INFLUENCE OF AGE ON SUSCEPTIBILITY AND ON IMMUNE RESPONSE OF MICE TO EASTERN EQUINE ENCEPHALOMYELITIS VIRUS

By ISABEL M. MORGAN, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, May 15, 1941)

In the summer of 1938 an outbreak of encephalomyelitis in man in the vicinity of Massachusetts was proved to be due to the virus of equine encephalomyelitis, Eastern strain (1). An epidemic of encephalomyelitis in horses had appeared a month before and continued during the human outbreak (2). It was thought, although not proved, that the virus was transmitted by mosquito vectors (3). The age distribution indicated that the highest incidence was among the youngest children. Thus, more than a third of the thirty-eight recognized cases were in children under 2 years of age, half under 5 years, and more than two-thirds under 10 years of age (2). This age distribution is of special interest in connection with previous reports of an increasing resistance with age of animals to peripheral injection of equine encephalomyelitis virus as well as of certain other neurotropic viruses. In each case cerebral susceptibility was found not to be influenced by age.

Kleine (4 *a*) reported that young geese are susceptible to subcutaneous and intramuscular injection of fowl plague virus; Kraus and Schiffmann (4 *b*) found adult geese resistant to the virus introduced by these peripheral routes. However, both young and adult animals are susceptible to subdural inoculation of active virus. Andervont (5) noted that although young and old mice are equally susceptible to intracerebral injection of herpes virus, adult mice are no longer susceptible to a peripheral injection of virus to which young mice succumb. Theiler (6) found a similar change in susceptibility with age of mice to yellow fever virus. Olitsky and his collaborators (7, 8 *a*) observed an increasing resistance of growing animals to peripheral injection of the viruses of vesicular stomatitis and of Western and Eastern equine encephalomyelitis, although, as with the other viruses referred to, young and old animals are equally susceptible to cerebral injection of the virus. King found differences in age susceptibility between fresh and fixed Eastern strains (9). Sabin and Olitsky (8 *a*) in a study of the pathogenesis of equine encephalomyelitis virus, observed that after intramuscular injection of active virus in young mice the virus, in most instances, is carried in the blood stream, is deposited on the nasal mucosa, and enters the brain by way of the fibrils of the olfactory nerve (*cf.* Hurst, 8 *d*). Furthermore, when the nasal mucosa has been blocked by spraying with zinc sulfate solution,

intramuscularly injected virus fails to produce encephalitis in most of the animals, but in the exceptional ones which succumb the virus is found to have entered by routes other than the olfactory. In each case, however, the virus travels along a closed nerve pathway. In a comprehensive study of vesicular stomatitis virus, Sabin and Olitsky (8 *b*) found that virus injected intramuscularly in the leg muscles of young mice multiplies locally and invades the sciatic nerve and spinal cord. In old mice, no such local multiplication occurs and, although the active virus persists, no invasion of the sciatic nerve nor of the central nervous system takes place.

Age is known to be a factor in determining also the degree of immune response of which an animal is capable.

As early as 1897, Metchnikoff (10) found that when alligators are injected with tetanus toxoid large animals respond with a higher concentration of antitoxin in their serum than small ones. Freund (11) demonstrated an increasing antibody production with increasing age of rabbits in response to a variety of antigens: formolized typhoid bacilli, sheep red cells, horse serum, and egg albumin. Baumgartner (12) presented a thorough review of the subject up to 1932. In studies on *Trypanosoma lewisi*, Culbertson and Kessler (13 *a*) observed that with increasing age of rats a greater antibody response as well as greater resistance to living trypanosomes develops; Kolodny (13 *b*) added data on a greater rate of antibody formation with age. Morgan (14) found that the ability of mice to be immunized with formolized virus of Eastern equine encephalomyelitis increases with age, as tested by cerebral resistance, as well as by degree and rate of antibody formation. Casals (15) reported diminishing susceptibility with age of mice to peripheral injection of rabies virus and an increasing immunizability with age.

The experiments to be reported here were carried out to find what relationship exists between the influence of age of mice on their susceptibility to peripheral introduction of the virus of Eastern equine encephalomyelitis and on their capacity to elicit an immune response.

Methods and Materials

Active Virus.—Mouse brain infected with the virus of Eastern equine encephalomyelitis (E.E.E.) was used in the experiments. This strain was obtained 6 years ago from Dr. C. TenBroeck and has been through more than 200 mouse brain passages in this laboratory. The Western strain of equine encephalomyelitis virus (W.E.E.) was obtained 8 years ago from Miss B. Howitt. Stock virus, dried from the frozen state by the Flosdorf-Mudd lyophile method, was kept *in vacuo* in rubber-stoppered glass vials in the refrigerator. Before use, it was passaged intracerebrally in normal mice, and the brains of at least three mice prostrate or recently dead were removed at autopsy. These were weighed and ground without abrasive; meat infusion broth at pH 7.4 was added to make a 10 per cent suspension. After light centrifugation, 5 minutes at 2,000 R.P.M., the supernate was removed. This was designated 10^{-1} E.E.E. virus; tenfold dilutions made in broth from this were then 10^{-2}, 10^{-3}, etc.

Formalin-Inactivated Virus.—To a 20 per cent broth suspension of mouse brain infected with E.E.E. virus was added an equal volume of 1 per cent formalin (containing 37 per cent formaldehyde) in 0.85 per cent NaCl solution, thus making a 10 per cent infected mouse brain suspension in 0.5 per cent formalin. The preparation was kept at room temperature in the dark for 2 days and then placed in the refrigerator at 4°C. For a vaccine to be acceptable, the virus, before the addition of formalin, must have been infective to a titer of 10^8 when injected intracerebrally into normal mice. After a week in the refrigerator, and again when first used for vaccination, the vaccine was tested intracerebrally in mice to determine whether all active virus had been destroyed. It may be stated that by the most stringent tests we have never been able to detect active virus in such a formolized preparation.

Mice.—Albino mice, Rockefeller Institute strain, were used throughout. In the stock colony, the date of birth of each litter of mice is recorded. Young of the stock mice are weaned at 21 days of age; in the course of experiments they were frequently weaned at 14 days. In the stock colony, mothers with young are fed purina fox chow pellets and given water from a water bottle. When young are weaned, a slice of bread soaked in water is added daily to the pellet diet. When mice are taken from the stock, and kept for an experiment, an equal part of pasteurized grade B milk is added to the water in which the bread is soaked. Pellets are still in the diet, but the amount needed is less. The average weight of young mice of various ages used in the following experiments is given below.

Age, *days*	2	5	7	10	13	15
Average weight, *gm*	2.0	3.5	4.9	5.5	8.5	10.6
No. of mice as basis for average weight	76	107	118	22	24	28

Routes of Injection.—Intracerebral. A short incision was made to one side of the midline in the scalp of a mouse under ether anesthesia. With a fine trephine a hole was bored through the skull in the angle formed by the sagittal and fronto-parietal sutures, about 2 mm. from each; through this 0.03 cc. dilution of virus was injected. In younger mice the trephine was not used. Accidental deaths as a result of this method of injection were very few.

Intraperitoneal. The needle was inserted subcutaneously for a short distance, and then plunged into the peritoneal cavity; on removal, the needle was gently turned to draw the skin together at the site of injection. Various doses were used in the intraperitoneal injections, but it may be mentioned that even the youngest mice used, 2 days old, could retain 0.1 cc. of injected vaccine with little loss.

Intramuscular. 0.1 cc. injections of vaccine were made into the muscles of the thigh of one hind limb. This dose was well retained.

Bleeding.—When relatively large amounts of blood were needed, anesthetized mice were bled from the heart. If it was necessary for mice to survive, they were bled by snipping off a piece of tail. Sufficient blood was obtained, even from young mice, provided they had been kept in a warm place before bleeding.

Intraperitoneal Injection of Active Eastern Equine Encephalomyelitis Virus in Mice of Various Age Groups

The susceptibility of mice of various ages to the intraperitoneal injection of active E.E.E. virus was tested. Since the last report from this laboratory (8) on susceptibility of mice to E.E.E. virus given by the peritoneal route, two changes have taken place: (1) The virus has been through a greater number of mouse brain passages, and (2) the average weight of mice of a given age has gradually increased.

Groups of mice 14 days, 22 days, 1 month, and 6 months of age were injected intraperitoneally with 0.25 cc. 10^{-3} dilution of active E.E.E. virus. The amount of virus, when tested intracerebrally in normal adult mice, proved to be 1,000,000 cerebral lethal units. The outcome is shown in Fig. 1.

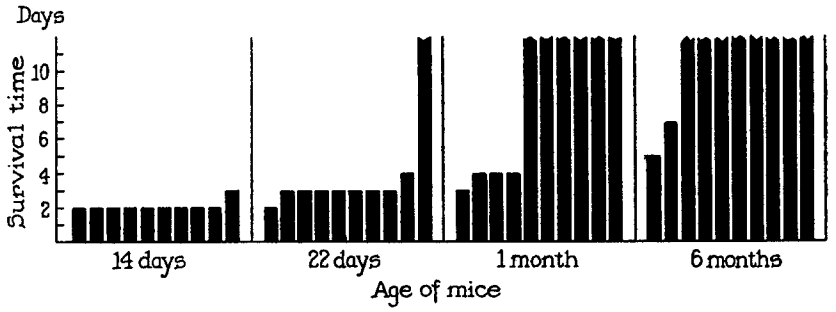


FIG. 1. Susceptibility of mice of various ages to intraperitoneal injection of active E.E.E. virus (1,000,000 cerebral units).

None of the ten mice 14 days old survived the intraperitoneal injection of active virus. One of ten, 22-days-old; six of ten, 1-month-old, and eight of ten, 6-months-old mice, survived to the end of the 12-days-observation period. Deaths due to virus have not been noted beyond this time. With increasing age, the average incubation period of those which succumbed was 2, 3, 4, and 6 days respectively. Incubation period is used in the sense of time between injection and death, which followed closely upon first symptoms of central nervous system involvement. It should be added that the incubation period for any given age is shorter after intracerebral than after intraperitoneal injection of active virus.

With increasing age, mice showed a diminishing susceptibility to intraperitoneal injection of active virus. Of those which succumbed, the length of incubation period increased with age. These results are consistent with previous reports (7 *b*, 8 *a*).

Antibody Response to Active Virus in Mice of Various Age Groups

The antibody response of mice of various ages to intraperitoneal injection of active E.E.E. virus was studied, in order to determine whether the age of

the surviving mice affected the amount or rate of formation of serum-neutralizing antibodies.

Additional groups of mice of the ages shown in Fig. 1 were injected intraperitoneally with the same dose of 0.25 cc. 10^{-3} dilution of E.E.E. virus. Surviving mice were bled from the heart on the 3rd, 4th, 5th, and 8th days after injection. Sera were pooled according to age and time after injection; each pool contained sera from at least three mice. There was not a sufficient number of survivors of the 14-days-old group to bleed on the 3rd day, nor of 22-days-old mice after the 3rd day. Test for antibody was carried out by the intraperitoneal serum-neutralization test of Olitsky

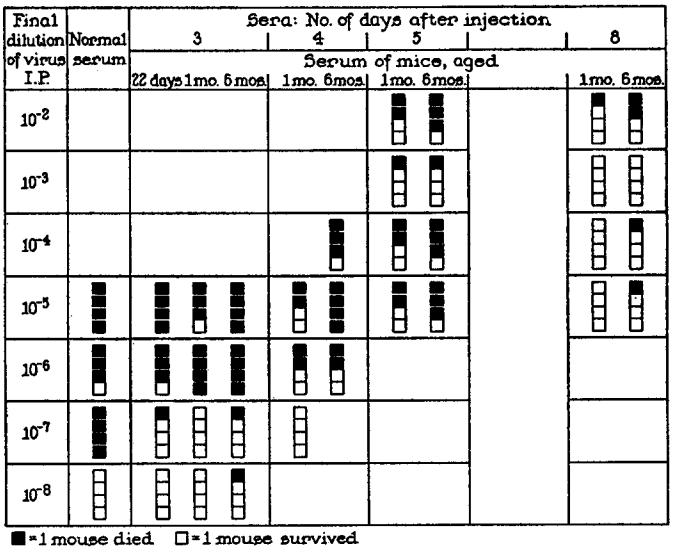


FIG. 2. Development of serum-neutralizing antibodies in mice of various ages in response to intraperitoneal injection of active E.E.E. virus.

and Harford (16). 0.03 cc. of each mixture of serum and dilution of virus was injected intraperitoneally into each of four mice. The ratio of serum to virus dilution was 4 to 1; for example, 0.2 cc. of serum was mixed with 0.05 cc. of virus dilution (final dilutions of virus are recorded); 0.03 cc. of this mixture was injected. The results are given in Fig. 2.

The titer of virus in the presence of normal serum was 10^7, and was 10^6 in the presence of serum taken on the 3rd day from 22-days-old, 1-month-old, and 6-months-old mice. This difference in titer does not, however, establish the presence of antibody at this time. In our experience a difference in titer must be more than tenfold to be significant when so few mice are injected. The essential point is that there was no difference in protective capacity of the three test sera. On the 4th day, the sera of the 1-month-old and 6-months-old mice showed perhaps a slight protective effect, but again no difference was apparent between the test sera. By the 5th day, the sera of the 1-month-old and 6-months-old mice protected about half of the mice at dilu-

tions of virus ranging from 10^{-2} to 10^{-5}. It may be noted that this protection of half of each group over a range of virus dilutions, rather than the clear-cut end-point found with very strong or very weak antisera, is characteristic of a serum of moderate antibody content. By the 8th day, sera of mice of the two surviving age groups protected at least half the number of mice which received the 10^{-2} dilution of virus, or 100,000 peritoneal units of virus, as compared with the normal serum control.

Thus, there was no difference demonstrable in antibody response of *surviving* mice of various age groups after intraperitoneal injection of active virus. However, it must be kept in mind that there is inherently a selection of mice available for this antibody study. The survivors are the more resistant animals of each group and, in the younger groups especially, are hardly representative. Expressed in another way, mice surviving an intraperitoneal injection of active virus, regardless of age, were indistinguishable in rate of antibody development.

Immune Response of Young Mice to Formolized Virus

Since there were difficulties inherent in the direct approach to the study of immune response to active virus with increasing age, a more indirect approach was resorted to, by vaccinating young mice with inactivated, formalin-treated virus. In a previous study of immune response with age (14), it had been observed that although 2-days-old mice, after vaccination with formalin-treated virus, exhibited little or no antibody and no cerebral immunity, nevertheless they resisted 1,000 times more virus introduced intraperitoneally than their non-vaccinated litter mates. This finding was investigated further in the following experiments.

Immunization had to be achieved before the mice were 15 days old, since beyond that age normal mice rapidly lose their susceptibility to peripheral injection of the virus. Using an interval of 8 days between the beginning of vaccination and test with active virus, it was possible to start vaccination when mice were 2, 5, and 7 days old and test for resistance with active virus when 10, 13, and 15 days old. Mice of these age groups were vaccinated with a 10 per cent E.E.E. virus-infected mouse-brain suspension in 0.5 per cent formalin in which no active virus was detectable by cerebral test. They were injected intraperitoneally with three doses of 0.1 cc. of inactivated virus on alternate days. This dosage was well retained, even by the youngest animals. Since all mice received the same amount of vaccine, the ratio of dose to body weight was smaller in the larger animals. However, since it has already been shown (14) that the immune response of older mice is greater in spite of the proportionally small amount of antigen, the unequal dosage per body weight may be disregarded.

Cerebral and Peritoneal Resistance.—A comparison was made between the degree of peritoneal and of cerebral resistance induced by intraperitoneal vaccination with formalin-inactivated virus.

Eight days after the beginning of vaccination, at least six vaccinated mice of each age group were bled from the heart and the sera pooled according to age. Of the remaining vaccinated mice, some were tested for resistance to active virus injected intracerebrally and some intraperitoneally. Non-vaccinated litter mates served as controls for each route of injection. The results presented in Fig. 3 are based on data from three experiments, each of which included all groups.

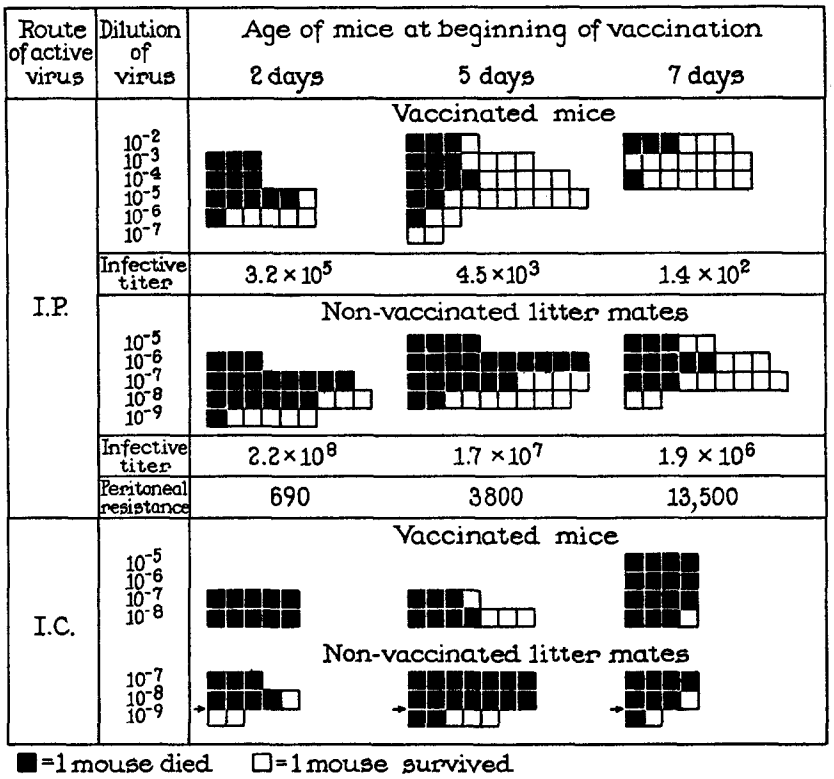


FIG. 3. Resistance to intraperitoneal and intracerebral injection of active E.E.E. virus after intraperitoneal vaccination with formolized E.E.E. virus.

The cerebral titer of E.E.E. virus, as measured by the 50 per cent end-point of death in normal 10-, 13-, and 15-days-old mice, was 10^8. This titer was not changed as a result of vaccination of mice of the same ages. In mice 10, 13, and 15 days old the 50 per cent end-point of virus injected intraperitoneally, as calculated by the Muench accumulation method, was 2.2×10^8, 1.7×10^7, and 1.9×10^6, respectively. As a result of vaccination begun 8 days previously, when mice were 2, 5, and 7 days old, the peritoneal titer was 3.2×10^5, 4.5×10^3, and 1.4×10^2, respectively, indicating a peritoneal resistance, as measured by difference in titer, of 690, 3,800, and 13,500, each compared with its non-vaccinated control. The lack of sharp end-point in the vaccinated, intermediate age group, which was apparent in each of the tests

contributing to this figure, recalls the partial protection, over a wide range of virus dilutions, of groups of mice receiving virus plus serum containing only a moderate amount of antibody (Fig. 2, 5th-day serum). This partial group resistance may be an expression of the differences between individuals which become apparent at this intermediate level of resistance of the group. It may be added that mice which succumbed in spite of vaccination died after the shorter incubation period characteristic of young non-vaccinated mice rather than that of adult mice after intraperitoneal injection of active virus.

The titer of E.E.E. virus injected intracerebrally was the same in mice of the ages studied. Vaccination with formolized virus failed to induce cerebral resistance. Although the susceptibility of normal mice to intraperitoneally injected E.E.E. virus decreased slightly with age from 10 to 13 to 15 days, as measured by titer of virus, the amount of peritoneal resistance induced by formalin-treated E.E.E. virus increased progressively with these small increments of age. The susceptibility of the young vaccinated mice recalls that of normal adult mice; that is, although fully susceptible to virus injected cerebrally, the majority resisted peritoneally many times the amount of virus fatal to non-vaccinated young mice.

Antibody Response.—Sera of mice vaccinated at the same time as those tested for cerebral and peritoneal resistance were studied for neutralizing antibody.

Sera taken 8 days after the beginning of vaccination of mice 2, 5, and 7 days of age were tested for capacity to neutralize E.E.E. virus. The intraperitoneal serum-neutralization test (16) was used. Mice 13 to 14 days of age were injected intraperitoneally with 0.03 cc. of mixtures of equal parts of serum and virus dilutions. The results reported in Fig. 4 represent five different tests, each of which included all three test sera as well as control normal serum. 50 per cent end-points were calculated by the Muench accumulation method.

The difference in titer of virus in the presence of test serum and that in normal serum was taken as the measure of neutralizing capacity of the serum. These were found to be 5.9, 25.6, and 123 respectively for sera of mice 2, 5, and 7 days old at the beginning of vaccination.

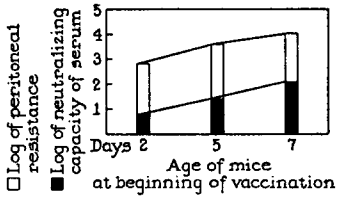
Correlation of Peritoneal Resistance with Antibody.—When the logarithm of the peritoneal resistance induced by vaccination of mice 2, 5, and 7 days old was plotted, as in Fig. 5, and compared with the logarithm of neutralizing capacity of a sample of serum, they were found to be almost parallel. The ratio of peritoneal resistance to neutralizing capacity of a serum sample varied from 110/1 to 148/1. Thus antibody content, as measured by serum-neutralization tests, paralleled peritoneal resistance at a lower level and was therefore found to be a less sensitive measure of immune response than intraperitoneal inoculation of active virus. The fact that the degree of peritoneal resistance

induced by vaccination was paralleled by antibody concentration in the serum suggested that the induced resistance was of the nature of a specific immune response.

Dilution of virus I.P.	Serum of normal mice	Sera of vaccinated mice		
		Age at beginning of vaccination		
		2 days	5 days	7 days
10^{-3}				
10^{-4}				
10^{-5}				
10^{-6}				
10^{-7}				
10^{-8}				
Infective titer	4.2×10^6	7.1×10^5	1.8×10^5	3.4×10^4
Neutralizing capacity		5.9	25.6	123

■=1 mouse died □=1 mouse survived

FIG. 4. Serum-neutralization test. Sera of mice of three age groups vaccinated with formolized E.E.E. virus.



Age of mice	2 days	5 days	7 days
Peritoneal resistance	690	3800	13,500
Neutralizing capacity of serum	5.9	25.6	123

FIG. 5. Correlation of resistance to active E.E.E. virus by the intraperitoneal route with neutralizing capacity of serum of mice after vaccination with formolized E.E.E. virus.

Is the Resistance Induced by Vaccination Local or Systemic?—It was of interest to know whether the resistance induced by intraperitoneal vaccination with formolized virus was local or systemic,—systemic in the sense of resistance of tissues other than the central nervous system.

One group of 7-days-old mice was vaccinated by intraperitoneal injection of formolized virus as before, and another group by intramuscular vaccination. The mice were injected three times on alternate days with 0.1 cc. of formalin-inactivated E.E.E. mouse brain virus suspension. Active virus was injected into half of each vaccinated group by the same route as that used for vaccination and half by the other route. The results of two experiments are presented in Fig. 6.

Mice vaccinated intraperitoneally were no better protected against active virus injected by the intraperitoneal than by the intramuscular route; and

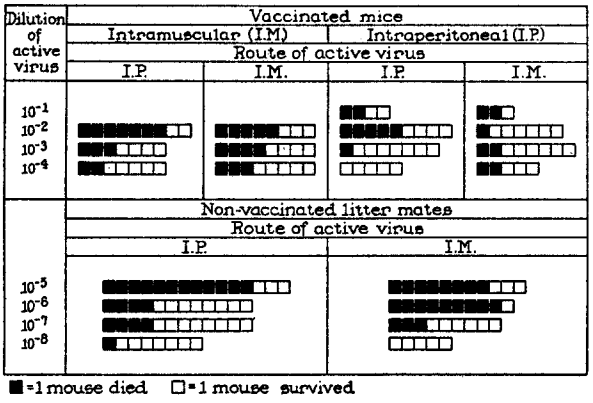


FIG. 6

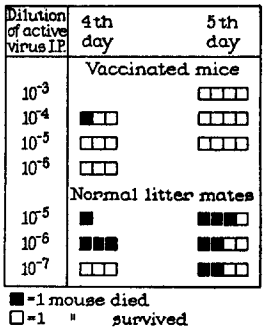


FIG. 7

FIG. 6. Comparison of resistance to active E.E.E. virus by intraperitoneal and by intramuscular routes after intraperitoneal or intramuscular vaccination with formolized E.E.E. virus.

FIG. 7. Immunity to active E.E.E. virus by the intraperitoneal route 4 to 5 days after beginning of vaccination of 10-days-old mice with formolized virus.

conversely, in mice vaccinated intramuscularly the resistance to active virus given by the same route was no greater than to virus by the intraperitoneal route. Thus, the resistance induced by vaccination proved to be not local, but a general, systemic immunity.

Time of Appearance of Immunity to Active E.E.E. Virus Given by the Intraperitoneal Route.—It was desirable to choose mice as old as possible for vaccination, since the immune response has been shown to increase with age, yet of such age that control litter mates at the time of test would still be susceptible to active virus by the peritoneal route.

With these limitations in mind, vaccination was begun when mice were 10 days of age. Half of the number of mice born on a given day were vaccinated; the others remained as non-vaccinated controls. Vaccination consisted of three intraperitoneal injections of 0.1 cc. of formalin-inactivated E.E.E. virus on consecutive days. Tests

for resistance to active E.E.E. virus injected intraperitoneally were carried out on the 2nd, 3rd, 4th, and 5th days after the beginning of vaccination. 4th- and 5th-day groups were tested simultaneously, vaccination having been begun on consecutive days when the mice in each group were 10 days old. The young mice were kept with their mothers until tested with active virus when they were, respectively, 12, 13, 14, and 15 days of age.

Immunity to active virus by the peritoneal route was demonstrable on the 4th and 5th days after beginning of vaccination, as charted in Fig. 7.

The difference between the titer of virus in non-vaccinated mice and that in vaccinated mice on the 4th day was at least 1,000-fold; on the 5th day, more than 10,000-fold. Other experiments indicated a small amount of resistance on the 2nd and 3rd days. However, this proved to be non-specific when mice vaccinated with the Eastern strain were compared with Western-vaccinated as well as with non-vaccinated control mice. The specificity of the immunity present on the 4th day was tested in the following experiments.

Specificity of Immunity Induced by Vaccination with Formolized W.E.E. or E.E.E. Virus.—The Western strain of equine encephalomyelitis virus, the virus most closely related to the Eastern strain, is known to be immunologically distinct from the Eastern, when compared in artificially immunized laboratory animals (17 *a*) as well as in convalescent horses (17 *b*). Therefore the specificity of the immunity induced by vaccination with formolized Eastern strain compared with that by the Western was tested by intraperitoneal injection of active virus of the homologous or heterologous strain.

One-third of a large group of 10-days-old mice was vaccinated with formolized Eastern strain, one-third with formolized Western, and a third left as non-vaccinated controls. Vaccination was carried out by three intraperitoneal injections of 0.1 cc. of formalin-inactivated virus on consecutive days. On the 4th day after the beginning of vaccination, when mice were 14 days old, they were tested by the intraperitoneal injection of active Eastern strain of virus.

In another experiment, one-third of a group of 7-days-old mice was vaccinated with formolized Eastern strain, one-third with formolized Western, and the remainder kept as controls. Vaccination consisted of three intraperitoneal injections of 0.1 cc. of formolized virus on alternate days. 8 days after the first injection of vaccine, the mice, then 15 days old, were tested with active W.E.E. virus intraperitoneally. The results of the two experiments are presented in Fig. 8.

The difference between the titer of active Eastern strain of virus injected intraperitoneally into non-vaccinated control mice and that in vaccinated mice on the 4th day after the beginning of vaccination with the homologous strain was 100- to 1,000-fold. The titer of the same virus in mice vaccinated with the heterologous (Western) strain was ten-fold less than in the non-vaccinated control mice. This may represent a small amount of non-specific resistance,

and, indeed, such resistance was found in vaccinated mice on the 2nd and 3rd days after beginning of vaccination, regardless of whether the vaccine was of the homologous or heterologous strain. However, the resistance induced by the 4th day as a result of vaccination with the homologous strain was significantly greater than that by the heterologous strain and was therefore of the nature of a specific immunity.

By the 8th day after the beginning of vaccination of mice 7 days old, a high degree of immunity to active virus of the Western strain (1,000,000 peritoneal units) had been induced by means of the homologous vaccine. This proved to be a highly specific immune response when compared with the lack of resistance of mice vaccinated with the heterologous strain. The degree of im-

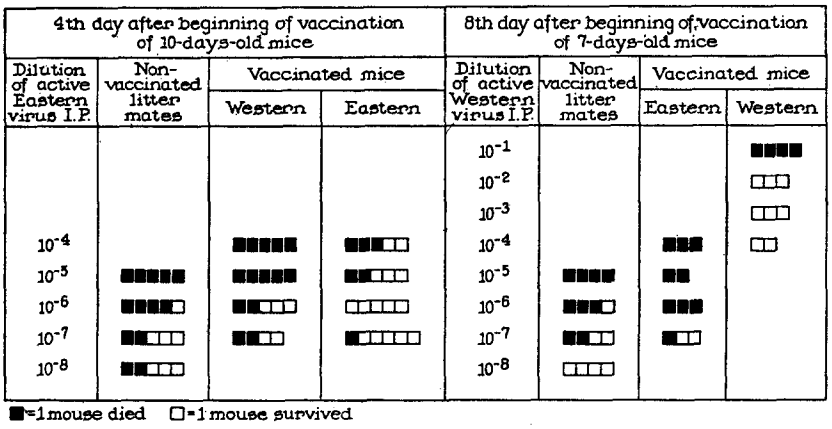


FIG. 8. Specificity of immunity induced by vaccination with formolized W.E.E. or E.E.E. virus as tested by intraperitoneal injection of active virus.

munity on the 4th and 8th days can hardly be compared since the intervals used and the ages of the animals differed.

These experiments proved that the resistance induced by vaccination with formalin-inactivated virus and tested by intraperiteoneal injection of active virus was a true immunity specific for the Eastern or the Western strain of equine encephalomyelitis virus.

Infectivity of Blood of Mice after Intraperitoneal Injection of Active Virus

The susceptibility of young vaccinated mice to injection of active virus by cerebral and peripheral routes, compared with that of adult normal and young normal mice, has been studied. It was then of interest to find how much virus was recoverable from the blood of each of these groups after intraperitoneal injection of active virus, since virus introduced peritoneally reaches the central nervous system by way of the blood stream (8 *a*, *d*).

10^6 cerebral units of active E.E.E. virus were injected intraperitoneally into three groups of mice. One group consisted of normal 2-months-old mice; another, of normal 15-days-old mice, and the third, of 15-days-old mice which had been vaccinated, beginning at 7 days of age, by four intraperitoneal injections of 0.1 cc. of formalin-inactivated E.E.E. virus on alternate days. The six mice in each group were bled from the tail; the blood samples of each group were collected in a test tube containing a drop of sterile solution of 1/500 heparin in saline. Bleedings were made at 2 hours, 24 hours, and 48 hours after the injection of active virus, and at 6 days in the two surviving groups. 0.03 cc. of each pool of blood, and dilutions in broth where indicated, was injected intracerebrally into three mice. The fate of the injected mice as well as the infectivity of blood at intervals are presented in Fig. 9.

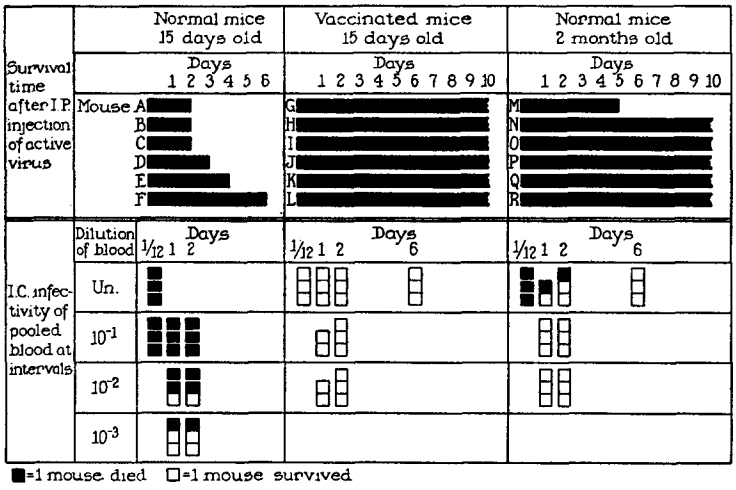


FIG. 9. Effect of intraperitoneal injection of active E.E.E. virus in mice and subsequent infectivity of the blood.

Thus, all six non-vaccinated 15-days-old mice died within 6 days after injection of active virus, none of the six vaccinated 15-days-old mice died, and one of the six normal 2-months-old mice died on the 5th day. This is consistent with previous experience; that is, young mice were susceptible to active virus by the intraperitoneal route, whereas the majority of old mice were resistant; furthermore, young mice, as a result of vaccination, became immune to the active virus given by this peripheral route.

Active virus was recoverable from the blood of the young normal group 2 hours after intraperitoneal injection; at 24 and 48 hours, a 100-fold dilution of blood was fatal to two of three mice and a 1,000-fold dilution to one of three. In contrast to this, no active virus was recovered from the blood of the young vaccinated mice at these intervals, nor at 6 days. Sufficient virus was present at 2 hours in the blood of the adult normal group to kill all of three mice injected intracerebrally, but only a minimal amount at 24 and 48 hours; one of two and one of three injected mice re-

spectively died. None was recoverable at 6 days. However, since one animal died on the 5th day, the recovery of a minimal amount of virus up to that time may have been due to virus in the blood of this individual rather than to a low concentration of virus in the blood of all.

Thus, after intraperitoneal injection of active virus in young normal mice, virus was demonstrable in 100- and 1,000-fold dilutions of blood for at least 2 days; all animals succumbed. In adult normal mice virus was recoverable in the blood at 2 hours, a minimal amount at 24 and 48 hours, and none at 6 days; the group as a whole was resistant to injection of active virus. Finally, as a result of vaccination, no virus appeared in the blood of young immunized animals, all of which survived. This is an example of another way in which young vaccinated animals in their response to active virus more closely resembled adult than young normal mice.

RÉSUMÉ AND DISCUSSION

The experiments reported deal with the influence of age on increasing capacity to elicit an immune response as correlated with decreasing susceptibility to peripheral injection of a neurotropic virus. The reaction of mice to Eastern equine encephalomyelitis virus was studied.

After intraperitoneal injection of active virus into mice of various age groups, a larger proportion survived with greater age, and of those which succumbed, the greater the age, the longer the incubation period. In the serum of surviving mice of the various age groups no difference in rate of development of serum-neutralizing antibodies was found. It should be recalled that survivors were not available in the younger groups on the 5th day when antibodies were first clearly demonstrable in the serum of adults. Since it was inherently impossible to obtain representative groups of each age at intervals after injection with active virus, immune response to formalin-inactivated virus was studied. Young mice, after vaccination with inactivated virus, developed peritoneal resistance in the absence of cerebral resistance at a time when their serum showed little or no demonstrable antibody (14). The degree of peritoneal resistance induced in young mice increased with very small increments of age. This increase in resistance was paralleled by increase in serum-neutralizing antibodies. Thus the amount of serum-neutralizing antibody was found to be an index of peritoneal resistance after vaccination. It was, however, an insensitive indicator, since the neutralizing capacity of a sample of serum was consistently lower than the peritoneal resistance of the animal, as shown by comparing the amount of virus neutralized with the amount resisted. The peritoneal resistance induced was found not to be local, *i.e.*, confined to the peritoneum, but rather a systemic immunity of non-nervous tissue, specific for the Eastern strain of equine encephalomyelitis virus.

An explanation offered for an acquired resistance of growing animals to peripheral injection of certain neurotropic viruses in the absence of humoral antibodies and of previous exposure to infection was the development of barriers with age which served to arrest the progression of virus (7 *b*). Since no anatomical changes were observed to account for these barriers, they were considered hypothetical barriers, or silent areas beyond which the virus could not pass. In the present experiments an attempt was made to find to what extent the greater rate of immune response of older animals might account for their insusceptibility to virus given peripherally. A parallel was drawn between the reaction of young vaccinated mice and adult normal mice to peripheral injection of active virus. Young vaccinated mice simulated adults in that although fully susceptible to virus given by the intracerebral route, they resisted large doses of virus by the intraperitoneal route. It should be remembered, however, that their serum contained minimal amounts of neutralizing antibody.

Another comparison between adult mice, and young mice vaccinated and non-vaccinated, was based on the recovery of virus from their blood after intraperitoneal introduction of active virus. Since virus is known to invade the central nervous system by being deposited from the blood onto the nasal mucosa, it was of interest that a minimal amount of virus was found in the blood of adults, none in that of young vaccinated, and a large amount in that of young non-vaccinated mice. The lack of large amounts of circulating virus in the blood of adult mice would explain why invasion of the central nervous system in most individuals failed to take place, although it was fully susceptible at the time. The possibility must not be overlooked that failure to recover large amounts of virus may have been due in part to an insusceptibility of non-nervous tissues of the adult. Although immune response is set in motion at the time of injection of active virus (14), at just what point it becomes effective is difficult to define. It has been shown that 10-days-old mice had developed a specific immunity to active virus injected intraperitoneally by the 4th day after beginning of vaccination. Older animals, capable of a more rapid immune response, would be expected to have developed an effective immunity within that time.

Although the central nervous system of old and young mice was found equally susceptible to Eastern equine encephalomyelitis virus, with increasing age there was a decreasing susceptibility to virus introduced by the peritoneal route. Evidence has been presented to show to what extent the more rapid and greater immune response of older animals might have influenced the outcome of such peripheral injection.

No generalization is intended that young animals immunized by means of other viruses would always present a greater resistance of non-nervous tissue compared with that of the central nervous system. This relationship has

been found to obtain for Eastern equine encephalomyelitis virus, a virus to which nervous tissue is the most susceptible. The relative resistance of the tissues of the host to each virus would have to be studied. For rabies virus, another neurotropic virus, there is already evidence (15) of decreasing susceptibility with age of mice, and furthermore mice are more readily immunized with increasing age. As with Eastern equine encephalomyelitis virus, immunity is more readily induced to peripheral than to cerebral injection of virus.

Other factors which influence degree of immune response may be mentioned. That hormonal factors may influence immunizability has been brought out by Hodes (18*a*) who found a reduction in the capacity of mice to elicit an immune response to St. Louis encephalitis virus during pregnancy. Sabin and Duffy (18*b*) have demonstrated that diet may affect the age at which susceptibility of mice to peripheral injection of the neurotropic virus of vesicular stomatitis changes. It would be of interest to know whether the difference in diet affects the degree to which animals may elicit an immune response.

Although relatively few cases of infection of man with the virus of equine encephalomyelitis have as yet occurred, it is of interest to consider what bearing the experiments reported may have on the human disease. The age difference in the milder infection by the Western strain is not marked (19). In the Massachusetts epidemic (2) caused by the Eastern strain, children were predominantly affected. The majority of cases terminated in death, and in those which survived permanent cerebral injury occurred (20 *a*). Although Fothergill (20 *b*) was unable to find antibodies in the sera of adult contacts available for study, he stated that his negative findings did not exclude the possibility of inapparent infection in adults. Antibodies to the Eastern strain have not only been demonstrated in the serum of recovered cases but also in the serum of apparently healthy individuals associated with laboratory work on the virus (21). In summary, infection due to equine encephalomyelitis virus, Eastern strain, has proved fatal to children, or has left permanent cerebral injury, whereas in adults, inapparent infections have occurred as evidenced by serum-neutralizing antibodies in the absence of symptoms.

SUMMARY

The experiments described in this paper were carried out with the Rockefeller Institute strain of albino mice and with the Eastern strain of the virus of equine encephalomyelitis.

1. The observation was confirmed that with increasing age of mice there occurred a decrease in susceptibility to intraperitoneal injection of active virus; also, the length of incubation period of those which succumbed increased with age.

2. The mice of various age groups which survived an intraperitoneal injection of active virus were indistinguishable in their antibody response.

3. Young mice, vaccinated with formalin-inactivated virus when 2, 5, and 7 days old, gave an immune response to such a degree that they showed (*a*) measurable peritoneal immunity which increased with small increments of age, (*b*) no cerebral resistance, and (*c*) detectable amounts of neutralizing antibody in their sera which paralleled, though at a considerably lower level, their peritoneal resistance.

4. The peritoneal resistance induced as a result of vaccination was shown to be not local, but a general, systemic immunity, specific for the Eastern strain. Such a peritoneal resistance was demonstrable by the 4th day after beginning of vaccination of 10-days-old mice.

5. After intraperitoneal injection of active virus, large amounts of virus were recoverable from the blood of non-vaccinated young mice; none was found in the blood of vaccinated young mice; a minimal amount was detectable in the blood of non-vaccinated adult mice.

6. The bearing of age on the degree of immune response of which mice are capable and on their susceptibility to the virus has been discussed.

BIBLIOGRAPHY

1. Fothergill, L. D., Dingle, J. H., Farber, S., and Connerley, M. L., *New England J. Med.*, 1938, **219,** 411. Webster, L. T., and Wright, F. H., *Science*, 1938, **88,** 305.
2. Feemster, R. F., *Am. J. Pub. Health*, 1938, **28,** 1403.
3. Kelser, R. A., *J. Am. Vet. Med. Assn.*, 1938, **92,** 195.
4. (*a*) Kleine, F. K., *Z. Hyg. u. Infectionskrankh.*, 1905, **51,** 177. (*b*) Kraus, R., and Schiffmann, J., *Centr. Bakt., 1. Abt., Orig.*, 1907, **43,** 825.
5. Andervont, H. B., *J. Infect. Dis.*, 1929, **44,** 383.
6. Theiler, M., *Ann. Trop. Med. and Parasitol.*, 1930, **24,** 249.
7. (*a*) Olitsky, P. K., Cox, H. R., and Syverton, J. T., *J. Exp. Med.*, 1934, **59,** 159. (*b*) Olitsky, P. K., Sabin, A. B., and Cox, H. R., *J. Exp. Med.*, 1936, **64,** 723.
8. (*a*) Sabin, A. B., and Olitsky, P. K., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38,** 595, 597; (*b*) *J. Exp. Med.*, 1937, **66,** 15, 35; (*c*) 1938, **67,** 201, 229. (*d*) Hurst, E. W., *Brain*, 1936, **59,** 1.
9. King, L. S., *J. Exp. Med.*, 1940, **71,** 95.
10. Metchnikoff, E., *Ann. Inst. Pasteur*, 1897, **11,** 801.
11. Freund, J., *J. Immunol.*, 1930, **18,** 315.
12. Baumgartner, L., *Yale J. Biol. and Med.*, 1934, **6,** 403.
13. (*a*) Culbertson, J. T., and Kessler, W. R., *Am. J. Hyg.*, 1939, **29,** Section C, 33; (*b*) Kolodny, M. H., *Am. J. Hyg.*, Section C, 1940, **31,** 1.
14. Morgan, I. M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42,** 501; 1940, **44,** 473.
15. Casals, J., *J. Exp. Med.*, 1940, **72,** 445, 453.
16. Olitsky, P. K., and Harford, C. G., *J. Exp. Med.*, 1938, **68,** 173.
17. (*a*) TenBroeck, C., and Merrill, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31,** 217. (*b*) Shahan, M. S., and Giltner, L. T., *J. Am. Vet. Med. Assn.*, 1935, **86,** 764.

18. (*a*) Hodes, H. L., *J. Exp. Med.*, 1939, **69,** 533. (*b*) Sabin, A. B., and Duffy, C. E., *Science*, 1940, **91,** 552.

19. (*a*) Platou, R. V., *Am. J. Dis. Child.*, 1940, **60,** 1155. (*b*) Davis, J. H., *J. Pediat.*, 1940, **16,** 591. (*c*) Howitt, B. F., *Science*, 1938, **88,** 455. (*d*) Buss, W. C., *Ann. Rep. Kern County Dept. Pub. Health*, Bakersfield, California, 1939–40, 79. (*e*) Fothergill, L. D., Holden, M., and Wyckoff, R. W. G., *J. Am. Med. Assn.*, 1939, **113,** 206. (*f*) Helwig, F. C., *J. Am. Med. Assn.*, 1940, **115,** 291.

20. (*a*) Farber, S., Hill, A., Connerley, M. L., and Dingle, J. H., *J. Am. Med. Assn.*, 1940, **114,** 1725. (*b*) Fothergill, L. D., in Virus and rickettsial diseases, Cambridge, Massachusetts, Harvard University Press, 1940, 677.

21. (*a*) Olitsky, P. K., and Morgan, I. M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41,** 212. (*b*) Beard, J. W., Beard, D., and Finkelstein, H., *Science*, 1939, **90,** 215; *J. Immunol.*, 1940, **38,** 117.