IMMUNOLOGICAL STUDIES WITH HERPES VIRUS WITH A CONSIDERATION OF THE HERPES-ENCEPHALITIS PROBLEM.

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I.

Since 1923 one of us has been attempting to transfer virus from human encephalitis cases to animals, using rabbits for most of the experiments and for a few of them monkeys. In the course of this work it has become necessary to make a thorough study of the virus of herpes febrilis, for the obvious reason that this virus has been identified with that of encephalitis lethargica by a great many experienced workers in the past (1). We will deal with our attempts to obtain a virus transmission from man to animals in a later section of this paper. Before approaching this phase of the problem, however, we wish to report briefly upon studies made with the herpes virus itself, and more particularly upon phenomena of immunity which can be experimentally studied with this virus more easily than with most other filterable agents. Moreover, some of the phenomena encountered in this study have, we believe, distinct bearing upon the encephalitis-herpes question.

Our work has been done with three strains of herpes, all of them isolated from vesicles on laboratory workers, some of them carried through several years. Unlike some of the British virus, our own strains have been extremely resistant when preserved in glycerol, one of them recently causing typical death in a rabbit, without material prolongation of the incubation time, after $11\frac{1}{2}$ months in 50 per cent neutral glycerol in the ice box.

Dosage of virus has been a matter to which we have given considerable thought, and in which we have tried to give attention not only

to the actual amounts of brain substance and dilution used, but also to the possibility that the virus might be irregularly distributed throughout the brain. In addition to the method of emulsifying weighed amounts of brain in definite volumes of salt solution, we have followed a method first used in this laboratory by Breinl, in which an entire brain or entire hemisphere was emulsified by grinding in sterile sand, gradual additions of salt solution being made up to 100 cc., and subsequent dilutions made from this. The dilutions in such procedure were calculated from the total brain as a basis. Thus one one-hundred-thousandth of such a dilution would indicate approximately a hundred-thousandth part of the entire brain.

In working with the virus, the interesting observation was made that if prolonged grinding in sand was carried out, and the suspension thus obtained was centrifuged at high speed for an hour or longer until the supernatant fluid was almost clear, rabbits inoculated with such supernatant fluid died often a day or two earlier than those inoculated with the sediment, indicating—to our minds—that in the brain the virus is intracellularly localized and that it is to some extent discharged from the cells in the process of trituration. It is not impossible that the intracellular position of the virus may have some bearing on the irregular results obtained by various observers in experiments upon *in vitro* neutralization by immune sera. Another way of obtaining virus relatively free from cells, which, however, we have not used in these experiments except to determine the possibility, is by tapping the cisterna magna when the animal begins to show symptoms, such spinal fluid being virulent at the time.

Active Immunization.

On this subject little can be added to what is already known, and our results have been consistent in themselves and in harmony with previous reports. The method we have used chiefly has consisted of a preliminary inoculation upon the cornea, followed by intracerebral[1] test doses at varying intervals. Active immunity has regularly followed survival from infection of the eye, appearing not earlier than $2\frac{1}{2}$ weeks after the eye inoculation and lasting up to as long as 6

[1]For intracerebral inoculation ether anesthesia was used.

months. Later tests have not so far been made. Active immunity can also be obtained by preliminary skin inoculation as practised by Perdrau, and by intratesticular inoculation.

Neutralization of Herpes Virus with the Sera of Actively Immune Animals.

The literature on this question has been contradictory, some workers denying the neutralizing value of convalescent and immune sera; others, notably Flexner, having no apparent difficulty in such neutralization. Our first three experiments were entirely negative, although we titrated our infecting doses down to five times the minimum fatal amount. In some of these experiments, indeed, the rabbits inoculated with virus-serum mixtures died sooner than the saline controls. This we have now determined is due to a rapid deterioration of the virus when incubated with salt solution, a deteriorating effect prevented in the presence of normal serum.

These negative results agreed in general with those published by Perdrau, but were inconsistent with those of Flexner and others. We therefore continued with them, thinking that probably such differences might be due to fortuitous variations in the sera of individual actively immunized rabbits, depending perhaps upon the method of active immunization, the severity of the herpetic process, or the time factor.

Subsequent experiments were done by a simple technique in which about 50 minimum fatal doses of the virus, that is 0.1 cc. of a 1:10,000 dilution of fresh or glycerolated herpetic brain, were incubated with 1 cc. of either the active or inactivated serum of rabbits that had been immunized by a corneal inoculation and subsequently tested for immunity by an intracerebral one. The mixtures were kept at $37\frac{1}{2}^{\circ}$ from 5 to 6 hours, and amounts of 0.25 cc., representing, thus, something over 10 fatal doses, were intracerebrally injected into normal rabbits. Controls with incubated saline and normal serum suspensions were used.

First Experiment.—The serum of the actively immunized rabbit was obtained 25 days after the second injection. In this experiment the saline control died in 9 days and the rabbit receiving the immune serum-virus mixture gradually lapsed into a lethargic condition from which it recovered in 3 weeks. This

rabbit will be discussed below. No normal serum control was made in this experiment.

Second Experiment.—The serum here used was taken 52 days after the second or intracerebral inoculation of the actively immune animal. The normal serum control died typically on the 4th day, the saline control after 6 days, and the immune serum animal died on the 8th day.

Third Experiment.—The serum used in this case was taken 59 days after the second or intracerebral virus inoculation of the immunized animal. The experiment was somewhat varied from the others in that both activated and inactivated immune and normal serum mixtures were made in order to determine any differences produced by inactivation. The rabbits receiving the mixtures in which immune serum, active and inactive, had been used survived; those in which normal active and inactive serum were used died on the 8th day, while the animal receiving the incubated mixture of virus and saline died after a protracted illness lasting 15 days, and the inactivated virus controls died on the 8th and 11th days, respectively.

From these and similar experiments we may conclude that the serum of rabbits actively immunized by corneal inoculation and subsequently reinjected intracerebrally develop definite protective bodies in their serum which probably do not appear sooner than the 3rd and 4th weeks in sufficient quantity to yield a clean-cut experiment.

Experiments like those above, however, are merely examples of the general type obtained. The results are irregular, thus accounting for discrepancies in the literature, but we do not believe that this need necessarily be accounted for by fluctuations in the potency of the sera themselves.

Irregularities are rather due, we believe, to the fact that in the ordinary technique of using brain material for virus, even when accurately measured dilutions are used, some of the virus is intracellular and some extracellular, and the serum protective bodies, whatever they may be, may therefore be blocked from complete contact with the intracellularly localized fraction. We have discussed this in our introduction, where we mentioned the fact that when virulent brain material is triturated for an hour in sterile sand, taken up in Ringer's solution, and centrifuged for an hour or more, the supernatant fluid, which is clear and relatively cell-free, kills a little more quickly than do equivalent volumes of the sediment.

To some extent the potency of sera, we think, may be enhanced by

what may be spoken of as hyperimmunization. We have treated a number of rabbits after they had developed active immunity with repeated intracerebral injections and large intraperitoneal injections of potent virus, and in this way have obtained sera which exhibited considerably more regularity in the neutralization of herpes virus. Comparative quantitative results cannot yet be made with sufficient accuracy to permit more than a very definite probability in favour of the enhancement of potency by such a method.

Immunization with Brain Extracts.

There has been some discussion in the literature concerning the possibility of the location of the immune substance in the suceptible cells; namely, those of the central nervous system. Here, again, there have been considerable contradictions, Perdrau (2), indeed, finding that the brain extracts of an immunized animal, far from immunizing, increased the virulence of the virus that had been in contact with it. His results in the latter respect may have been due to the salt solution effects that we have mentioned above.

Having determined that the brain suspensions of an immune animal do not retain active virus, we carried out a number of experiments analogous to those carried out with serum in which we incubated active virus with brain extracts of immune animals.

The brain extracts were made by grinding an entire hemisphere with sand for ½ hour, gradually adding 20 cc. of saline. The mixture was shaken for 2 hours, then set in the incubator for an hour, and again shaken for 15 minutes while warm. In some cases it was extracted in the ice chest for longer periods, but in others this was omitted. Finally, the material was centrifugalized at high speed and the supernatant fluid used.

First Experiment.—In this experiment the brain of an immune rabbit was taken out 74 days after the second injection and the rabbit bled at the same time. In other respects the experiment was carried out in a manner similar to that used with the serum tests. In this experment, curiously enough, there was no protection whatever by the brain extract, and the serum did nothing but prolong the life of the rabbit for 3 days beyond the control, and 2 days beyond the brain extract one, dying on the 8th day.

Second Experiment.—In this experiment, in which the brain was removed from the immune animal 62 days after the second inoculation, the result was approximately the same. Again, the serum protected, prolonging life until the 10th day, 4 days beyond the normal serum control; the brain extract rabbit dying on the 7th day.

These two experiments may therefore be regarded as practically negative.

The following experiment was carried out in connection with those on the protective properties of hyperimmunized rabbit serum.

The brain of a rabbit which had received an eye inoculation on August 31 and subsequently four intracerebral inoculations, the last one on December 21, was taken out 21 days after the last injection. Blood was obtained at the same time. The method of extraction was essentially similar to the one described above. Mixtures were made in which 1 cc. of the fresh brain extract was added, respectively, to 0.15, 0.1, and 0.05 cc. of a 5 per cent fresh virus suspension. A control was made of 1 cc. of the serum of the same rabbit with 0.2 cc. of the virus, and salt solution controls set up with 0.05 cc. of the virus, that is the smallest dose, mixed with the brain. After an incubation of 4 hours, 0.2 cc., respectively, of the mixtures was injected into rabbits. The control died in 9 days; the two rabbits receiving brain extract plus the larger doses of virus died in 5 and 10 days, respectively, while the rabbit receiving the smallest amount of virus plus brain extract, an amount equivalent to that received by the saline control, survived, as did the rabbit receiving the hyperimmune serum plus four times the amount of virus which killed the control and four times the amount of virus which killed one of the brain extract rabbits.

This experiment and another like it seem to prove conclusively that, while the brain extracts of hyperimmunized rabbits may contain a limited amount of protective substance, this is considerably less than that contained in the circulating blood of the same animal.[2]

Passive Immunization with Serum by Injecting the Serum Both Intravenously and into the Cisterna Magna the Day before Injecting the Virus.

All the experiments done in this direction up to the present time have been completely negative. Neither the intravenous administration of sera known to possess protective action in considerable quantities nor the injection of $\frac{3}{4}$ to 1 cc. of such sera into the cisterna magna the day before intracerebral infection has afforded even the slightest protection to the rabbits so treated.

[2] Incidentally a considerable number of complement fixations have been done in which filtrates of normal herpetic brains were used as antigen, and attempts have been made at agglutination reactions in virulent filtrates with the serum of immune animals. All of these efforts have remained entirely negative in result.

Are Rabbits Which Survive Neutralized Serum-Virus Mixtures Actively Immune?

In a number of experiments we have subjected rabbits that have survived serum-virus mixtures as above described to subsequent intracerebral inoculation with virus alone. In no case so far have these rabbits shown signs of resistance above the normal.

We report this type of experiment because we believe it adds additional proof to the other observations made that immunity does not follow unless there is an active process at least initiated in the animal during immunization.

Immunization with Phenolized Virus.

In considering the literature on immunity in connection with the filterable viruses, one gains throughout an impression consistent with the observations noted in this paper, that dead virus does not protect, or—in other words—that virus which does not give rise to at least a slight or moderate disease process is not followed by immunity. This, of course, is consistent with the many observations recorded in poliomyelitis, and observers who have studied this are unanimous in stating that monkeys who have failed for one reason or another to "take" may be actively infected in a subsequent experiment. The exception to this rule seems to be the now widely employed method of prophylactic rabies treatment by the Semple method, in which phenolized virus is subcutaneously administered. We have attempted to apply this method to protection against herpes virtually by the same method used by Semple in hydrophobia.

10 per cent suspensions, that is 5 gm. per 50 cc., of fresh herpes virus were made in a 1 per cent carbolic solution. This was incubated for 6 hours, shaken twice while incubating, and left in the refrigerator overnight. This was diluted on the following day with an equal volume of salt solution, making a ½ per cent carbolic suspension.

In attempting protection with phenolized virus made in this way, it seemed to us of the greatest importance to determine whether the carbolic acid killed or merely attenuated the active agent. This determination is incidentally of fundamental importance in appraising the immunological significance of the Semple method in rabies. We

therefore inoculated rabbits intracerebrally with 0.3 cc., respectively, of phenolized virus made as above, 6 hours, 36 hours, 48 hours, and 5 days after the phenol had been added. None of these rabbits, not even the one inoculated with the 6 hour phenolized material, showed any symptoms, even a temperature, and all remained healthy and well. None of them were immunized by the procedure. The only one that died was the 5 day one which died of diarrhea and pneumonia some time after the inoculation.

We can only conclude that the phenol used in the manner indicated by Semple kills the herpes virus.

Two series of three rabbits each were then treated with daily injections of the phenolized virus for 14 days. Test injections with virulent material were made 1, 2, and 3 weeks after the last dose of the phenolized material. In no case was there the slightest protection. Here again, therefore, we are in a position to conclude that no protection whatever is afforded by dead virus.

Believing that possibly the manner of administration might make a difference, we reinoculated intracerebrally some of the rabbits in which the phenolized virus had been administered in the same manner to test whether it was dead or not, and found that these rabbits, likewise, were unimmunized, even though the phenolized material had been given intracerebrally.

II.

The Herpes-Encephalitis Question.—In the course of the last 3 years we have attempted to transfer a virus from human encephalitis to animals with material of some eight cases.[3] In at least four of these the pathological examination seemed as reasonably certain in indicating encephalitis lethargica as this is possible, at the present time, by histological examination. In two of the cases where the possibility of poliomyelitis existed, this was ruled out by negative intracerebral monkey inoculation.

Experiments with Rabbits.—A large number of rabbits were used in these experiments, but we do not describe these inoculations in detail

[3] We have used spinal fluid from a number of other cases which, however, on later study could not be considered as representing probable encephalitic infection.

because, rigidly analyzed, they must all be regarded as negative. A good many of our rabbits died, and in some of the earlier cases we obtained a few rabbits that died in series up to the fourth generation from the original case. Careful autopsies and cultures in some of these cases revealed other possible causes of death and never have we obtained a typical herpetic syndrome in such animals. While a not inconsiderable number of the rabbits in these experiments died without morphological or cultural evidence of bacterial infection, our inability to carry on a virus in series, the complete absence of anything like an herpetic syndrome, and our later suspicions of possible spontaneous encephalitis justified us in rejecting any suggestions that we had killed any of these animals with a virus emanating from the human encephalitis case.

In our recent cases, in which the rabbits were more closely observed than before, we often obtained a type of languid and almost somnolent condition in many of the inoculated rabbits which was characterized by completely relaxed attitudes in the cages and immobility continuing for hours when the animals were not disturbed. Such animals, however, were always quite alert and normal when roused into activity either mechanically or by the proffer of food, and few of them died, even though observed for long periods. The two or three that did die usually had diarrhea, and transfer of material from their nervous systems did not produce disease in other animals. We believe it likely that the somnolent condition described may represent the clinical picture given by Kling and others which, however, as observed by us, was entirely unconvincing. We may summarize our attempts of directly transferring human encephalitic virus to rabbits with the statement that never, in spite of the use of a large number of animals, have we in any single instance produced anything simulating the herpetic syndrome in such animals, and the fortunate freedom of our experiments from spontaneous rabbit encephalitis has resulted in very few deaths of rabbits which in any manner would have tended to mislead us into thinking that we had made a successful transfer.

Attempt to Repeat Perdrau's Method of Repeated Inoculations.—Perdrau has recently described a procedure with which he reports successes in starting an herpetiform virus in rabbits by repeated inoculations of glycerolated encephalitic brain given on alternate days,

three inoculations to each rabbit, alternately on the skin, into the brain, and again upon the skin. He has varied this in a number of different ways. He also reports success with intracerebral inoculations of an encephalitis virus which had been preserved in glycerol for 43 days in cases in which the fresh, unglycerolated virus gave negative results. He furthermore succeeded by inoculating, together with the human encephalitic brain material, suspensions of the brain of herpes immune rabbits in which, by his own control tests as well as in repetitions of our own, no surviving herpes virus can be detected.

We tried Perdrau's method with material from three cases in two of which there seemed to be no possible question as to clinical or pathological diagnosis, and in one of these cases we persisted with the repeated inoculation technique quite beyond Perdrau's original report, administering in some cases as many as five injections. In no case did we either succeed in producing an herpetiform syndrome nor did we obtain any other type of disease in the rabbits which might have been attributed to a transmissible virus. Again, however, we noticed the prolonged somnolence, temporary loss of appetite, and that slight departure from normal which has been described above. In no case, however, did these rabbits develop the temperature changes which we have found so uniform in successful herpes inoculations.

In view of the failure of direct repetition of Perdrau's experiments, and considering the results with partial immunization of herpetic rabbits which will be recorded below, we thought it entirely logical to subject the animals treated by repeated injection of the encephalitic virus to subsequent herpes inoculation, with the idea of determining whether or not the development of a partial immunity would indicate the presence of perhaps an attenuated herpes-like virus in the encephalitis brains.

Altogether fourteen rabbits treated by the Perdrau method or some modification of it, all of them having received at least four consecutive inoculations of encephalitic material, were reinoculated with measured doses of herpes virus at periods sufficiently long after the last inoculation to warrant the expectation of some immunity. All these animals died at periods sufficiently close to those at which the controls succumbed to exclude the possibility of their having been even partially immunized by the injection of the encephalitic brain.

Partial Immunization.—Among the most interesting of the results of our experiments are those in which, either purposefully or by accident, the partial immunization of the rabbits to herpes virus was produced. These observations were begun a year ago in experiments carried out in this laboratory by Dr. Breinl.[4] They have since been extended in various ways. They are similar in result to some of those reported by Perdrau. Partial immunization was obtained either by the injection of sublethal doses of herpes virus, by which we mean the injection of 0.1 cc. of a 1:50,000 to a 1:200,000 dilution of a brain emulsion fatal in amounts of 1:10,000, or they were obtained by immunization with serum-virus mixtures. In one case the effects to be described were observed after the first inoculation, when a virus was injected which had been incubated with salt solution and thus attenuated, as described in a preceding section. It is not always possible to produce such rabbits at will, but when successful, the animals, after a slight preliminary temperature, develop gradual drowsiness and weakness at about the time that the controls die, and may go to sleep, remaining in this comatose condition anywhere from 4 to 8 days before death. Animals similar to these have been described by Perdrau. The brains of such rabbits are virulent.

One rabbit in this series is particularly interesting. This was a rabbit of about 2500 gm. which was injected on October 21 with 0.1 cc. of a 1:10,000 herpes virus obtained from the fourth brain passage of this particular material after 20 days in glycerol. The virus had been incubated for 5 hours at 37°C. before injection, which—as other experiments show—materially attenuates the virus. The animal at first showed no symptoms, but 3 weeks after inoculation it was found in a lethargic state, sleeping most of the time. Slight stimulation rapidly removed the drowsiness into which the animal at once relapsed as soon as stimulation was interrupted. The animal remained in this condition for 3 weeks, when a gradual recovery to normal occurred. During this entire course there was no fever, and careful examination elicited neither tremors nor paralysis. Muscular weakness seemed to be associated with the drowsiness, even when temporarily roused. On January 25, 1926, the animal was tested for immunity with 0.15 cc. of a 5 per cent virus suspension of a potent virus. 5 days later it developed a flaccid paralysis of the hind legs. There was no fever, but there was typical

[4]It was unfortunate that Dr. Breinl had to leave at a time when this work was started. Experiments referred to in the text were subsequently extended from observations made while he was still in this laboratory.

grinding of the teeth. A control done with the same virus at the same time died on the 30th with typical symptoms. On January 31 the condition took the form of a gradually ascending paralysis, the animal lying on its side and still moving its front legs, the head slightly bent to one side, as with a spasm. This condition continued throughout February 1. Death occurred on February 2, the entire clinical picture from beginning to end being as parallel as possible in clinical story with Case 9 of our human encephalitis series.

These experiments were considered of great importance because, in spite of all of the preceding negative evidence, the clinical picture in these cases is so strikingly similar to the conditions observed in diseased human beings that they rather open the possibility that the encephalitis and herpes virus may still be related, but that in passing through the nervous system of man and remaining there for some time, the herpes virus may have become so attenuated for rabbits that it can no longer be started in these animals unless exceptionally fortunate cases are encountered. Such exceptional circumstances might explain the ease with which earlier investigators obtained rabbit transmission, as contrasted with the negative results of Flexner and Amoss' (3) experiments and our own. Moreover, it is not at all impossible that the positive findings of herpes virus in the central nervous systems of human beings not suffering from encephalitis, as well as the innocuousness of encephalitis virus administered to men, as in Busacca and Bastai's (4) experiments, might be explained by an immunity to the virus existing in many people. For it is more than probable that human beings may develop a considerable resistance against the deeper penetration of a virus so ubiquitous in our environment and so frequently causing lesions of minor importance on the skin and mucous membranes. We mention these things because it is necessary in a subject as important as this one to consider all points of view as broadly as possible. Moreover, the extraordinary attenuation of fixed rabies virus for man furnishes a closely pertinent analogy.

SUMMARY.

In the preceding experiments observations have been reported upon the nature of herpes virus which confirm the suspicion that the virus is intracellularly located in the infected nervous system.

In regard to the immunological conditions existing in this disease, our experiments have reaffirmed that herpes virus can be neutralized with the serum of actively immunized animals and have offered an explanation for the irregularity of the results of others, as well as our own.

It has been found that brain extracts possess some virus-neutralizing power, but considerably less than the serum of the corresponding animals.

Attempts at passive immunization with neutralizing serum were uniformly negative, even when the serum was introduced into the cisterna magna 12 to 24 hours before infection with the virus.

It has been shown that active immunity can be attained only when some degree of reaction to the living virus has occurred. Rabbits which survived neutralized serum-virus mixtures did not acquire immunity nor did those treated with virus phenolized to the extent of actual destruction. This point suggests a reinvestigation of the Semple method of rabies immunization.

In so far as our studies touched upon the herpes-encephalitis problem we have uniformly failed in attempts to transfer herpes virus directly from man to rabbits. These results are in contradiction to those of most of the earlier workers, but in keeping with the recent reports of Flexner and Amoss.

Attempts to overcome the difficulty of transfer by the recently published technique of Perdrau were unsuccessful. Furthermore, animals repeatedly treated with human encephalitis material, either fresh or glycerolated, as practised in the Perdrau method, failed to acquire the slightest degree of immunity to subsequent herpes inoculation.

By the inoculation of very small doses or by infection of partially immunized rabbits, as described above, we have succeeded in modifying the characteristic herpetic syndrome in rabbits in a manner which simulates many of the clinical features of human encephalitis.

Our own experience forces the conclusion that no valid proof exists upon which can be based an assertion concerning the identity of the virus of herpes with that of encephalitis lethargica. Either the two viruses are entirely unrelated, or else prolonged sojourn in the central nervous system of man attenuates the virus for rabbits to an extent

analogous to that in which rabies virus is attenuated for man by passage through rabbits. The isolated successes of Levaditi and of Doerr and their assistants might thus be regarded as fortunate exceptions in which material incompletely attenuated had been at their disposal.

We suggest this point of view as an alternative working hypothesis largely because the results we are reporting, as well as those of Flexner and Amoss, are in flat contradiction to the reported successes of earlier workers and the more recent experiments of Perdrau. The experiments of the latter, as described, cannot be explained by the occasional existence of spontaneous encephalitis in his rabbits, nor by the assumption that a herpes virus fortuitously coexisted with that of lethargic encephalitis in his material, inasmuch as this material alone at first injection or in the unglycerolated state failed to infect. It is also possible to conceive that human beings may, by repeated skin infections, attain a not inconsiderable partial immunity to herpes virus, which would explain the nature of the clinical course (as in our partial immunity rabbits) as well as the innocuousness of direct injections of herpetic virus into man, as reported by Bastai and Busacca, and the finding of herpes virus in human beings not suffering from lethargic encephalitis.

These suggestions are discussed in order to give this important problem the broadest possible consideration.

For the time being, however, such reasoning cannot be taken as more than a logical possibility impressed upon us by our partial immunization experiments.

All other experimental evidence obtained by direct inoculations with the limited material at our disposal tends to render identity of the two varieties of viruses unlikely.

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