B and St. Louis agents, at the same time attempting to establish a possible relationship between the two latter viruses. They tested a great many sera from certain regions in Africa for the presence of neutralizing antibodies; tests were carried out also with animal hyperimmune sera. These investigators concluded that the three viruses possessed some common immunological properties: Sera from animals immunized with Japanese B virus that contained neutralizing antibodies against the Japanese virus neutralized both St. Louis and West Nile viruses; St. Louis immune sera that neutralized St. Louis virus also neutralized the virus of West Nile encephalitis but not that of Japanese B encephalitis. Finally, sera from animals immunized against West Nile virus protected against the West Nile agent but not against Japanese or St. Louis viruses. Hence Smithburn inferred that the Japanese virus was a more complex antigenic entity than the St. Louis virus, and that the latter was in turn more complex than the West Nile virus. Cross-protection tests in his laboratory showed no indication of crossing among these three viruses. In addition, Havens *et al.* (4), employing the complement fixation test, failed to find any relationship among the Japanese, St. Louis, and West Nile type of encephalitis viruses.

Shortly after their isolation of the Russian spring-summer encephalitis virus (5), Smorodintseff and his associates undertook comparative studies with other viruses of the central nervous system type, by means of neutralization tests on human and animal sera. They reached the conclusion that the Russian and Japanese B viruses were related in that Russian immune sera protected against Japanese virus, while Japanese immune sera either did not protect at all against Russian virus or did so only slightly. Russian and St. Louis viruses were found by them to be distinct.

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The problem of the immunological relationships among viruses has been investigated extensively in our laboratory by means of complement fixation, neutralization, and cross-resistance tests and the results obtained are reported here.

Materials and Methods

Viruses.—The following viruses have been compared in our tests: Russian spring-summer encephalitis, louping ill, St. Louis encephalitis, Japanese B encephalitis, West Nile encephalitis, and Western equine encephalomyelitis (W.E.E.). The sources of the various strains employed are given below.

Russian Spring-Summer Encephalitis.—This strain was obtained from the National Institute of Health through the cooperation of Drs. R. R. Parker and H. R. Cox in Montana. It had been transported to their laboratory in ticks from that of Dr. M. P. Chumakov in Moscow. This strain had undergone only a few mouse passages; in our laboratory it was passaged two or three times.

Louping Ill.—The louping ill virus used in our studies was the Moredun strain. It had been received from Scotland in 1931 by Dr. T. M. Rivers and turned over to our laboratory for further study in 1932, where it has been maintained since that time. The number of intracerebral passages this strain has undergone is not known, but there have probably been many.

St. Louis Encephalitis.—This strain was isolated in 1933 from a human case by Dr. Webster and maintained in our laboratory by means of intracerebral mouse passage. In 1937 it was sent to Dr. G. O. Broun in St. Louis and later, in 1940, returned to us. This strain, designated as B 33, was used in preference to our original St. Louis No. 3 strain because of its better immunizing qualities as well as the smaller number of intracerebral mouse passages it had undergone. Although no regular count was kept of the number of passages, it was undoubtedly high.

Japanese B Encephalitis.—The virus used in our tests was a strain isolated in Tokyo in 1939 and sent to Dr. Webster in May, 1940, by Dr. R. Kobayashi. It has been maintained in this laboratory by alternating mouse passage with storage in glycerine. The number of mouse passages it has undergone is not known.

West Nile Encephalitis.—Our strain of West Nile virus was obtained through the courtesy of Dr. Max Theiler of the International Health Division of The Rockefeller Foundation. The agent had been isolated from the blood of a febrile individual in the West Nile district, Northern Province of Uganda, and was described by Smithburn and coworkers (3) in 1940. The virus has had only two intracerebral mouse passages in our laboratory.

Western Equine Encephalomyelitis.—The W.E.E. virus employed in these studies was isolated from a specimen of glycerolated brain tissue from a fatal human case in the Manitoba epidemic (6). It has been designated as the McMillan strain and has had three or four intracerebral mouse passages since its isolation.

All of these viruses are kept in our laboratory in the form of a 10 per cent mouse brain suspension in distilled water and stored in a dry-ice box at −76°C. As needed, a sample of the stock solution is inoculated intracerebrally into young Swiss mice. Thus, by reverting to the stock solution, the number of intracerebral mouse passages can be kept to a minimum. This method of preservation has been followed since

1940. Prior to that time, the viruses had been propagated by alternating intracerebral mouse inoculation with storage in the refrigerator in 50 per cent glycerine.

Animals.—Hamsters and guinea pigs were used for the production of hyperimmune sera in some cases, but in general W-Swiss (7) mice were employed throughout. Young mice, 20 to 25 days of age, were used for passage of virus; older mice, 50 to 60 days of age, for production of hyperimmune sera or for vaccination. Groups of mice in the several tests were as uniform as possible in regard to age and weight.

Vaccines.—In the course of the production of hyperimmune sera as well as in tests for resistance to infection, avirulent vaccines were used. These vaccines were prepared as follows: Batches of thirty mice, 20 to 25 days of age, were injected intracerebrally with a 10^{-2} suspension of a given virus. When prostrate, animals were sacrificed, their brains removed, weighed, and emulsified in sufficient physiological saline to give a 10 per cent brain tissue emulsion. A mechanical blendor was utilized for emulsification. Next, ½ per cent commercial formaldehyde was added to the brain emulsion and the mixture kept in the refrigerator in glass-stoppered bottles. The material was tested for virulence by intracerebral inoculation into young mice and when it proved to be no longer virulent, it was ready for use. Usually 10 to 15 days were long enough to inactivate the virus completely. Some batches of vaccine were used within 15 to 20 days of preparation; others after 4 or 5 months.

Hyperimmune Sera.—Hyperimmune sera were obtained from mice, guinea pigs, and hamsters. Mouse sera were chiefly employed for studies on hyperimmune sera, even though the mouse, because of its small size, was not the most satisfactory animal for this purpose. It was desired to test the hyperimmune sera for complement-fixing as well as neutralizing antibodies. As is well known (8), homologous brain tissue must be employed for immunization to provide sera suitable for use in the complement fixation reaction and since the mouse was the only available animal susceptible to intracerebral inoculation of all the viruses under study, mice had to be used in preference to other larger laboratory animals.

Mice hyperimmune sera were obtained in the following manner: A group of mice from 60 to 70 days old were divided into seven batches of forty mice each. Each batch of animals were immunized with one of the viruses under study and the last batch left untreated as controls. Mice were vaccinated according to the following schedule:—

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

When the course of vaccination was concluded, the mice were bled from the heart under ether anesthesia; the bloods were pooled, allowed to clot, centrifuged, and the sera stored in a dry-ice box in lusteroid tubes. Bleedings were done on the following days, reckoning from the time of the final immunizing injection: 10th, 25th, 50th, and 140th, respectively. The control mice were bled at approximately the same time. The sera were tested for both complement-fixing and neutralizing antibodies, as described below.

Hyperimmune Sera from Hamsters and Guinea Pigs.—Homologous hyperimmune sera were obtained from hamsters against the viruses of St. Louis, Russian spring-sum-

mer, and Japanese B encephalitis. These viruses killed young hamsters when injected intracerebrally in dilutions of 10^{-2}, thereby enabling us to prepare formolized, inactive suspensions of hamster infected brain, as well as virulent suspensions of hamster brain with which to carry out homologous immunization. Such sera were tested for both complement-fixing and neutralizing antibodies.

Hyperimmune sera were obtained from guinea pigs immunized with mouse infected brain against the viruses of Russian spring-summer, St. Louis, West Nile, and Japanese B encephalitis, W.E.E., and louping ill. These sera were tested only for neutralizing antibodies.

No strict schedule for immunization and bleeding of hamsters and guinea pigs was adhered to. Hamsters were given two injections of 1 cc. each of formolized vaccine followed by three to four injections of 1 cc. each of virulent hamster infected brain in dilutions of 10^{-2}. They were bled from the heart at different times, starting about 5 weeks from the time vaccination had been instituted. Titers of sera were kept at a high level by occasional intraperitoneal injections of virulent hamster brain.

Guinea pigs were hyperimmunized by repeated intraperitoneal injections of virulent mouse brain tissue in dilutions of 10^{-3} or 10^{-2} in 4 cc. amounts. About 5 weeks after the start of vaccination, when the animals had received from four to six injections, they were bled for the first time. Titers of these sera were likewise kept at a high level by occasional intraperitoneal injections of live virus.

Complement Fixation Tests.—Complement fixation tests employing brain tissue antigens can now be carried out extensively inasmuch as factors heretofore disturbing have been eliminated. The method followed has been described previously (9). The essential steps were as follows: Antigens were prepared from each virus by freezing and thawing a 10 per cent infected mouse brain suspension and then centrifuging the material in an angle-head centrifuge at 5000 R.P.M. The supernatant fluid, to which merthiolate was added to prevent contamination with molds or bacteria, constituted the antigen. It could be stored in the refrigerator for several months without alteration in stability. Fresh guinea pig serum, properly diluted, constituted the complement. Finally, the hemolytic system was made up of a 3 per cent suspension of packed sheep cells and rabbit anti-sheep hemolysin. The sera to be tested for complement-fixing antibodies were inactivated for 20 minutes at the appropriate temperature (60° C. for mouse serum; 65°C. for hamster serum). The test was carried out in the following manner: 0.25 cc. of antigen plus 0.50 cc. of complement equivalent to 2 units plus 0.25 cc. of the serum in twofold dilutions, beginning with either undiluted serum or serum in a dilution of 1:2, were placed in small test tubes and incubated for 18 hours at −4°C. Then the hemolytic system, consisting of 0.25 cc. of sheep cells plus 0.25 cc. of hemolysin containing 3 M.H.D., was added. The tubes which then contained a volume of 1.5 cc. of fluid were incubated for 30 minutes at 37°C. and the reaction was read. The titer of a serum was regarded as the highest dilution giving a 2 plus or better fixation. Adequate controls were included in the test in order to make certain of complete specificity. Each one of the hyperimmune sera for the six different viruses was tested simultaneously against the six different antigens. Thus complete cross-reaction under identical conditions for all the antigen-antibody combinations was secured.

Neutralization Tests.—Mouse, hamster, and guinea pig hyperimmune sera were

tested for neutralizing antibodies against the six different viruses in the following way: One of the viruses from the stock suspensions was inoculated intracerebrally into four mice. When these animals were prostrate, their brains were removed and made up into a 10 per cent suspension in diluent consisting of nine parts of physiological saline and one part of normal rabbit serum. This virus suspension was then centrifuged at 2000 R.P.M. for 10 minutes, the supernatant pipetted off and diluted in tenfold dilutions, starting with a dilution of 1:50 and carrying the dilutions as far as necessary, depending on the expected titer of the virus. Then 0.20 cc. aliquot portions of these virus dilutions were placed in tubes measuring 100 × 8 mm.; as many sets of tenfold dilutions were provided as there were sera to be tested, plus one for the control serum. Next the hyperimmune sera were added, each tube of a set, as well as the control, receiving 0.20 cc. of a given serum. Thus there was present in each set of tubes a mixture of constant amount of hyperimmune serum and tenfold dilutions of virus. The tubes were then shaken and placed in a water bath at 37°C. for 2 hours. After incubation the mixtures were injected intracerebrally in 0.03 cc. amounts into W-Swiss mice 20 to 25 days of age; four mice were used per dilution. The mice were observed for a 21-day period and the survivors, which were considered as having been protected, discarded.

Cross-Resistance Tests.—After vaccination mice were tested for resistance to infection by intracerebral and intraperitoneal routes. For the intracerebral test, animals 60 to 70 days old at the beginning of the experiment were employed; they were vaccinated against each virus by means of two injections of formolized 10 per cent mouse brain vaccine prepared as described above, followed by three injections of active virus. The latter material had been kept in the dry-ice box as a 10 per cent suspension of infected mouse brain tissue in distilled water. When needed the suspension was thawed, diluted to the appropriate strength, and injected immediately. The schedule of vaccination for the intracerebral test was as follows:—

1st day—10 per cent formolized vaccine, 0.25 cc. intraperitoneally; *3rd day*—10 per cent formolized vaccine, 0.25 cc. intraperitoneally; *12th day*—virulent brain emulsion in dilution of 10^{-4}, 0.5 cc. intraperitoneally; *16th day*—virulent brain emulsion in dilution of 10^{-2}, 0.5 cc. intraperitoneally; *20th day*—virulent brain emulsion in dilution of 10^{-2}, 0.5 cc. intraperitoneally.

On the 35th day from the beginning of the test, that is, approximately 2 weeks after the last injection had been given, the mice were injected intracerebrally with the selected virus in 0.03 cc. amounts of a series of tenfold dilutions; five mice were injected per dilution. In all the resistance tests, intracerebral as well as intraperitoneal, additional mice were set aside at the start of the experiment and left unvaccinated to serve as controls.

For the intraperitoneal test, mice 20 to 25 days old were used. They were vaccinated against each virus by means of two subcutaneous injections of 0.25 cc. each of formolized 10 per cent mouse brain vaccine given on 2 consecutive days. The total amount of vaccine administered was thus 0.5 cc. 15 days from the time of the first injection, the mice were infected intraperitoneally by injection of 0.5 cc. amounts of virus in tenfold dilutions; four mice were injected per dilution.

The virus used for infection of the mice, in both intracerebral and intraperitoneal tests, was a suspension that had been freshly prepared for each experiment. For this

purpose four to six mice were injected intracerebrally with a 10^{-2} suspension of virus taken from the dry-ice box. When sick or prostrate they were sacrificed, their brains removed, and usually three of these brains were ground in a mortar, emulsified in 10.8 cc. of saline-serum and a 10^{-1} suspension of mouse infected brain was thus yielded. This suspension was centrifuged at 2000 R.P.M. for 10 minutes and the supernatant further diluted in tenfold dilutions.

Infected mice were observed for 21 days and then discarded. Animals surviving that long were considered as having resisted the infection.

Evaluation of Neutralization and Cross-Resistance Tests.—The evaluation of the amount of protection given by a particular serum or of the degree of resistance conferred by a vaccine was determined by the Reed and Muench method (10). In a given test the 50 per cent mortality titer was calculated for the controls and for each of the different groups. This was the highest dilution of virus that killed 50 per cent of a group of mice and is represented in the accompanying tables by the logarithm of the denominator of the dilution. The amount of protection given by a serum is expressed by the ratio between the 50 per cent mortality titer of the serum and the 50 per cent mortality titer of the control: this ratio is designated in the tables as *Neutralization index.* Likewise, the amount of resistance conferred by a vaccine is represented by the ratio between the 50 per cent mortality titer of the vaccinated mice and the 50 per cent mortality titer of the controls: this ratio is designated as *Resistance index.* Both neutralization and resistance indices express the maximum number of M.L.D. of protection given by a serum or vaccine as compared to the controls, for which the value of the index is always 1. Neutralization and resistance index values of less than 10 are without significance and are considered negative; values from 10 to 49 are barely significant and are called equivocal and finally, values of 50 or more are regarded as significant and designated positive.

This method of evaluating results proved to be satisfactory for the neutralization and intracerebral resistance tests; in these the titer of a given virus in the controls in individual tests showed little variation. Moreover, the titrations were regular and uniform, as the protocols demonstrate. Hence the indices present in both instances a fairly approximate numerical value which can be reproduced, within limits, in repeated tests. On the other hand, in the case of intraperitoneal resistance tests, the method of evaluating results described above was not satisfactory for some viruses on account of the irregularity in titrations and the low titers obtained. Still the index values have been retained in the tables as an indication of the relative effect of the different vaccines. In order to simplify the tables, the values of neutralization and resistance indices have been expressed by the nearest whole digit when the index proved to be less than 50; by the nearest 10, when the index was between 50 and 100; by the nearest 50, when the index was between 100 and 1000, and by the nearest 100, when the value of the index was above 1000.

EXPERIMENTAL

The experiments to determine the relationships between the viruses of Russian spring-summer encephalitis, louping ill, St. Louis encephalitis, Japanese B encephalitis, West Nile encephalitis, and Western equine encephalomyelitis

were carried out with the aid of complement fixation, serum neutralization, and cross-resistance tests.

Complement Fixation Tests.—Mouse hyperimmune sera were used chiefly in the present tests.

Experiment 1.—280 W-Swiss mice 60 to 70 days of age were divided into seven groups of forty mice each. Group 1 were vaccinated with Russian encephalitis virus, No. 2 with louping ill, No. 3 with Japanese B, No. 4 with St. Louis, No. 5 with West Nile, No. 6 with W.E.E., and No. 7 were left untreated as controls. The vaccination consisted of two injections of formolized virus and three of live virus, as described under Materials and methods. Next, 10, 25, 50, and 140 days from the time of the last injection the mice in each group were bled and their sera tested for complement-fixing antibodies. The complement fixation tests were planned so that sera obtained on a particular date could be tested simultaneously against all antigens and identical conditions were thus maintained for all sera tested. The results obtained are presented in Table I. This table represents a summary of four tests, each carried out on a different date.

In the 10th day test, the Russian serum gave a titer of 1:64 against Russian antigen, of 1:32 against louping ill antigen, and it was negative against Japanese B, St. Louis, West Nile, and W.E.E. antigens; louping ill serum showed titers of 1:32 and 1:64 respectively against Russian and louping ill antigens and was negative against the remaining antigens. Japanese B serum did not react with Russian, louping ill, or W.E.E. antigens; it showed a titer of 1:128 with its own antigen, and titers of 1:8 and 1:4 against St. Louis and West Nile antigens respectively. St. Louis serum reacted to a titer of 1:64 with the homologous antigen, of 1:8 with Japanese B antigen, and was negative in the case of the other antigens. West Nile serum had a titer of 1:64 with West Nile antigen, of 1:8 with Japanese B antigen, and was negative when tested against the other antigens. Finally, W.E.E. serum reacted only with its own antigen to a titer of 1:128.

In the 25th day test, Russian serum reacted with Russian antigen to a titer of 1:32 and with louping ill antigen to a titer of 1:16; louping ill serum reacted with louping ill antigen to a titer of 1:64 and with Russian antigen to a titer of 1:32. Neither Russian nor louping ill serum reacted with the Japanese B, St. Louis, West Nile, or W.E.E. antigens. Japanese serum which showed a titer of 1:64 with Japanese B antigen, gave a titer of 1:8 with West Nile antigen and of 1:2 with St. Louis antigen; no reaction was elicited against the other antigens tested. St. Louis serum, which reacted with titers of 1:64, 1:8, and 1:2 with St. Louis, Japanese B, and West Nile antigens respectively, did not react with the remaining antigens. West Nile serum had a titer of 1:64 with its homologous antigen and of 1:8 with Japanese B antigen; it was negative in the case of the four remaining antigens. Finally, W.E.E. serum again reacted with its own antigen, giving a titer of 1:64, while the reaction was negative with all the other antigens.

In the 50th day test, the specific titers had a lower value than previously. Russian serum reacted to a titer of 1:16 with Russian antigen and of 1:8 with louping ill; it was negative against the other antigens. Louping ill serum was positive with Russian

and louping ill antigens, showing titers of 1:8 and 1:16 respectively. Japanese serum reacted only with Japanese antigen, to a titer of 1:16. St. Louis serum was positive with St. Louis and Japanese antigens to titers of 1:32 and 1:4 respectively; this serum

TABLE I

Complement Fixation Tests with Mouse Hyperimmune Sera

Immune mice		Antigens					
Day of bleeding	Serum	Russian encephalitis	Louping ill	Japanese B encephalitis	St. Louis encephalitis	West Nile encephalitis	W.E.E.
10th	Russian encephalitis	1:64*	1:32	0	0	0	0
	Louping ill	1:32	1:64	0	0	0	0
	Japanese B encephalitis	0	0	1:128	1:8	1:4	0
	St. Louis encephalitis	0	0	1:8	1:64	0	0
	West Nile encephalitis	0	0	1:8	0	1:64	0
	W.E.E.	0	0	0	0	0	1:128
25th	Russian encephalitis	1:32	1:16	0	0	0	0
	Louping ill	1:32	1:64	0	0	0	0
	Japanese B encephalitis	0	0	1:64	1:2	1:8	0
	St. Louis encephalitis	0	0	1:8	1:64	1:2	0
	West Nile encephalitis	0	0	1:8	0	1:64	0
	W.E.E.	0	0	0	0	0	1:64
50th	Russian encephalitis	1:16	1:8	0	0	0	0
	Louping ill	1:8	1:16	0	0	0	0
	Japanese B encephalitis	0	0	1:16	0	0	0
	St. Louis encephalitis	0	0	1:4	1:32	0	0
	West Nile encephalitis	0	0	1:2	1:2	1:8	0
	W.E.E.	0	0	0	0	0	1:32
140th	Russian encephalitis	1:8	1:4	0	0	0	0
	Louping ill	1:4	1:8	0	0	0	0
	Japanese B encephalitis	0	0	1:4	0	0	0
	St. Louis encephalitis	0	0	0	1:8	0	0
	West Nile encephalitis	0	0	0	0	1:2	0
	W.E.E.	0	0	0	0	0	1:16

0 = negative reaction in dilution of 1:2 or higher.

* Highest dilution of serum giving a 2+ or better fixation.

was negative against the other antigens. West Nile serum had a low titer (1:8) against its homologous antigen and low titers also (1:2) with Japanese and St. Louis antigens; it was negative when tested against the other antigens. W.E.E. serum had a titer of 1:32 with W.E.E. antigen and was again negative with the remaining five antigens.

In the 140th day test, all titers were low and no crossing was found except in the case of Russian and louping ill sera. Russian serum reacted in dilutions of 1:8 and

1:4 with Russian and louping ill antigens respectively. Louping ill serum showed titers of 1:4 and 1:8 against Russian and louping ill antigens. The titers reached by the other sera were as follows: Japanese B, 1:4; St. Louis, 1:8; West Nile, 1:2, and W.E.E., 1:16.

The results of complement fixation tests on sera from batches of mice observed over a definite period revealed the existence of a pronounced and constant relationship between Russian and louping ill viruses; while between Japanese B, St. Louis, and West Nile viruses there was a less pronounced but nevertheless definite relationship. The Russian and louping ill viruses did not exhibit any relation to any of the other viruses tested, nor did Japanese B, West Nile, or St. Louis virus react with Russian or louping ill virus. Finally, W.E.E. virus proved to be unrelated to the five other viruses tested.

TABLE II

Complement Fixation Tests—Hamster Hyperimmune Sera

Serum	Antigens		
	Russian encephalitis	Japanese B encephalitis	St. Louis encephalitis
Russian encephalitis	1:128	0	0
Japanese B encephalitis	0	1:64	1:8
St. Louis encephalitis	0	1:2	1:16

Footnotes as in Table I.

In each case the heterologous titer of the Russian and louping ill sera amounted to one-half their homologous titer. Such a relationship between these two viruses has been studied extensively elsewhere (11). Crossing among the Japanese B, West Nile, and St. Louis viruses was much less pronounced than that between the Russian and louping ill viruses. It was most evident when the homologous titers were high; then the Japanese serum reacted with Japanese, West Nile, and St. Louis antigens, the St. Louis serum with the St. Louis and Japanese antigens, and the West Nile serum with the West Nile and Japanese antigens. Only an occasional cross-reaction occurred between St. Louis and West Nile viruses and, when present, it was of a low titer.

Complement Fixation Tests with Hamster Hyperimmune Sera.—Additional information bearing on the relationship among these central nervous system viruses was gained by testing hamster hyperimmune sera for the presence of complement-fixing antibodies.

Hamsters were immunized in the manner described under Materials and methods with virus propagated in hamster brain tissue. 2 months from the time of the initial vaccination their blood was tested for complement-fixing antibodies. The results are shown in Table II.

The Russian serum, which reached a titer of 1:128 with its antigen, failed to react with either Japanese or St. Louis antigen. The Japanese serum showed a titer of 1:64 with Japanese antigen and of 1:8 with St. Louis antigen; it failed to react with Russian antigen. Finally, St. Louis serum reacted in dilutions of 1:16 and 1:2 with St. Louis and Japanese antigens respectively, while it elicited no reaction with Russian antigen.

Neutralization Tests. Mouse Hyperimmune Sera.—Tests for neutralizing antibodies were carried out on the same samples of mouse sera as were used for the complement fixation tests described in Experiment 1. The sera from the 140th day bleeding were omitted because it was felt that no pertinent information would be gained from them. Due to the nature and size of the experiment, the neutralization tests could not be done under conditions parallel to those observed for the complement fixation tests, that is, testing simultaneously all sera drawn on a given date for neutralizing antibodies against all the viruses. Rather a number of separate tests had to be performed in which several sera—from seven to fourteen—including normal mouse control sera were tested for neutralizing antibodies against one virus. Hence the results presented in Table III constitute a summary of twelve different tests. Since the titer shown by a given virus in the presence of normal control serum in the different tests did not vary beyond a tenfold range, the various tests have been tabulated together. The technique for the neutralization tests, as well as the method used in evaluating results, has been described earlier in the paper.

Table III is a summary of all the neutralization tests. The figures express the neutralization indices of each serum against each virus. No detailed description of the table appears necessary, but the following facts emerge.

With the exception of the W.E.E. sera, all sera tested gave a low homologous protection. The protection shown by the W.E.E. sera was specific in all instances. Russian serum, which had a low index at the time of the first bleeding, subsequently developed increasing protection against Russian virus. Only at the time the Russian serum showed its highest index against Russian virus, did it protect against louping ill virus, and then only weakly. No protection was given by Russian serum against any of the remaining viruses. Louping ill serum produced, in general, a good protective effect against louping ill virus and weak protection against Russian virus; it did not protect against the remaining viruses tested. Japanese immune serum was positive for Japanese virus on only one occasion, when the neutralization index reached 1000. It failed to protect against St. Louis virus. The 10th day sample which lacked protective effect against Japanese virus showed a low but definite protection against both Russian and West Nile viruses. Mice immunized against St. Louis virus were slow in developing neutralizing antibodies against the homologous antigen and when antibodies did finally appear their level was low—neutralization index of 100. St. Louis serum gave no protection against

Japanese virus and in a single instance showed weak protection against West Nile virus. West Nile serum gave moderate, though consistent, protection against the homologous virus. In one instance the 25th day sample gave slight protection against Russian virus. West Nile serum did not protect at all against either Japanese B or St. Louis virus in our tests.

TABLE III

Neutralization Tests with Mouse Hyperimmune Sera

Hyperimmune mice		Neutralization index of serum against the viruses of:					
Day of bleeding	Serum	Russian encephalitis	Louping ill	Japanese B encephalitis	St. Louis encephalitis	West Nile encephalitis	W.E.E.
10th	Russian encephalitis	**100**	2	1	1	17	—
	Louping ill	35	**800**	1	1	25	3
	Japanese B encephalitis	**50**	−1	32	2	**80**	—
	St. Louis encephalitis	5	−1	7	22	**50**	2
	West Nile encephalitis	17	1	8	−1	**150**	1
	W.E.E.	12	−1	1	−1	3	**8,500**
25th	Russian encephalitis	**650**	21	7	5	9	2
	Louping ill	**90**	**60**	7	−1	2	−4
	Japanese B encephalitis	13	2	**1,000**	1	4	−4
	St. Louis encephalitis	−2	−3	5	22	5	−2
	West Nile encephalitis	**60**	−3	13	22	**300**	−8
	W.E.E.	29	−5	7	2	−1	**18,600**
50th	Russian encephalitis	**2,200**	**100**	5	2	3	2
	Louping ill	**200**	**1,000**	1	1	−1	4
	Japanese B encephalitis	15	1	7	3	3	4
	St. Louis encephalitis	40	3	3	**100**	1	2
	West Nile encephalitis	2	−2	10	5	**450**	3
	W.E.E.	8	1	1	5	4	**13,500**

See description in text.
— = not tested.

The results in Table III lend only partial support to the conclusions drawn from the results of complement fixation tests shown in Table I. It is true that the neutralization tests pointed to the existence of some relationship between the Russian and louping ill viruses, but its extent was not as marked as that which was found by means of complement fixation tests. On the other hand, the neutralization tests with mouse sera gave no indication of a relationship among the viruses of Japanese, West Nile, and St. Louis encephalitis. In the case of W.E.E. virus, neutralization as well as complement fixation tests showed that it was wholly unrelated to the other viruses tested.

Hamster and Guinea Pig Hyperimmune Sera.—Since the levels of neutraliz-

ing antibodies in mouse hyperimmune sera were in general low, thus probably precluding the detection of relationships among different viruses, individuals from other animal species were hyperimmunized and their sera tested for neutralizing antibodies in the hope that higher homologous protection would be obtained.

Hamsters and guinea pigs were hyperimmunized in a manner already described, the hamsters with infected hamster brain tissue and guinea pigs with infected mouse brain tissue. No attempt was made to study the development of neutralizing antibodies over a prolonged period; rather we aimed to obtain sera of high homologous titer whose protective power against heterologous viruses could be tested. Hamsters were immunized against Russian, Japanese,

TABLE IV

Neutralization Tests with Hamster and Guinea Pig Hyperimmune Sera

Hyperimmune serum	Neutralization index of serum against the viruses of:					
	Russian encephalitis	Louping ill	Japanese B encephalitis	St. Louis encephalitis	West Nile encephalitis	W.E.E.
Russian encephalitis, guinea pig	1,700	1,000	5	−2	7	70
Louping ill, guinea pig	100	2,100	5	−3	—	7
West Nile encephalitis, guinea pig	−2	—	300	−1	1,000	5
W.E.E., guinea pig	3	4	1	−1	6	20,000
Russian encephalitis, hamster	2,100	2,100	10	3	—	1
Japanese B encephalitis, hamster	5	2	1,000	70	150	6
St. Louis encephalitis, hamster	2	28	14	22,000	70	1

See description in text.

— = not tested.

and St. Louis viruses; guinea pigs against Russian, louping ill, Japanese, St. Louis, West Nile, and W.E.E. viruses. Sera were tested on several occasions for neutralizing antibodies against the different viruses, with consistent results. Table IV presents a summary of a number of tests.

The neutralization indices shown in Table IV demonstrate the following points: Russian guinea pig and hamster hyperimmune sera protected equally well against Russian and louping ill viruses, with indices from 1000 to 2100. The Russian sera, however, did not protect against any of the other viruses tested. Louping ill guinea pig immune serum which gave good protection (index of 2100) against louping ill virus, showed a lower yet significant protection (index of 100) against Russian virus. This serum protected against no other virus. W.E.E. guinea pig immune serum showed high and specific protection against W.E.E. virus, with a neutralization index of 20,000. Japanese hamster serum showed a protective index of 1000 against Japanese virus, of 150 against West Nile, and of 70 against St. Louis virus. The last two indices,

although low, are within the significant range. However, Japanese serum failed to protect against any of the remaining viruses. St. Louis hamster hyperimmune serum which showed strong protection against St. Louis virus, with an index of 22,000, gave only weak protection against West Nile virus and none against the other viruses. Finally, a West Nile guinea pig immune serum protected well against West Nile virus, with an index of 1000, gave only slight protection against Japanese virus, with an index of 300, and none against the remaining viruses.

These observations on guinea pig and hamster immune sera showed (*a*) that the Russian and louping ill viruses were closely related to each other but were unrelated to the other viruses studied; (*b*) Japanese, St. Louis, and West Nile viruses were slightly related as follows: Japanese hyperimmune serum protected mildly against West Nile and St. Louis viruses; St. Louis serum gave some protection against West Nile and none against Japanese virus, and West Nile serum gave weak protection against the Japanese but none against St. Louis virus. These three viruses showed no crossing with either Russian, louping ill, or W.E.E. viruses in neutralization tests. (*c*) Finally, W.E.E. immune serum did not protect against any except its homologous virus.

Cross-Resistance Tests.—The final step in our comparative study of central nervous system viruses was the determination of cross-protection afforded by vaccination. Two methods were used for testing resistance after vaccination, namely, infection by intracerebral and by intraperitoneal routes. Swiss mice, regardless of age, were highly susceptible to intracerebral injection of all viruses studied. In this respect the intracerebral method was the one of choice, even though slight degrees of resistance may be missed because the test is so rigorous. Since protection against peripheral infection was more easily achieved, additional vaccinated mice were tested by this route. However, because of the low susceptibility of W-Swiss mice to some of the viruses, especially St. Louis, given by this route, the intraperitoneal test too is limited. As indicated by the results reported, the cross-resistance tests brought out the existence of a close relationship between Russian and louping ill viruses and thus confirmed the results obtained in the complement fixation and neutralization tests. On the other hand, no relationship was found among Japanese, St. Louis, and West Nile viruses, in contradistinction to results shown by both complement fixation and neutralization tests.

Intracerebral Resistance Tests.—

Experiment 2.—This experiment consisted of six separate tests, one for each virus. For each test, groups of W-Swiss mice from 50 to 60 days of age were divided into seven batches of from twenty-five to forty mice. These batches were vaccinated as follows: No. 1, with Russian virus; No. 2, with louping ill; No. 3, with Japanese; No. 4, with St. Louis; No. 5, with West Nile; and No. 6, with W.E.E. virus; the seventh

batch were left unvaccinated as controls. Details of the vaccination procedure have already been given. About 35 days from the start of vaccination, each group of mice were injected intracerebrally with one of the six viruses.

TABLE V

Cross-Resistance Test. Mice Vaccinated with Different Viruses and Tested for Intracerebral Resistance to West Nile Virus

Mice vaccinated with:	Fate of mice following injection of virus in dilution:									Titer of virus in vaccinated or control mice	Resistance index
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
Russian encephalitis			5/5*	5/5	5/5	5/5	0/5	0/5		7.50	5
Louping ill			5/5	5/5	5/5	5/5	2/5	0/5		7.84	2
Japanese B encephalitis	5/5	5/5	5/5	5/5	5/5	4/5	0/5	0/5		7.38	6
St. Louis encephalitis	5/5	5/5	5/5	5/5	5/5	4/5	0/5	1/5		7.47	5
West Nile encephalitis	0/5	0/5	0/5	2/5	0/5	0/5	0/5			<2.00	1,500,000
W.E.E.			5/5	5/5	5/5	4/5	3/5	0/5		8.00	1
Unvaccinated controls					5/5	5/5	3/5	0/5	0/5	8.17	

* 5/5 = five out of five mice died following injection.

TABLE VI

Cross-Resistance Test. Mice Vaccinated with Different Viruses and Tested for Heterologous Resistance by Intracerebral Inoculation

Mice vaccinated with:	Resistance index of vaccinated mice against viruses of:					
	Russian encephalitis	Louping ill	Japanese B encephalitis	St. Louis encephalitis	West Nile encephalitis	W.E.E.
Russian encephalitis	1,100,000	18	1	5	5	1
Louping ill	39	240,000	1	5	2	1
Japanese B encephalitis	−1	5	420,000	7	6	2
St. Louis encephalitis	−2	<8	−2	10,000,000	5	2
West Nile encephalitis	−1	<8	1	2	1,500,000	2
W.E.E.	−4	<8	2	3	1	15,000,000

See description in text.

The complete results of one of the tests are given in Table V; they show that only the mice vaccinated with West Nile virus were protected against intracerebral infection with West Nile virus, the titer of the resistance index being 1,500,000. None of the other vaccinated mice showed any resistance against West Nile virus given intracerebrally.

Tests similar to that with the West Nile virus were carried out with the other viruses under study and the results obtained were similar to those reported in Table V. They have been summarized in Table VI.

The data show that in each case a specific immunity was achieved with

high-titer protection, while in no instance was there any indication of cross-resistance. Even the Russian and louping ill viruses which were found to be closely related in all the other tests did not exhibit here any indication of crossing.

Intraperitoneal Cross-Protection Tests.—

Experiment 3.—This experiment was done along lines similar to those followed in Experiment 2. Experiment 3 was composed of six different tests, one for each virus. In each test groups of W-Swiss mice, 20 to 25 days old, were divided into seven batches, each containing between twenty and thirty-two mice. These batches were vaccinated against Russian, louping ill, Japanese B, St. Louis, West Nile, and W.E.E.

TABLE VII

Cross-Resistance Test. Resistance of Mice Vaccinated with Different Viruses to Intraperitoneal Infection with Japanese B Encephalitis Virus

Mice vaccinated with:	Fate of mice following injection of virus in dilution:							Titer of virus in vaccinated and control mice	Resistance index
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Russian encephalitis		4/4	0/4	0/4	3/4	1/4		3.00	10
Louping ill		4/4	1/4	2/4	1/4	1/4	1/4	4.00	1
Japanese B encephalitis	4/4	0/4	1/4	0/4	0/4			1.63	250
St. Louis encephalitis		4/4	2/4	3/4	0/4	3/4		4.45	−3
West Nile encephalitis		4/4	0/4	2/4	0/4	1/4		2.87	14
W.E.E.		4/4	3/4	0/4	2/4	2/4	2/4	4.50	−3
Controls		4/4	2/4	2/4	1/4	1/4	1/4	4.00	

Footnote as in Table V.

viruses respectively; the seventh batch were left untreated as controls. The vaccine employed and the course of vaccination have been described under Materials and methods. 2 weeks after vaccination the mice were infected by intraperitoneal injection of 0.5 cc. of each virus in tenfold dilutions.

Table VII gives the results of one of the tests, that with Japanese B virus. Unlike the reaction following infection by the intracerebral route, intraperitoneal infection produced, in general, irregular titrations not only in the mice of the vaccinated groups but also in those of the control groups. The test with the Japanese virus illustrates this point well: In the control group, all mice given the 10^{-2} dilution died; then, from the 10^{-3} to the 10^{-7} dilutions there was a scattering of deaths, making the determination of the 50 per cent endpoint of mortality difficult. The method of Reed and Muench was followed in all cases, even though the significance of the 50 per cent mortality titers and consequently that of the resistance index may be of doubtful absolute value because of the

irregularity in the titrations. Still, as an indicator of trends these numerical values are significant.

Table VIII summarizes the results of all the intraperitoneal cross-resistance tests. The outcome of that with the St. Louis virus proved valueless because the mice were not sufficiently susceptible to intraperitoneal infection to permit a titration. In the control group, only the mice given the 10^{-1} dilution died, while only one of four given the 10^{-2} dilution died. The table shows that with the viruses of W.E.E., West Nile, and Japanese B a specific resistance was produced with a resistance index of 10,000 plus, 450 plus, and 250 respectively, and that there was no trace of crossing. The Russian and louping ill viruses showed a high degree of homologous resistance with titers of 32,000,000 and

TABLE VIII

Cross-Resistance Test. Mice Vaccinated with Different Viruses and Tested for Heterologous Resistance by Intraperitoneal Inoculation

Mice vaccinated with:	Resistance index of mice against viruses of:					
	Russian encephalitis	Louping ill	Japanese B encephalitis	St. Louis encephalitis	West Nile encephalitis	W.E.E.
Russian encephalitis	32,000,000	300	10	1	−1	−40
Louping ill	320,000	10,000	1	1	1	1
Japanese B encephalitis	<10	−1	250	−7	1	−3
St. Louis encephalitis	<10	−6	−3	2	1	−32
West Nile encephalitis	<10	1	14	1	>450	−16
W.E.E.	<10	−32	−3	2	−2	>10,000

See description in text.

10,000 respectively, and a considerable amount of crossing. Such a result had been obtained previously (11). However, neither Russian nor louping ill virus crossed with the other viruses tested.

The outcome of the intraperitoneal cross-protection tests can best be summarized as follows: Russian and louping ill viruses showed a considerable degree of cross-resistance, while they failed to exhibit any for the remaining viruses; W.E.E. virus gave a high specific immunity and showed no crossing with the other viruses studied; Japanese B and West Nile viruses showed only a moderate amount of homologous resistance. Although in our tests these two viruses elicited no cross-reaction when tested against the other viruses, it should be borne in mind that had the amount of homologous resistance been higher, heterologous protection might have appeared. Finally, the test with St. Louis virus was without significance on account of the low susceptibility of W-Swiss mice to our strain of this virus.

DISCUSSION

The present experiments and others reported in the literature indicate that there are antigenic constituents common to two or more of the viruses studied in the present work, the existence of which allows a grouping based on immunological reactions. In the case of the St. Louis, Japanese B, and West Nile encephalitis viruses, the amount of common antigen is probably low, since immunological reactions amongst the three are not elicited readily. On the other hand, it appears that louping ill and Russian spring-summer encephalitis viruses have a closely connected antigenic composition, since a relationship between them has been demonstrated under many different conditions.

The fact that two or more of the viruses have been found to possess common antigens may, in certain situations, make a diagnosis of human cases of encephalitis difficult; for example, if both louping ill and Russian viruses are implicated in a laboratory infection. In general, however, the fact that the homologous reactions are stronger than the heterologous, when added to the circumstances of place and season, should simplify a specific diagnosis of the natural infection.

The present studies on the relation between six central nervous system viruses do not by any means exhaust the field. Other known viruses may be related to the ones studied here, and it may be found that new viruses will fit into the groups. Moreoever, it is possible that the number of instances in which viruses are shown to be antigenically related amongst themselves will prove to be greater than in the case of bacteria, since the smaller size and probably simpler composition of the former should provide more opportunities for the same structure to repeat itself in different viruses.

SUMMARY AND CONCLUSIONS

From observations carried out with the viruses of Russian spring-summer encephalitis, louping ill, W.E.E., and the Japanese B, St. Louis, and West Nile types of encephalitis, the following facts and inferences have been derived.

1. Russian encephalitis and louping ill viruses showed a close relationship by complement fixation, neutralization, and intraperitoneal cross-resistance tests. Intracerebral cross-resistance tests, on the other hand, failed to reveal any connection between them. Neither Russian nor louping ill virus appeared to be related to the remaining viruses tested.

2. Japanese B, St. Louis, and West Nile types of encephalitis, as a group, showed a certain degree of group relationship, but it was not so close as that between the Russian and louping ill viruses. In complement fixation tests, besides homologous reactions, Japanese serum brought about reactions with both St. Louis and West Nile antigens; St. Louis serum reacted with Japanese antigen, and West Nile serum with Japanese antigen. In neutralization

tests with mouse sera, no relationship was found amongst these three viruses, while in similar tests with either hamster or guinea pig serum,—which gave higher homologous titers,—it was found that Japanese serum protected against West Nile and St. Louis viruses, St. Louis serum protected against West Nile virus, and West Nile serum against Japanese virus. In intracerebral and intraperitoneal cross-resistance tests, no relationship was found to exist between these three viruses. Moreover, the Japanese B, St. Louis, and West Nile viruses appeared to be unrelated to any one of the three other viruses tested.

3. W.E.E. virus stood apart in all tests as unrelated to any of the other viruses studied.

4. The homologous titers of complement-fixing antibodies in mouse sera showed a gradual decline with the passing of time after vaccination, and this loss of homologous titer was paralleled by a similar drop in the titer of the heterologous reactions. In the case of the Japanese B, St. Louis, and West Nile viruses, with which at the start the amount of crossing was not high, a point was reached beyond which heterologous reactions could no longer be detected.

5. A comparison of the specific levels of complement-fixing and neutralizing antibodies for the viruses in mouse hyperimmune sera showed their rate of persistence to differ. Complement-fixing antibodies which had highest titers on the 10th day, diminished gradually until, when tested on the 50th day, all titers had reached levels from one-fourth to one-eighth of their values on the 10th day. On the other hand, the levels of neutralizing antibodies for the same samples of serum were, on the 50th day, as high as or higher than those found on either the 25th or 10th days, save in the case of the Japanese B virus.

6. The state of immunity of animals following vaccination with the viruses discussed was found to be different at a given time, depending on the method employed to determine it. Thus, mice vaccinated with St. Louis virus had positive complement-fixing antibodies on the 10th day and no neutralizing antibodies. The state of immunity changed in the course of time. For this reason it is felt that in order to detect whether two viruses are related or not, multiple observations are necessary, over a considerable time and employing all available methods of immune comparison.

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