

THE STABILITY OF VARIOLA VIRUS PROPAGATED IN EMBRYONATED EGGS

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(Received for publication, June 24, 1943)

Since the publication of Edward Jenner's (1) "An inquiry into the causes and effects of the variolae vaccinae" in 1798 it has been assumed that certain transformations occur with the viruses of the pox group, either under natural conditions or as the result of experimental manipulation. Particular attention has been focused on the transformation of variola virus to vaccinia virus by animal passage. While variola virus is non-pathogenic on initial inoculation into the skin of domestic and laboratory animals, except in the case of the monkey, there is a considerable amount of evidence indicating that it can be passaged and that by successive passage in the skin of the calf and in the skin or testis of the rabbit it is eventually changed into a virus which resembles vaccinia in producing an extensive dermal reaction in these animals. There is no question that vaccinia virus has often been obtained by this passaging of variolous material. The pertinent question is, however, where the terminal virus originated. As pointed out by Horgan (2) in his extensive review of the situation, the work of this nature has usually been conducted in laboratories which prepare vaccinia virus vaccines in large quantities for prophylactic use and is hence under suspicion because of accidental seeding of that virus, particularly in experimental animals. The possible recovery of vaccinia virus from the testis of normal rabbits in laboratories where it is not freely handled is indicated by the recent report of Pearce (3) who isolated it from the testes of two healthy rabbits which had had no known exposure to vaccinia.

Some years ago we began a study of variola virus in another connection and isolated two bacteria-free strains which were subsequently maintained by transfer in embryonated eggs. In view of the assumed variola-vaccinia transformation it seemed of interest to inquire into the behavior of these bacteriologically sterile egg-propagated strains on testicular passage in an initially refractory animal, namely, the rabbit.

Source of the Viruses

The first of the two strains of variola virus used in the following experiments was isolated in 1940 from glycerinated crusts obtained from China.¹ It was subsequently

¹The variolous material was obtained through the courtesy of Dr. C. E. Lim of the Peking Union Medical College and Dr. J. H. Jordan of the Shanghai Municipal Council.

subjected to 44 transfers in embryonated eggs. The second virus was an older strain isolated in 1938 from a case of smallpox which occurred in Minnesota, and was similarly transferred 200 times (4).

Behavior of the Viruses in Embryonated Eggs

The two strains of variola virus behave identically on allantoic inoculation in 10 day embryonated hens' eggs and the lesions produced enable them to be readily differentiated on inspection from a typical strain of vaccinia virus (the New York City Board of Health vaccine).

A 10 per cent suspension of the vaccinia virus in normal salt solution produces an initial proliferation of the ectodermal epithelium of the chorioallantoic membrane, which is rapidly followed by widespread necrosis and degeneration. On the 3rd day after incubation at 37°C. the inoculated membrane shows a thin, oval, diffuse area of reaction, sharply demarcated from the normal tissue. The embryo is almost invariably dead at this time. Innumerable elementary bodies are usually demonstrable in films impregnated with silver by the Morosow method. If the suspension is highly diluted discrete areas of hyperplasia are formed and the embryo may be alive on the 3rd day.

The two strains of variola virus in equivalent concentration produce a similar growth of epithelium but there is little or no necrosis and the tissue cells continue to proliferate for some time, resulting in a markedly thickened area of reaction very different in appearance from that produced by vaccinia virus. Elementary bodies are less readily demonstrable in films. The embryo remains active, though virus may be recoverable from it, and if incubation is continued it sometimes hatches.

Neither of the two strains of variola virus after transfer in embryonated eggs produces more than a transient hyperemia on skin inoculation in susceptible rabbits.

It seemed reasonable to suppose that the embryonated egg itself might furnish the stimulus for the transformation of variola to vaccinia virus, since the embryo is to some extent refractory to it, at least in comparison with vaccinia, while the chorionic membrane is equally susceptible to both. Prolonged passage of the Minnesota strain, however, indicated that such was not the case. This virus showed no alteration of its behavior in the egg after 200 transfers. The reaction produced by it after prolonged passage was indistinguishable from that of the Chinese strain when first isolated.

Survival of the Viruses in the Testis of the Rabbit

The Chinese Strain of Variola.—In these experiments the reaction of embryonated eggs to the inoculation of testicular suspensions was used as the criterion for the presence or absence of the specific virus. The results of the first few tests were essentially negative. The virus was demonstrable in the testes of rabbits killed on the 3rd day after injection but failed to survive on subsequent passage. It was not recoverable locally from rabbits killed on the 5th day.

In the above experiments and those which follow the primary inoculum was 0.5 cc. of a 10 per cent egg membrane suspension in saline, the membrane being removed on the 3rd day after inoculation and ground in a glass tissue grinder. All of the membranes were shown to be bacteriologically sterile, as were also the testicular suspensions, by the inoculation of horse blood agar slants. Egg transfers 12 through 20 were used in the first tests. The egg membrane suspensions were injected directly into the left testis of normal rabbits from a colony maintained at The Rockefeller Institute in Princeton, New Jersey. These rabbits were subsequently maintained under strict quarantine in individual cages. It may be said that vaccinia virus had only occasionally been handled in these laboratories for a period of several years when this work was begun. At autopsy on the 3rd to the 5th day and in later experiments on the 7th day, both testes were removed and weighed. A small piece of the left testis (injected) was removed for histological study and the remainder finely ground. Three dilutions in saline were made: 1:5, 1:50, and 1:500. Approximately 0.05 cc. of each dilution was inoculated in two 10 day embryonated eggs, making a final concentration of 1:100, 1:1000, and 1:10,000. The eggs were opened and examined on the 3rd to the 5th day. If a second rabbit passage was made, 0.5 cc. of the 1:5 dilution of the above suspension was injected as before, the final concentration being 1:10.

Whereas the specific virus was demonstrable in the testes of rabbits killed on the 3rd day after primary injection in the preceding experiments, it was not recoverable from those killed on the 5th day or from passaged rabbits. In a subsequent experiment a rabbit injected with a suspension of the 24th egg transfer showed variola virus in 2 of the 3 dilutions when killed on the 3rd day. A second rabbit passage was made and by chance the animal was held through the 7th day and then killed. An observation was made at this time which may account for the earlier failure to demonstrate the virus on passage. It was found that a single egg transfer was not an adequate test for the presence of variola virus. The egg membranes inoculated from the testis of the second passage rabbit, killed on the 7th day, showed no visible reaction with any of the 3 dilutions. A second egg transfer was made using a 10 per cent membrane suspension from the eggs inoculated with the 1:100 dilution of the testis. These eggs showed the characteristic focal reaction of variola in the membranes when opened on the 3rd day, the embryos being normal and active.

This injection series was subsequently maintained for 10 successive passages at intervals of 7 days. Survival of the specific virus in the testis was determined by the subinoculation of embryonated eggs, 2 transfers being made with each passage. As indicated in Table I, 2 of the 11 passage rabbits showed the specific virus in 2 of the 3 testis dilutions on direct egg inoculation. In 6 animals it was demonstrable in all 3 dilutions. In 3 rabbits, those of passages 2, 5, and 10, the presence of virus was indicated only in the 2nd egg transfer. Except for a few eggs which were obviously contaminated with bacteria, the embryos were alive and normal on the 3rd to the 5th day.

The 11 rabbits in this passage series were all normal in appearance when killed on the 7th day. The injected testes were freely movable and showed no macroscopic lesions, save a slight trauma at the site of inoculation. Histological examination failed to reveal any indication of specific orchitis. The comparative weights of the paired testes from each animal are shown in Table I. The injected testis was heavier than the uninjected one in 10 of the 11 animals, but the difference was probably of little significance in view of the amount of inoculum. In 4 rabbits the left testis was a deeper pink than the

TABLE I
Comparative Weights and Virus Content of the Testes from Rabbits Injected with the Chinese Strain of Variola Virus

Passage No.	Days to autopsy	Weight of injected testis <i>gm.</i>	Weight of uninjected testis <i>gm.</i>	Weight difference <i>gm.</i>	Reaction in membrane of inoculated eggs		
					10 ²	10 ³	10 ⁴
1	3	0.86	0.40	+0.46	+	+	-
2*	7	2.17	2.18	-0.01	-	-	-
3	7	1.71	1.47	+0.24	+	+	-
4	7	1.50	0.96	+0.54	+	+	+
5	7	1.63	1.47	+0.16	-	-	-
6	7	2.40	2.10	+0.30	+	+	+
7	7	2.11	1.79	+0.32	+	+	+
8	7	2.02	1.81	+0.21	+	+	+
9	7	3.21	2.93	+0.28	+	+	+
10	7	2.65	2.58	+0.07	-	-	-
11	7	2.95	2.32	+0.63	+	+	+

* Passages 2, 5, and 7 showed a specific reaction in the membrane on subinoculation in embryonated eggs.

right. It was apparent that the residence of variola virus in the testis of the rabbit was not accompanied by any distinctive pathological alteration.

The reaction in the embryonated eggs was quite uniform throughout the entire series of 11 passages. The 10th testis suspension, which showed a questionable reaction on primary inoculation in eggs, was carried through 4 additional transfers. There was no indication in these eggs or in those of the other rabbit passages of the necrosis and lethal action on the embryo which characterize vaccinia.

The Minnesota Strain of Variola.—Similar experiments were made with the Minnesota strain of variola virus which had then been transferred 183 times in embryonated eggs. The specific virus was not recovered from the testes of rabbits killed on the 7th day after intratesticular injection. The virus was locally demonstrable in rabbits killed on the 3rd and the 5th day but attempts

to maintain it in the testis by successive passages were unsuccessful. At 5 day intervals the virus was demonstrable through the 3rd passage but not thereafter, and at 3 day intervals it survived only the initial injection.

The rabbits used in these tests were injected with egg membrane suspensions of the 183rd through the 200th transfers of the Minnesota strain. The testes of the 3 rabbits killed on the 7th day showed only minor differences in weight and color, and in suspension failed to produce any reaction in embryonated eggs.

The testes of the 2 animals killed on the 3rd and the 5th day, respectively, were also similar in appearance but produced a typical variolous reaction in embryonated eggs.

The first rabbit in the passage series killed at intervals of 5 days was the only animal in the entire group of experiments to show significant macroscopic changes in the injected testis. The left organ was 1.3 gm. heavier than the right, the superficial blood vessels were markedly distended, and there was a large red area on the surface. It was sterile bacteriologically but contained the specific virus in fair concentration, all 3 dilutions producing a reaction in embryonated eggs. The testes of the 2nd and 3rd passage rabbits killed on the 5th day showed only minor differences in appearance and were essentially normal. Virus was demonstrable but was evidently present in low dilution as only a few focal areas were produced by the 1:100 dilution. The testes of passage rabbits 4 and 5 were also similar in appearance but failed to produce any reaction on 2 successive egg transfers. The series was then discontinued.

Variola virus was demonstrable only in the testis of the first rabbit in the series killed at 3 day intervals. Three subsequent passages were made but the testicular suspensions were all inactive on inoculation in embryonated eggs, 2 transfers being made.

Stability of the Chinese Strain on Passage in the Rabbit Testis

The preceding observations on the Chinese strain of variola virus indicated survival in the testis of the rabbit for at least 11 passages with no apparent species alteration during this period of continued residence. Additional experiments were carried out to determine whether any change in the nature of the virus could be detected by other procedures.

Saline suspensions of the testes from the rabbits of passages 9, 10, and 11, shown to contain variola virus by the subinoculation of embryonated eggs, were inactive in the scarified skin of susceptible rabbits. The reaction in each animal was limited to a slight and transient local hyperemia, apparent on the 2nd day and lasting through the 4th. There was no subsequent crusting or scab formation.

In these tests the hair was removed from the side of the rabbit with an electric clipper. Areas approximately 0.5 inch square were lightly scratched with a surgical needle and 0.1 cc. of the suspension rubbed into them, any surplus fluid after several minutes' exposure being drained off with a capillary pipette.

The first intimation of an altered reaction by the passaged Chinese strain of variola was obtained on skin inoculation of the virus after it had been transferred from the testis to embryonated eggs. Rabbits inoculated in the skin with the earlier egg transfers from passages 9, 10, and 11 showed a significantly intensified local reaction. As such it was not characteristic of vaccinia virus, but since it might represent the activity of virus in the process of transformation particular attention was paid to it.

Subsequent tests on these egg membrane suspensions containing the passaged Chinese strain of variola failed, however, to demonstrate vaccinia or a vaccinia-like virus. Skin suspensions from rabbits which showed the local reaction mentioned above were inactive on passage to the skin of susceptible rabbits. Moreover, on recovery from the reaction there was no demonstrable protection afforded to vaccinia virus later introduced in the skin.

Earlier work (5) having shown that vaccinia virus ultimately predominated on transfer in embryonated eggs when inoculated in low concentration together with a high concentration of variola virus, successive egg transfers were made of the passaged Chinese strain with the view of concentrating vaccinia virus should it be present in the inoculum. Ten successive egg transfers were made of the virus from the testis of the 10th passage rabbit. There was no departure from the typical reaction of variola in any of the inoculated eggs. The later egg transfers also failed to produce the intensified skin reaction in rabbits which characterized the earlier membrane suspensions and were likewise inactive in the skin of a calf.

Three rabbits inoculated intradermally with the 2nd egg membrane transfers from the testes of passages 9, 10, and 11 showed a distinct local hyperemia diffused through the scarified area. It was first observed on the 2nd day and faded gradually after the 5th or 6th day. The skin was somewhat thickened, flabby, and sensitive to the touch. On the 13th day there was some slight crusting over the inoculated area.

Two rabbits inoculated intradermally with suspensions of skin removed on the 5th day from rabbits which had received the 4th and 5th egg transfers, respectively, of virus from the testis of passage 10 (the skin in each case being hyperemic when removed) showed only a transient local reaction with slightly increased color which faded after the 2nd day and no thickening.

One rabbit inoculated intradermally with the 2nd egg membrane transfers of the testes from passages 9, 10, and 11, and one rabbit similarly inoculated with the 3rd egg transfer of the testis from passage 10 alone were tested intradermally with vaccinia virus 3 months after recovery from the initial reaction. The first rabbit showed a typical graded reaction with vaccinia virus in dilutions from 10^4 through 10^6 , and the second through 10^7 , as did also a previously normal rabbit inoculated with the same dilutions of virus.

The embryonated egg series started with the testis from the 10th rabbit passage was continued at intervals of 3 to 5 days through the 10th transfer, the inoculum being a 10 per cent membrane suspension in saline. There was no necrosis of the

inoculated membrane on any of the eggs and the embryos were all active. Rabbits were inoculated intradermally with egg transfers 2 through 10. All of these suspensions through the 8th produced a local reaction with varying degrees of color but little or no thickening of the skin. There was no significant reaction with the 9th and 10th transfers, merely the slight transient flush seen with variola virus not previously passed in the rabbit testis and sometimes also with membrane suspensions from normal uninoculated eggs.

DISCUSSION

After 24 transfers in embryonated eggs the Chinese strain of variola virus was established in the testis of the rabbit and maintained for 10 successive passages at intervals of 7 days, the actual period of residence being 73 days. In the presence of the specific virus, indicated by subsequent inoculation of embryonated eggs, the testis showed neither macroscopic nor microscopic changes suggestive of the pathology associated with the viruses of the pox group. It seems evident, however, that the virus multiplied in the rabbit testis during this period. The series of 11 passages representing a tenfold dilution with each passage is comparable to a dilution series through at least 10^{11} which is well beyond the activity limit of an egg membrane suspension. In spite of the absence of specific lesions it is probable that multiplication occurred within the tissue cells of the host. Cells from the chorioallantoic suspension introduced with the inoculum might conceivably have furnished the necessary substrate in the first passage but not in subsequent ones which were made with suspensions of the testis.

The Minnesota strain of variola virus showed no evidence of alteration in the direction of vaccinia virus as the result of long continued transfer in embryonated eggs. Unlike the Chinese strain, however, attempts to maintain it in the rabbit testis were unsuccessful. The virus was recoverable on the 3rd and 5th days after initial injection but not on the 7th. It failed to survive beyond the third passage on successive transfer at 5 day intervals. It had been transferred 180 times in embryonated eggs when these experiments were begun, whereas the Chinese strain had been transferred only 12 times. Since neither strain was inoculated intratesticularly in rabbits prior to its propagation in the egg, we have no real basis for a comparison of their subsequent behavior in the testis. The two strains may have differed initially in this respect. The Minnesota strain, however, was subjected to a much more rigid selection than the Chinese strain by reason of prolonged residence in the embryonated egg and may, accordingly, have emerged with certain altered characteristics. Whatever the explanation, the two strains of variola virus as tested showed a significant difference in their adaptability.

The Chinese strain of variola virus failed to produce a reaction indicative of pox in any of the rabbits of the passage series though it was demonstrable by

the subinoculation of embryonated eggs. The testis suspensions from the last 3 animals in this series were likewise inactive on skin inoculation in susceptible rabbits. Membrane suspensions of embryonated eggs inoculated from the testes of these rabbits did, however, produce an inconspicuous though definite reaction in the skin of rabbits. It was thought that this reaction might well represent the first step in the transformation of variola to vaccinia virus, similar to that reported by Horgan (2) on direct skin transfer from the rabbit testis. The reaction was not intensified by subsequent passage and repeated tests both in rabbits and embryonated eggs failed to demonstrate any virus other than variola. We have no adequate explanation for this transient skin reaction, but since vaccinia virus was not recoverable we feel justified in assuming that it did not represent a vaccinia transformation. It is possible that some other infective agent present in the testis suspensions was carried along with the variola virus in the embryonated egg and finally lost. It seems unlikely that this explanation is applicable to vaccinia virus which is significantly more active than variola in the egg, as indicated by our earlier experiments on the simultaneous inoculation of these two viruses in unbalanced concentration (5).

We interpret the preceding experimental findings as indicative of a marked stability on the part of variola virus in spite of drastic experimental manipulation. A difference was observed in the behavior of the two strains employed which may be referable to this manipulation, but in our opinion no change was observed which exceeded the limits of species identity. In view of the findings of other investigators, in particular the well controlled work of Horgan, it should be emphasized that the viruses used in our experiments were not strictly comparable since they were subjected to the selective action of the embryonated egg prior to animal inoculation. We believe, however, that the stability of variola virus, herewith reported, should be considered in critically appraising the transformation hypothesis.

SUMMARY

After 24 transfers in embryonated eggs a strain of variola virus (Chinese) was established in the testis of the rabbit and maintained for 11 passages at intervals of 7 days. Residence in the rabbit testis was not accompanied by any significant alteration in the species identity of the virus.

A second strain of variola virus (Minnesota) was transferred 180 times in embryonated eggs with no apparent change in its behavior. Subsequent attempts, however, to maintain this strain in the rabbit by serial testicular passage were unsuccessful.

These observations are discussed in relation to the so called transformation of variola to vaccinia virus by animal passage.

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