Age Factor in Adaptation of Vaccinia Virus to Mouse Brain. (29465)

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In order to examine a possible correlation between viral neurotropism and oncolytic activity, one of the agents which has been under scrutiny in this laboratory is the IHD strain of vaccinia virus. Earlier studies(1) revealed that a neuropathic line of this virus was highly oncolytic. It became of interest to determine the oncolytic manifestations of vaccinia virus in a gradient of viral sublines ranging from non-neurotropic to highly neurotropic. In the course of developing these sublines, by serial intracerebral passage, a unique age-associated resistance factor was encountered in the animal host. The present report describes the characteristics of this factor, and the manner in which the desired viral gradient was finally derived.

Materials and methods. Mice. The mice employed were of the N. I. H. general-purpose strain. Their ages varied, as indicated, according to the experimental procedure. Where mice over 3 weeks of age were required, females were employed exclusively.

Virus. The virus referred to as IHD-L is a line of the IHD strain of vaccinia virus, maintained since 1909 at the Lederle Laboratories by alternate passage through calves, rabbits and humans. It was never passed through the central nervous system of any animal species, and we found it capable of proliferating in the brain of the newborn mouse only, following intracerebral inoculation. This virus was received through the courtesy of Dr. V. J. Cabasso.

Virus assay. This procedure has been described(1), and involved the counting of viral pocks on the chorioallantois of the chick embryo. The count is expressed as pock-producing units (p.p.u.) per 0.05 ml of a given virus preparation. In the text, where the 4-day infectivity levels are stated in connection with serial intracerebral passage, it should be understood that this refers to the virus titer per 0.05 ml of a 20% brain suspension.

Intracerebral passage of virus. Up to the point of adaptation of the virus to 5-week-old mice, 200 p.p.u. were inoculated at each passage. In any series where the virus died out this obviously was not possible beyond a certain point. After adaptation to 5-week-old mice the virus was passed as an undiluted 20% brain suspension. The inoculum varied from 0.01 ml with suckling mice to 0.04 ml with adult mice. At each passage the animals were sacrificed in 4 days, and the brains ground with Alundum to give a 20% suspension in 0.85% NaCl. A virus assay was made at each passage.

Results. Early studies emphasized the great resistance of the adult mouse to neurotropic manifestations by the IHD-L virus. The virus would not multiply in the brain of the 6-week-old mouse, regardless of inoculum size. A variety of factors known to reduce host resistance were studied. Cortisone, for example, was administered intraperitoneally to 5-week-old mice at minus 2 days, minus 1 day, on the day they received the virus intracerebrally, and each day thereafter for 7 days. The dose at each injection was 1 mg. Viral multiplication did not occur.

X-ray radiation was also employed, under standardized conditions. A dose of 900 r was required to produce a 50% lethal effect at 7 days postradiation of 6-week-old mice. Three days after radiating mice different virus doses were administered intracerebrally, and infectivity levels followed for 5 days. No viral replication occurred.

Studies with 6-thioguanine in West Nile virus-inoculated mice(2) suggested that this compound might have application in the present case. Employing 6-week-old mice, it was established that an intraperitoneal dose of 2.7 mg would produce a 50% lethal effect

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TABLE I. Intracerebral Growth Curves in Mice Inoculated with IHD-L Virus and Lines of This Virus from Serial Intracerebral Mouse Passages 162, 230 and 235.

Day after inoculation*	Virus titers† Virus IHD-L	IHD-L-162	IHD-L-230	IHD-L-235
	Age of inoculated mice			
	1 day	5 wk	5 wk	5 wk
1	2.9	Neg	1.0	1.9
2	4.7	1.5	2.1	4.3
3	4.9	2.0	5.0	5.7
4	5.9	4.2	4.5	5.1
5	6.0	3.4	4.5	5.1
6	6.2	2.4	—	—

* For each growth curve, each animal received a virus dose of 200 p.p.u. Four animals were sacrificed per day.

† The titer is expressed as the log of the p.p.u. per 0.05 ml of a 20% suspension of the brain tissue removed on a given day.

at 6 days after administration. Two days following this dose virus was inoculated intracerebrally. The daily specimens subsequently collected gave no evidence of viral multiplication.

It was observed that the IHD-E virus(1), a neurotropic line, was much more heat-resistant than the IHD-L virus. Knowing this, we tried to enhance the adaptation of the IHD-L virus by heating it at 60°C. This procedure failed repeatedly, even as far along as the 58th intracerebral passage in the series to be described shortly. At passage 71 in this same series male mice were substituted for females, but this too had no influence. The strain of mouse was also considered at passage 71. The N. I. H. mouse was replaced by CFW and CF#1 mice. No viral multiplication could be obtained in 6-week-old mice of either strain. The CFW mouse is of particular interest, since it is a stock selectively inbred for susceptibility to neurotropic viruses.

The preceding results are outlined briefly, since they are of a negative nature. From them, however, the resistant character of the host is quite evident. In view of the failures with young, adult mice, the susceptibility of younger animals was examined. Multiplication of the IHD-L virus, after intracerebral inoculation, took place only in newborn mice. The growth curve in 1-day-old mice is shown in Table I. Of the 37 inoculated animals, 24 were sacrificed in the study, 4 were dead on the 6th day, and the remaining 9 mice were dead by the 10th day.

Serial intracerebral passage was then begun in 1-day-old mice. The virus level in the 20% brain suspensions from these passages remained in the vicinity of 1×10^6 p.p.u. per 0.05 ml. After 20 passages the virus was tested for its ability to multiply in mice of ages 11 days, 3 weeks, 4 weeks, and 6 weeks. Successful continuing passage could be made only in the 11-day-old mice, which were then employed up to and including passage 41. At this point it was found that 3-week-old mice were susceptible, and these were used through passage 50. The 4-day virus titers of passages 21 through 50 ranged from 1×10^3 to 1×10^6. At passage 51 unsuccessful efforts were made to increase the ages of the mice to 4, 5 and 6 weeks. The use of 3-week-old mice, therefore, was continued through passage 61. At passage 62 another attempt was made to carry the virus in 5-week and 6-week-old mice, but without success. It was possible, however, to continue in 4-week-old mice. At passage 74 the ages of the mice were stepped up to 5, 6 and 8 weeks. Only the 5-week-old mice supported viral proliferation, and animals of this age were employed through passage 77. Virus titers of passages 51 through 77 varied from 1×10^4 to 1×10^5.

From here, the virus was carried through 2 passages in 6-week-old mice, and 5 passages in 8-week-old mice, bringing it to passage 84, at which it was lost. Returning to passage 83, having a titer of 1×10^2, passage 84 was made in 2-week-old mice in order to elevate the virus level, which rose to 5×10^5. Passages 85 to 99 inclusive were made in 5-week-old mice, using undiluted brain suspension rather than the 200 p.p.u. which had been used up to this point. This procedure was continued for the rest of the series. The virus level remained at about 1×10^4.

Passage 93 virus was tested in 8-week-old mice, and found to be incapable of continuing to pass. At passage 100 an effort was made to carry the virus in 6-week and 8-week-old mice, but without success. It was

TABLE II. Capacity of IHD-L Virus from Different Points in Intracerebral Passage Series to Propagate on Continued Intracerebral Transfer in Mice of Various Ages.*

Virus examined†	Age of mice: 1 day	11 days	3 wk	4 wk	5 wk	6 wk	7 wk	8 wk	10 wk	12 wk
Original virus	+	—				—				
Passage 20		+	—	—		—				
" 41			+	—						
" 50			+	—	—	—				
" 61				+	—	—				
" 73					+	—		—		
" 93					+			—		
" 99					+	—			—	
" 108						+	—	—		
" 123							+	—		
" 140								+	—	
" 161										+

* Virus specimens from the intracerebral series described in text.

† The sign + designates that the virus could be passed serially in brain. The sign — indicates that the virus was lost after 2 to 3 serial passages. The signs are given only for those ages of mice for which serial transferability of the virus was actually tested.

possible to make passages 100 to 108 inclusive in mice 5 weeks and 4 days of age, with infectivity levels of 1×10^4 to 2×10^5. At passage 108 passage attempts were made in mice 6, 7 and 8 weeks of age. The virus multiplied in the 6-week-old mice, and passages 109 through 113 were made in animals of this age. Infectivity levels remained close to 5×10^4. Passages 114 through 118 could be made in 7-week-old mice, but the virus level dropped to 1×10^2. Passages 119 through 123 were made in 5-week-old mice for the purpose of bringing the titer up to 4×10^5. At passage 124 the age of the animals was again increased to 7 weeks, and this time the passages could be continued. In a parallel series through 8-week-old mice the virus was lost. At this point it seemed that it might be possible to enhance the adaptation by adsorbing a suspension of the virus with a suspension of disintegrated brain tissue, and inoculating the sedimented tissue intracerebrally. This effort, at concentrating that segment of the virus population having the greatest affinity for central nervous system tissue, was repeated several times to no advantage.

Virus passage in 7-week-old mice was continued through passage 140, with the virus level ranging from 1×10^3 to 4×10^5. Passage 141 was moved up to 8-week-old mice, and after 2 further passages the virus titer dropped to 2×10^1. Because of this, the passages were repeated, starting with an undiluted, 20% suspension of chick embryo chorioallantoic membranes which contained confluent lesions from passage 141 virus. No difficulty was encountered in taking this virus through passage 151 in 8-week-old mice, but it could not be carried in 10-week-old mice. The 4-day virus levels reached 1×10^4 to 1×10^5.

At passage 152 the age of the mice was increased to 10 weeks, and with each passage from here through 161 it could be increased half a week, until the age of 14½ weeks had been reached. Virus titers ranged from 2×10^3 to 2×10^5. A summation of the study up to this point is presented in Table II.

A growth curve was determined with virus from passage 162, and may be seen in Table I. This was carried out in 5-week-old mice, an age of particular interest, since it was the basis for comparison of several lines of IHD virus in a previous study(1). Passages 162 through 205 were made in 12-week-old mice, with 4-day virus levels of 6×10^3 to 2×10^5. Five-week-old mice were employed for all passages after 205. The first evidence of a neuropathic effect was seen at passage 218, when the animals appeared quite ill at 4 days. Three of the 5 mice at passage 221 died 3 to 4 days after inoculation. Virus titers from passages 218 through 231 were close to 1×10^5. All 5 of the mice at passage 228 were dead in 3 to 4 days. A growth

curve of the passage 230 virus in 5-week-old mice is shown in Table I. Ten separate animals held for mortality observations were still alive after 3 weeks. At passage 232 a further increase in viral virulence was noted, and at passage 233 all 6 of the inoculated animals were dead in 2 days. This degree of virulence continued, and it became necessary to dilute the viral inoculum to obtain a survival of the animals for 3 to 4 days. At passage 235 another growth curve study was made (Table I). Of the 10 animals held separately for mortality observations, 1 died on the 3rd day postinoculation, 5 on the 4th day, 2 on the 5th day, and 2 on the 6th day. The degree of virulence of the passage 235 virus, and the nature of its growth curve in brain, indicated that this would be probably the maximum degree of adaptation obtainable. The virus, now at passage 273, has shown no tendency to become less virulent.

Discussion. This work indicates the importance and complexity of age factor in viral infection and adaptation. Careful scrutiny reveals that not only are the factors behind age influences essentially unknown, but apparently the subject of age influence on virus multiplication in general has never been reviewed adequately in a single article. Admittedly, this would be difficult, since many pertinent facts are undoubtedly scattered through a vast number of publications not dealing primarily with age. Among the more commonly recognized aspects of age influence is the role of the suckling mouse in studies on the arboviruses, ECHO viruses, and Coxsackie viruses. The significance of age in the manifestations of certain oncogenic viruses is also well known. In general, younger animals are more susceptible to viral action than older ones, but in the case of certain strains of poliovirus in mice, and with vaccinia virus in rabbits, the reverse is true.

The report of Underwood *et al* on Coxsackie A-21 virus(3) parallels our experience with vaccinia virus. This Coxsackie virus was adapted to Tan mice, but initially it could not be adapted to Rockland mice. Tan-passed virus was finally adapted to Rockland mice by a gradual increase in the age of the intraperitoneally-inoculated animals. Between passages 9 and 24 the age was increased from 3 days to 24 days. Attempts to by-pass this gradual increase were unsuccessful.

The situation presented here with vaccinia virus is unique in that of all the procedures employed to select out more virulent virus, or to alter the host response, the only one that accomplished the adaptation was a gradual increase in age of the host. This age-wise increase, with prolonged passage at each step, evidently has not been reported commonly in adaptation studies. The time element alone might well account for this. In the present study it required over 3 years to carry the IHD-L virus from the newborn mouse to a neuropathic state in the adult.

The neurotropic IHD-E virus which we described previously(1) had, according to Dr. Robert F. Parker (personal communication), a history of prolonged intracerebral passage in mice at the Yellow Fever Laboratory of the International Health Division of the Rockefeller Foundation. We have been unable to ascertain the details of the conditions under which this adaptation was accomplished, hence cannot compare the neurotropic potentials of the IHD-E and IHD-L viruses.

Summary. Various methods were examined in an effort to adapt a non-neurotropic line of the IHD strain of vaccinia virus to the central nervous system of the adult mouse. This was accomplished only by passing the virus serially in mouse brain, beginning with the 1-day-old mouse, and gradually increasing the age. The adaptation process was unique in that the age of the mouse could be increased only by small increments, with prolonged passage at each step.

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