FURTHER OBSERVATIONS ON THE SURVIVAL OF VACCINE VIRUS SEPARATED FROM LIVING HOST CELLS BY COLLODION MEMBRANES

By RALPH S. MUCKENFUSS, M.D.

(From the Department of Internal Medicine, Washington University, St. Louis)

(Received for publication, November 28, 1930)

In a previous communication (1) it was reported that vaccine virus diluted in a solution of one part serum and two parts Tyrode's solution survived incubation for 4 days at 37°C. if separated by a semipermeable membrane from a suspension of minced kidney tissue in a similar mixture of serum and Tyrode's solution. The survival of the virus, however, was not complete. If kidney cells that had been killed by repeated freezing and thawing were added to the vaccine virus under these conditions, the survival as determined by the intensity of the skin reaction in rabbits was greater, and seemed to be almost complete. The possibility was considered that the virus might increase under these conditions and this was the first point studied in the present series of experiments.

Methods and Materials

The technique was detailed in the previous report (1). Briefly, the apparatus consists of two tubes, one of which can be inserted into the other. A shoulder is made on the smaller tube and ground to fit the top of the larger tube, making a perfect joint. The lower end of the inner tube is left open, and a collodion sac is attached to it, thus making the assembled apparatus consist of two chambers separated by a collodion membrane. The collodion sacs were arbitrarily standardized by measuring the amount of water that would pass through in 3 minutes under a pressure of 1 m. of water. The membranes used in this series of experiments varied in permeability from 0.65 cc. to 1.09 cc. of water passing through the membrane in 3 minutes, with an average permeability of about 0.75 cc.

The neurovaccine of Levaditi, prepared by testicular inoculation was used exclusively. The testicles of rabbits were removed on the 4th day after inoculation and, after trituration with sand in a sterile mortar, about 10 cc. of Locke's solution was added. The testicular emulsion was centrifuged at high speed for

20 minutes and the supernatant fluid was pipetted off for use. The menstruum used throughout was a fluid composed of one-third normal rabbit serum, and two-thirds Tyrode's solution.

At the end of each experiment the different suspensions (triturated in a sterile mortar without sand if tissue was present) were tested for the presence of vaccine virus by inoculating 0.25 cc. into the shaved skin of a rabbit. Stained films of each preparation were examined and all those showing bacterial contamination were discarded.

In quantitative determinations, serial dilutions were made in Locke's solution in multiples of ten, and 0.25 cc. of each dilution was inoculated into the shaved skin of a rabbit.

Each experiment was controlled by adding virus to normal tissue in a mixture of serum and Tyrode's solution according to the technique of Maitland and Maitland (2), and incubating in test tubes and in Carrel flasks. The virus invariably survived under these conditions, and the reactions resulting from inoculation of the virus in the Carrel flasks were always greater than from that in the test tubes. This would be expected from the observations of Maitland and Laing (3) on the necessity of free access of air for the increase of the virus, since the ratio of surface to volume in Carrel flasks is greater than in test tubes.

Attempts at Passage in Series

Experiment 1.—Normal minced kidney tissue was placed inside the collodion sac of a dialyzing apparatus. In the outer chamber, vaccine virus was placed with kidney cells that had been killed by repeated freezing and thawing. This fluid containing virus had first been titrated by inoculating serial dilutions into the skin of a rabbit.

The dialyzing apparatus was opened after 4 days incubation at 37°C. A portion of the fluid in the outer chamber, which contained virus, was diluted 1:10 in a fresh suspension of killed cells and placed in the outer chamber of another dialyzing apparatus, with a fresh suspension of normal minced kidney tissue in the inner chamber. These passages were always made in duplicate. The virus removed from the outer chamber was titrated by intracutaneous inoculation of serial dilutions. The suspension of living cells from the inner chamber was always tested by intradermal inoculation to control the impenetrability of the membrane to vaccine virus.

This procedure was repeated with each passage.

The inoculated rabbits were observed daily, and the highest dilution showing a vaccinal reaction was considered to be the titer of the virus. By recording the highest active dilution it was possible to construct a curve showing the variation in concentration of the virus as the experiment progressed.

The results of two typical experiments are shown in Fig. 1. One line shows the curve that would be expected if survival were complete

and the concentration had diminished directly in proportion to dilution. The other two lines, recording the observations in two experiments, show that survival was not complete. With each passage there was a decrease in concentration, and the quantity of virus demonstrable

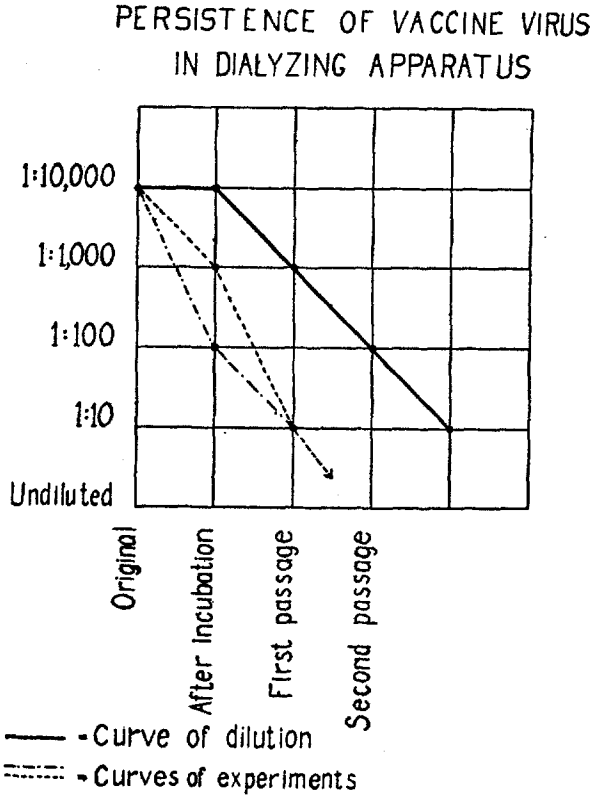

FIG. 1

at the end of each period of incubation was less than had been placed in the dialyzing apparatus.

It seems probable, therefore, that the intense reactions observed in the experiments previously reported represented the survival of a considerable part, but not all, of the virus.

The Effect of Tissue on the Intensity of the Reaction

In the original report it was shown that the reaction was more intense if virus was incubated with dead cells. In view of the obser-

vations of Duran-Reynals (4) on the influence of tissue extracts on vaccinal reactions, it seemed desirable to ascertain if the tissue played any part in the greater intensity of the reactions that had been previously observed. The work of Duran-Reynals was done with fresh tissue. Because of the 4 day period of incubation employed in this study, the action of fresh tissue was compared with that of tissue which had been incubated for 4 days. The action of normal cells was also compared with that of cells that had been killed by repeated freezing and thawing.

Experiment 2.—Two suspensions of minced kidney tissue similar to those previously used were prepared. Normal kidney tissue was used in one tube, and cells that had been killed by repeated freezing and thawing were used in the other. These tubes were incubated for 4 days. Two similar preparations were then made from fresh materials. Vaccine virus was then added to all of these tubes and also to a tube of serum and Tyrode's solution containing no tissue. After standing for a few minutes the emulsions containing tissue were triturated, and 0.25 cc. from each tube was inoculated into the shaved skin of a rabbit.

The rabbit was observed daily for 4 days and the reactions recorded.

While there was some increase in the intensity of the reactions in the presence of tissue, the increase was very slight, and there was no difference in the effects of the different tissue preparations.

This experiment was conducted with freshly prepared virus, and it seemed desirable to repeat the experiment using virus and cells that had been handled as nearly as possible in the same manner as had been done in experiments using the dialyzing apparatus.

Experiment 3.—In one dialyzing apparatus normal cells were placed in the inner chamber and vaccine virus in the outer chamber. In another, normal cells were placed in the inner chamber and a suspension of killed cells in the outer chamber. These, along with a suspension of normal cells in a test tube were incubated for 4 days. After incubation the virus from the outer chamber was removed and divided into three portions. To one portion an equal quantity of Locke's solution was added; to the second an equal quantity of the dead cells from the outer chamber of the second dialyzing apparatus was added; and to the third an equal quantity of the incubated suspension of normal cells. The suspensions containing cells were triturated and intracutaneous inoculations were made from each mixture.

Table I, which is typical of several such experiments, shows that the reaction was intensified by the addition of cells. In this experiment

the reagents were the same as in the inoculations from the dialyzing apparatus in previous experiments, but the virus and tissue were diluted to half their original concentration in placing them together.

The results of these experiments cannot be compared quantitatively with those of Duran-Reynals, as the quantities that he employed differ from those used in this study.

The Effect of Dead Cells on Survival

The action of kidney tissue in enhancing the skin reaction to vaccine virus made necessary a quantitative study of the survival of virus alone, as contrasted with the survival of virus to which killed cells had been added.

TABLE I

Control (virus plus Locke's solution)	++
Virus plus frozen and thawed kidney	+++
Virus plus normal minced kidney	+++±

TABLE II

Date	Virus plus dead cells	Virus without dead cells
Nov. 25, 1929	10^{-4}	10^{-3}
June 28, 1930	10^{-3}	10^{-3}
	10^{-3}	10^{-2}
	10^{-2}	10^{-2}

Experiment 4.—The same arrangement of the dialyzing apparatus, with living cells in the inner chamber, was employed. Virus alone was placed in the outer chamber of some, while virus with dead cells was placed in the outer chamber of others. After 4 days incubation the different virus preparations were titrated in the manner previously described.

The results of four such parallel titrations are recorded in Table II. In two instances the titer was higher when dead cells were present; in the other two the same titer was obtained.

While the difference is not striking, the method used can measure concentration only in multiples of ten, and the results suggest an increased survival of virus in the presence of dead cells. This sug-

gestion is further borne out by one experiment recorded in Table IV, in which no survival of the virus alone could be detected, although a definite reaction followed inoculation of virus that had been incubated with dead cells.

TABLE III

Container used for incubation	Vaccine virus	Vaccine virus plus dead cells	Vaccine virus plus extract of dead cells
Test tube	−	−	−
Dialyzing apparatus	−	+	?
Test tube	−	±	±
Dialyzing apparatus	++	+++	+

TABLE IV

Container used for incubation	Normal kidney plus vaccine virus (Maitland culture)	Vaccine virus		Vaccine virus plus dead cells		Vaccine virus plus cysteine hydrochloride		Vaccine virus plus dead cells plus cysteine hydrochloride	
Test tube	+++	−	−	−		−	−	−	?
Carrel flask	+++±			−		−	−	−	−
Dialyzing apparatus		+		++		++	++	++	++
Test tube	+	−	−	+		?	?	±	±
Carrel flask	++++			+		?	?	±	±
Dialyzing apparatus		+±	++	++±	++	+	+±	++	+++
Test tube	+	?	−	±		+±	?	±	?
Carrel flask	+++			−		−	−	±	±
Dialyzing apparatus		+±	+	+++	+++	+±	+±		+++
Test tube	+	−	−	−		−	−	−	−
Carrel flask	++			±		±	−	+±	+±
Dialyzing apparatus		±	±	++	++	+	±	+++	+++

The method used is inaccurate at best, and when virus is mixed with tissue, a special source of error must be considered. Even after several minutes trituration, some gross fragments of tissue were still present, and it is possible that these either adsorbed or took up in other ways a considerable amount of virus, making accurate dilution impossible.

The Effect of Tissue Extracts on the Survival of Virus

It seemed desirable to ascertain if the presence of the dead cells themselves was necessary for the increased intensity of the skin reactions. To study this question, extracts of the dead cells were prepared, and the action of the extracts was compared with the action of the extracted cells.

Experiment 5.—Minced kidney tissue that had been repeatedly frozen and thawed was added to Tyrode's solution in the proportion of 0.66 cc. of tissue to 12 cc. of Tyrode's solution. After thorough mixing, the suspension was allowed to stand for a few minutes and then centrifuged. The supernatant fluid was pipetted off, and serum and vaccine virus were added to it in the proportions previously described. The cells were resuspended in Tyrode's solution, and serum and vaccine virus added in the same manner.

These suspensions were incubated for 4 days on the opposite side of collodion sacs from live cells and then were tested by intracutaneous inoculation into rabbits.

The results were somewhat variable, but there was never any appreciable enhancement of the reaction as a result of the addition of the extracts, and in most instances the size of the reactions was diminished. In Table III the results of two typical experiments are recorded. Although the extracts were inactive, the extracted cells were still capable of causing an increased skin reaction. It is interesting to note that in the first experiment recorded in the table, the vaccine virus alone did not survive in the outer chamber of the dialyzing apparatus, although the virus with dead cells showed definite survival. This is the only experiment in which virus has not survived under the experimental conditions described.

The Effect of Cysteine

The observations of Mueller (5), Zinsser and Tang (6) and of Long and Olitsky (7) on the usefulness of cysteine in the preservation of viruses, together with the observations of Dubos (8) on its rôle in culture media used for the cultivation of pneumococci, made it desirable to ascertain if this substance could have any effect on the survival of vaccine virus under the experimental conditions used in this study.

Experiment 6.—A 10 per cent solution of cysteine hydrochloride (Pfanstiehl) in phosphate buffer at pH 7.6 was prepared and sterilized by filtration through a

Berkefeld N candle. The dialyzing apparatus was used as before, and in addition, the solution of cysteine hydrochloride was added to some of the virus preparations in the outer chambers so that the final concentration was 0.1 per cent or 0.15 per cent. After incubation for 4 days intracutaneous inoculations were made.

The results recorded in Table IV show that, while the skin reactions in a few instances were somewhat greater when cysteine hydrochloride had been added, this was not uniformly the case, and the effect was not great in any instance.

COMMENT

The results strongly suggest that the survival of vaccine virus made possible by the presence of live cells on the opposite side of a collodion membrane is further increased by admixing dead cells with the virus. It might be urged that the more intense skin reactions are due entirely to the effect of kidney tissue in enhancing the local reaction (Reynals factor). However, titration by serial dilution suggests an increased survival, and in one experiment survival occurred in the presence of dead cells while failing to occur in their absence. Extracts of dead kidney cells had no enchancing effect under the conditions of these experiments.

The reason for the survival of virus when incubated on the opposite side of a collodion membrane from a suspension of normal kidney tissue is uncertain. Two possibilities, however, suggest themselves. One is that some factor necessary for survival diffuses through the membrane from the living cells to the virus; the other is that the living cells utilize some substance, possibly oxygen, which if present in sufficient concentration would result in the inactivation of the virus.

SUMMARY

The survival of vaccine virus when incubated on the opposite side of a collodion membrane from a suspension of fresh minced rabbit kidney was not complete in these experiments, and passage in series was not successful. The degree of survival seemed somewhat greater if dead cells, killed by repeated freezing and thawing, were added to the virus during incubation, although the tissue was able to increase the intensity of the skin reactions. Extracts of dead kidney cells did not increase the degree of survival, as determined by the intensity of

the skin reaction. No significant or constant increase in the intensity of the skin reactions resulted from the addition of cysteine hydrochloride to the virus in the dialyzing apparatus.

BIBLIOGRAPHY

1. Muckenfuss, R. S., and Rivers, T. M., *J. Exp. Med.*, 1930, **51**, 149.
2. Maitland, H. B., and Maitland, M. C., *Lancet*, 1928, **2**, 596.
3. Maitland, H. B., and Laing, A. W., *Brit. J. Exp. Path.*, 1930, **11**, 119.
4. Duran-Reynals, F., *J. Exp. Med.*, 1929, **50**, 327.
5. Mueller, J. H., *J. Exp. Med.*, 1928, **48**, 343.
6. Zinsser, H., and Tang, F. F., *J. Immunol*, 1929, **17**, 3.
7. Long, P. H., and Olitsky, P. K., *Proc. Soc. Exp. Biol. and Med.*, 1930, **27**, 380.
8. Dubos, R., *J. Exp. Med.*, 1929, **49**, 559.