Attempts to Immunize Hamsters to West Nile Virus; Passive, Passive-Active and Active Methods.* (23104)

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As outlined previously(1,2) we have planned to test in laboratory animals the effectiveness of immunization against Japanese B (JBE), St. Louis (SLE), and Murray Valley (MVE) encephalitis viruses by primary immunization with living West Nile (WN) virus supplemented with an inactivated virus vaccine of one of the other types. The hamster proved to be a suitable host for test challenge by a peripheral route following immunizing procedures, thus simulating naturally acquired infection from a mosquito bite (2). Susceptibility of the hamster to MVE and WN was adequate without any modifying procedures(1,3), but to obtain adequate susceptibility for JBE and SLE challenges it was necessary to treat the hamsters with cortisone(2). Thus, an animal species other than man was available for suitable quantitative challenge after immunizing procedures. Monkeys, mice and other laboratory animals were not suitable.

The next problem was a method of producing the desired basic immunization with living WN virus, since hamsters are extremely susceptible to peripheral injection of this virus. High dilutions of WN virus were first employed to determine whether a dosage could be found which would produce an inapparent, immunizing infection. This was found to be impractical, for dosage-range between a fatal and an inapparent infection was entirely too narrow. Passive-active immunization was next tried and the unexpected results of both passive and passive-active immunization experience are reported here, together with the more expected type of results from use of a formalinized vaccine preparation. Also included in this paper are studies on effect of cortisone injections subsequent to immunization, on immunity previously acquired.

Materials and methods. A strain of WN virus obtained a number of years ago through the courtesy of Dr. Smithburn was used in its 30th mouse passage, prepared as a pool of frozen brain suspension. This pool had a titer of $10^{-8.6}$ when tested intracerebrally in mice and $10^{-8.5}$ subcutaneously in hamsters. Syrian hamsters from Lakeview Hamster Colony, N. J., were used when 8 to 10 weeks of age for the beginning of immunization procedures. Antisera were prepared in rabbits and in hamsters and their neutralization indices determined by the intracerebral mouse neutralization test(4). All sera were injected intraperitoneally, suitably diluted in a 0.5 cc volume in the hamsters to be protected. The selected virus dilution was given subcutaneously in a volume of 0.1 cc. Vaccine was prepared by mixing a freshly prepared 20% infected mouse brain suspension with an equal volume of 0.4% formalin. The mixture was kept at room temperature for a week with frequent agitation, then kept at +4°C in the refrigerator. A test to demonstrate the absence of detectable live virus was then made by intracerebral inoculation of mice, undiluted and at 10^{-1} after neutralization of the formalin.

Exp. 1. Passive-active immunization using one dose of WN immune rabbit serum. Immune rabbit serum with a neutralization index of 15,000 was employed in 5-fold serial dilutions ranging from 1:5 through 1:625. Each dilution was inoculated into each of a group of 6 hamsters. Twenty-four hours after serum inoculation each ham-

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TABLE I. Response to Subcutaneous Injection with 100 LD_{50} of WN Virus after Passive Immunization with WN Hyperimmune Rabbit Serum.

Serum dilution	Days of death	Mortality ratio
1: 5	27, 29	2/6
1: 25	14, 14, 16, 16	4/6
1: 50	15, 15, 15, 17, 17, 18	6/6
1: 75	9, 11, 12, 14, 14, 15	6/6
1:100	9, 10, 10, 10, 11, 12	6/6
1:125	9, 10, 10, 11, 11, 12	6/6
1:625	6, 8, 9, 9, 9, 10	6/6
Control	6, 7, 8, 8, 9, 10	6/6

ster was inoculated subcutaneously with 100 hamster LD_{50} of WN virus. An additional group of 6 hamsters was inoculated with the virus only. Five weeks after the virus inoculation the surviving hamsters were challenged subcutaneously with 100 LD_{50} of WN virus.

There was some degree of protection against the 1st injection of live virus in the group of hamsters which received the 1:5 dilution of serum and a small degree of protection in the group which received the 1:25 dilution, while there was no reduction of fatality in the other groups. (Table I) However, the immune serum prolonged the interval before death, and the greater the concentration of serum the more prolonged the period. This prolongation of life is observed even with the 1:125 serum dilution. Live virus challenge after 5 weeks, of the hamsters surviving from the 1:5 and 1:25 serum dilutions, gave the following results: 2 out of 4 hamsters died from the 1:5 serum and 0 of 2 died from the 1:25 group. This method did not appear to offer encouragement for producing passive-active immunization but it was felt that if a 2nd dose of serum was given at a suitable interval, many hamsters might survive among those whose lives were only prolonged in this experiment.

Exp. 2. Passive-active immunization using 2 doses of WN immune rabbit serum. Three groups of 5 hamsters each were treated in the following manner: In Group I each hamster received on the 1st day a 1:25 dilution of the same immune serum used in Exp. 1. On the 2nd day each was inoculated with 100 LD_{50} of WN virus as in the previous experiment. In Group II each hamster received serum and virus in a quantity and manner identical with Group I, but in addition was given another dose of serum in a 1:25 dilution 1 week after the virus inoculation. In Group III each hamster received virus only.

TABLE II. Response to Subcutaneous Injection with 100 LD_{50} of WN Virus after Passive Immunization with One and 2 Doses of WN Hyperimmune Rabbit Serum Diluted 1:25.

Serum doses	Days of death	Mortality ratio
1	15, 18, 18, 19	4/5
2	12, 12, 13, 20	4/5
0	8, 9, 9, 10, 10	5/5

This method of 2 doses of WN hyperimmune serum also failed to provide suitable passive-active immunization (Table II). The 2 surviving animals were not challenged. It was considered possible that the cause of failure of these methods was due to the rapid destruction and excretion of heterologous serum. WN hyperimmune hamster serum was then prepared in the hope of getting better results because of its lower rate of excretion.

Exp. 3. Passive-active immunization using WN immune hamster serum and a 10^{-1} dilution of virus. This experiment was done in a manner similar to that of Exp. 1, with an exception which was an oversight. They were inoculated with 0.1 cc of a 10^{-1} dilution of WN virus instead of a 100 LD_{50} dilution. Observation was continued in spite of this fact. WN hamster immune serum with a neutralization index of 870 was used. The serum dilutions employed are shown in Table III. Each group was composed of 10 animals. Three hamsters from each group were bled out just before virus inoculation of the remainder and these sera were titrated for WN neutralizing antibodies using the intracerebral mouse test. From the table it can be seen that the results in respect to survival were not

TABLE III. Response to Subcutaneous Challenge with 0.1 ml of 10^{-1} WN Virus after Passive Immunization with WN Immune Hamster Serum.

Serum dilution	Days of death	Mortality ratio
1: 5	9, 12, 15, 16	4/7
1:25	7, 9, 10, 10, 16	5/7
1:50	9, 10, 11, 11, 14	5/7
Control	8, 8, 9, 9, 9, 10, 10	7/7

TABLE IV. Log Neutralization Index* to WN Virus in Sera of Hamsters Sacrificed 24 Hr after Intraperitoneal Inoculation of Hamster Immune Serum.

Serum dilution	Log neutralization index: Hamster 1	Hamster 2	Hamster 3	Mean
1: 5	.9	1.0	2.0	1.3
1:25	.8	.8	1.1	.9
1:50	.3	.9	.7	.6

* Intracer. mouse test.

more encouraging than those of the earlier experiments using rabbit serum. It was thought that this result was quite possibly due to the use of a high concentration of virus. Results on serum neutralization tests (Table IV) suggest that antibody was present in the blood at a detectable level 24 hours after immune serum inoculation of the 1:5 and the 1:25 dilutions. The very few survivors (Table III), when subsequently challenged with 1000 hamster LD_{50} of WN virus 4 weeks after the 1st virus inoculation, were found to be resistant to disease.

Exp. 4. Passive-active immunization using one dose of WN immune hamster serum and 100 LD_{50} of virus. This experiment was done in a manner similar to the previous experiment, with the exception that 100 hamster LD_{50} of WN virus were used for inoculation. The serum dilutions employed are shown in Table V. Each group was composed of 10 animals.

The results of this experiment were no more encouraging than those of the earlier experiments. There were only 8 survivors, simply a slight prolongation of life in most instances. These remaining 8 hamsters survived when challenged with 1000 hamster LD_{50} WN virus 4 weeks after the 1st virus inoculation. From the results of the different experiments on passive-active immunization it was found that this method of immunization was impractical as a method of obtaining large numbers of surviving immune animals and active immunization of hamsters was tried.

TABLE V. Response to Subcutaneous Inoculation with 100 LD_{50} WN Virus after Passive Immunization with WN Immune Hamster Serum.

Serum dilution	Days of death	Mortality ratio
1:10	10, 11, 12, 13, 13, 13, 14	7/10
1:20	9, 9, 10, 10, 10, 11, 13, 13	8/10
1:30	9, 10, 10, 10, 10, 11, 11, 13, 13	9/10
1:40	7, 7, 7, 8, 9, 9, 13, 13	8/10
Control	7, 7, 7, 8, 8, 8, 9, 9, 10, 10	10/10

Exp. 5. Active immunization of hamsters to WN virus. Two groups of hamsters were employed. The 1st group received 1 injection of 0.5 cc of WN formalin inactivated vaccine intramuscularly, the 2nd group received 2 injections each of 0.5 cc of vaccine intramuscularly 2 weeks apart. Three weeks after the last vaccination or after the 1st in those receiving only 1 dose, the hamsters were inoculated subcutaneously with 100 subcutaneous hamster LD_{50} of WN virus to provide immunization with active virus in a manner which, it was hoped, would lead to survival of most animals.

From Table VI it can be seen that 2 doses of vaccine gave complete protection to 100 LD_{50} hamster doses of WN virus. Hamsters immunized with 1 dose of vaccine had a lower survival rate.

In a later experiment(5) in which 500 hamsters were basically immunized to WN virus, to be challenged later with JBE virus, the hamsters were inoculated each with only 1 dose of 0.5 cc of another lot of similarly prepared WN vaccine. One week later each hamster was given 100 hamster LD_{50} of WN virus subcutaneously. Only 5 out of the 500 hamsters died, *i.e.*, about 1% deaths. Serological tests done on a pool of sera of 5 of the surviving hamsters showed a complement fixing titer of 1:16 and a neutralization index of 1.9.

Exp. 6. Effect of cortisone on pre-existing active immunity. Fourteen hamsters were actively immunized to WN virus by 2 injections of WN vaccine given 1 week apart. Two weeks after the last injection each hamster was inoculated subcutaneously with 1000

TABLE VI. Response of Hamsters Immunized with One and 2 Injections of WN Vaccine and Inoculated Subcutaneously with 100 LD_{50} Hamster Doses of WN Virus.

Vaccine doses	Mortality ratio
1	3/10
2	0/10
0	5/ 5

TABLE VII. Effect of Cortisone on Previously Acquired Active Immunity of Hamsters.

Immunization	Cortisone treatment	WN virus inoc.	Mortality ratio
WN	Cortisone	Virus	1/4
WN	None	"	0/4
None	Cortisone	None	1/4
"	None	Virus	4/4

hamster LD_{50} of WN virus, and 2 weeks later each was inoculated with 10,000 hamster LD_{50} of the virus. These hamsters were divided into 2 groups of 7 each. Three hamsters were bled out from each group and their sera pooled and titrated to determine the neutralization index to WN virus. The 1st group of 4 remaining hamsters was treated with cortisone in a dose of 0.25 mg./g body weight (intentionally larger than the standard dose) given over 4 days and challenged subcutaneously with 300,000,000 hamster LD_{50} (0.1 cc of 10^{-1} dilution) of WN virus on the 2nd day of the experiment in a manner previously described(2). The 2nd group of 4 remaining animals was challenged with virus only. A control group of 4 non-immune hamsters was treated with the same dose of cortisone only, and a 2nd control group of 4 normal hamsters was inoculated with the same dose of the virus only. The hamsters were watched for a period of 3 weeks after challenge and then the surviving ones were bled and their sera were titrated separately for neutralizing antibodies.

Only 1 hamster from the immunized group which received cortisone died on the 6th day after virus inoculation. (Table VII). The autopsy done on that animal showed that the liver was mottled and friable and the spleen had many septic nodules. Culture of liver, spleen and blood on blood agar plates revealed hemolytic streptococci. The brain suspension of that hamster, after penicillin and streptomycin were added, was negative for bacterial growth and negative for virus when inoculated intracerebrally in mice. It was thus clear that this hamster did not die from the WN virus, but from bacterial infection, probably due to the high dose of cortisone given. Virus control animals all died as expected between the 7th and the 9th days. One cortisone control animal died of sepsis as in the immunized group. Thus there was no evidence of cortisone having interfered with the pre-existent immunity of the immunized group. Results of neutralization tests (Table VIII) reveal that there was no significant difference in the level of neutralizing antibodies between the cortisone treated and the non-cortisone treated immune groups post challenge. In fact, since there was no significant antibody rise in either group 3 weeks after challenge, it appears that all the hamsters were probably immune to the extent of resisting infection as well as disease.

Discussion. In an attempt to immunize hamsters to WN virus, both passive-active and active methods of immunization were tried. In passive-active immunization WN rabbit immune serum was first tried in 1 dose. Results were unsatisfactory in that there were only a few survivors even with the least diluted serum. In the higher dilutions only the incubation period was prolonged. Some hamsters surviving this method of immunization were found not to be immune when challenged later with live WN virus. In the case of the lowest serum dilution the serum apparently neutralized the virus and no infection took place; thus, at the end of the immunization period the hamsters were not immune. In the next higher serum dilution the small percentage of surviving hamsters was found later to be immune to live WN virus. The low survival rate during attempted active-passive immunization was first attributed to the fact that heterologous serum is rapidly destroyed and excreted. Two doses of serum were then employed, but this also was found to be unsatisfactory. Homologous serum was used

TABLE VIII. Effect of Cortisone on the Titer of Neutralizing Antibodies in Sera of Actively Immunized Hamsters after Virus Challenge.

	Log neutralization index*					
Cortisone treatment	Pre-challenge pool	Hamster No. 1	2	3	4	Mean†
Yes	3.1	2.8	3.6	3.0	—‡	3.1
No	3.1	3.3	3.2	3.2	3.7	3.3

* Intracer. mouse test.
† Geometric mean.
‡ This animal died from sepsis.

with the same poor results in so far as obtaining large numbers of survivors. However, in the dosages of virus and serum employed the few survivors did resist subsequent virus challenge.

In active immunization better results were obtained especially when 2 doses of formalin inactivated virus vaccine were used, followed 3 weeks after the last dose by 100 hamster LD_{50} of WN virus. Later(5), using 500 hamsters, it was found that even 1 dose of another lot of WN vaccine followed 1 week later by 100 hamster LD_{50} of WN virus provided a rapid and satisfactory method of immunization of hamsters to WN virus, with only 1% mortality during the procedure.

Since the principal purpose of this work was to prepare animals for subsequent subcutaneous challenge with certain other group B encephalitis viruses such as JBE and SLE which required cortisone treatment to render them susceptible, it appeared necessary to investigate the effect of cortisone on any preexisting actively acquired immunity. Kilbourne(6), working with influenza virus, demonstrated that mice with previously existing actively acquired immunity, when challenged with active virus and given large amounts of cortisone were rendered somewhat less immune. Even with smaller doses of cortisone, although most of the challenged mice survived, virus was found to persist in the lungs for 7 days while none was found in the lungs of those not receiving cortisone. Our tests with a small group of actively immunized hamsters treated over 4 days with such large doses of cortisone that 1 out of 4 from each of 2 groups died of bacterial sepsis, and challenged with very large doses of virus ($10^{8.5}$ LD_{50} when administered by the same route in normal hamsters) gave evidence of complete protection against the virus. Possibly, if less completely immunized animals had been used some quantitative differences might have become apparent. Further evidence indicating that the animals were even protected against inapparent infection from the large virus challenge together with cortisone is indicated by finding that there was no significant rise of neutralizing antibodies 3 weeks after virus was given in either the normal or cortisone treated animals. We did not test for persistence of virus as did Kilbourne. It is possible that our test demonstrating a lack of antibody rise should not be interpreted as lack of infection for cortisone would suppress this temporarily. However, we found in other work(2) that suppression of antibody rise by cortisone was not complete and did not appear to persist for long. Thus, since no rise was observed after 3 weeks, we believe it probable that infection was actually prevented.

Summary. Both passive-active and active methods of immunization of hamsters to WN virus were tried. The passive-active method gave very poor results; the active method gave satisfactory results. A procedure in which the hamsters are given 1 or 2 doses of 0.5 cc of freshly prepared WN vaccine, followed 1 week later by 100 hamster LD_{50} of WN virus, produced a satisfactory and rapid method of immunization. Hamsters immunized by vaccine plus active virus, when given large doses of cortisone and challenged with $10^{8.5}$ LD_{50} of virus survived. These hamsters and similar immunized control animals not given cortisone had all failed to develop increased neutralizing antibody titers 3 weeks following the virus challenge. These results are interpreted to indicate that cortisone probably did not substantially reduce previously acquired immunity to disease or to infection.

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