CONTRIBUTIONS TO THE PATHOLOGY OF EXPERIMENTAL VIRUS ENCEPHALITIS.

II. Herpetic Strains of Encephalitogenic Virus.

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In the course of the investigation of the etiology of epidemic encephalitis, the question of different varieties or strains of the encephalitogenic virus has often arisen.[1] The practice, up to a short time ago, has been to separate the so called encephalitis virus from the virus of febrile herpes and, in spite of their marked similarities, to regard them as fundamentally, or, as respects their etiological activities, distinct. It is true that this rule has been somewhat relaxed recently, but the essential question whether, within the two main classes of virus, substrains or varieties are pathogenetically distinguishable has remained an open one. And yet upon the answer returned to this question rests, in large measure, the pending decision as to complete or only partial identification of the supposed virus of encephalitis with the proved virus of herpes.

In two recent papers Levaditi[2] has reported upon experiments which have led him and his coworkers to conclude that the two classes of virus compose a group for which they propose the designation "herpetico-encephalitic." According to their conception, there may be distinguished within the group encephalitogenic as distinct from the ectodermic varieties of such a virus, the former showing a particular power of attaching itself to neural (brain) structures. It is, indeed, this especial neurotropic kind of the herpetico-encephalitogenic virus which constitutes the provocative virus of epidemic encephalitis. But, of itself, even this variety is impotent to excite encephalitis

[1] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

[2] Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1924, xc, 1372. Levaditi, C., Nicolau, S., and Poincloux, P., *Compt. rend. Soc. biol.*, 1924, xc, 1376.

in man, for which there is required, in addition, a peculiar susceptibility on the part of the nervous organs which only exceptional persons (or monkeys) display. In other words, in order that an epidemic of encephalitis may arise, there must coincide a mass modification of the soil (nervous system) and prevalence of a specially adapted variety of virus (herpetico-encephalitic). The conjunction of these two factors in many parts of the world in the last half dozen or more years is made responsible for the many outbreaks of epidemic encephalitis reported.

According to the point of view just outlined, certain virus strains derived from cases of encephalitis in man are to be regarded as possessing a special affinity for the central nervous organs. One such strain is *souche* (variety) C of Dr. Levaditi. Thanks to his courtesy, we possess this strain and have, therefore, been able to compare its mode of pathogenic action with that of certain strains of the virus of febrile herpes which we also possess. As in our hands the Levaditi strain has shown itself not to be more, but actually to be less encephalitogenic than certain true herpes strains, we are reporting our experiments because of their bearing on current notions of the etiology of epidemic encephalitis.

Sources of the Herpes Virus.

We have studied a considerable number of specimens of the herpes virus as obtained from different persons, and have determined that they show wide variations of activity when inoculated into rabbits.[3] Two strains have been distinguished from the rest as possessing high power to infect rabbits. One of these two strains has been chiefly used in carrying out the many experiments to be reported on, while the other has been employed chiefly as a check upon the former. The H. F. strain, the one principally studied, was taken from a fresh herpetic vesicle on the lip of a subject very prone to attacks of febrile herpes. The clear, straw-colored fluid was drawn into a Luer syringe containing sterile salt solution and injected intracranially into anesthetized rabbits. The fluid proved to be bacteriologically sterile. The injected rabbits developed typical symptoms of virus

[3] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli (in press).

encephalitis to which they succumbed. With this virus (brain) a long series of inoculations of the rabbit has been carried on.

The study of the H. F. virus has extended over a period of nearly 3 years, during which high activity has been maintained not only in direct passages but also after long glycerolation. The H. F. virus has been used in parallel tests with the J. B. virus as well as with Levaditi virus *souche* C, and a specimen of Doerr or Basel virus.[4] Moreover, a few comparative tests have been made with Goodpasture Strain M virus.[5] We are greatly indebted to Dr. Levaditi and Dr. Goodpasture, and Professor Doerr for kindly providing us with specimens of their viruses.

As our purpose in this paper is primarily to show that the H. F. virus is markedly neurotropic and, at the same time, readily implantable upon the cornea and skin of the rabbit, we shall content ourselves with recording illustrative protocols only. In the interest of brevity it may be stated here that the inoculation of the H. F. virus provokes in the rabbit those typical symptoms and associated histological lesions and cell inclusions now accepted as characteristic of the herpes and encephalitic kinds of virus.

Intracranial Inoculation.

Experiment 1. Suspension Inoculation.—From Mar. 23 to Apr. 14, 1922, the H. F. virus was passed through a successive series of four rabbits, by intracranial inoculation.

Rabbit 1. The original diluted vesicular contents inoculated. Violent symptoms appearing on the 6th day, the animal was etherized. The brain, which was congested, was sterile in ordinary cultures.

Rabbit 2. Inoculated from Rabbit 1. Violent and characteristic symptoms and death on the 4th day. Brain cultures sterile.

Rabbit 3. Inoculated from Rabbit 2. Symptoms and death on the 2nd day. Cultures of brain sterile.

Rabbits 4 and 5. Inoculated from Rabbit 3. Symptoms leading to death on the 6th day within 12 hours of each other.

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[4] As the fact may prove of interest in the future, we desire to record that in our hands the Doerr strain of virus has been less intensively and regularly active than even the Levaditi strain. Moreover, it has shown itself of low comparative glycerol resistance. In these two respects it resembles certain weak herpes strains studied by us.[3]

[5] Goodpasture, E. W., and Teague, O., *J. Med. Research*, 1923–24, xliv, 121.

Rabbits 6 and 7. Inoculated from Rabbit 5. Symptoms leading to death on the 4th and 5th days respectively.

In this series of rabbits the outstanding symptoms included torpor, hyperpyrexia, gnashing and salivation, tremor, convulsions, paralysis, urine retention, and head and shoulder turning. The sections of the brain examined microscopically showed the usual infiltrative lesions of the meninges and tissues, as described in the preceding paper.[5]

From the glycerolated brain tissues of these rabbits other rabbits were inoculated intracranially 6 or 7 months later, evoking the characteristic symptoms, among which may be especially mentioned an irregular, backward movement or walk, rhythmical erection and extension of the body on the hind legs, leading to a kind of somersaulting. Following the fall backward the animals became wildly excited, running or walking rapidly in circles, the direction always being toward the inoculated side.

The H. F. strain of virus, having been shown effective by direct intracranial injection of fresh and glycerolated tissue, was next tested by means of (*a*) paper and (*b*) Berkefeld filtrates.

It is common practice to speak of the active brain tissue as "virus." Of course, this tissue is merely the carrier of the real virus which has been implanted upon it. When the brain is emulsified and suspended in salt solution, a part of the virus goes over into the fluid. This part is less concentrated than the original tissue, and hence tends to infect more slowly, or after a greater incubation period. In a like manner the Berkefeld filtrate carries a less concentrated virus than does the paper filtrate. Because of this difference in concentration a Berkefeld filtrate may prove to be ineffective, while the paper filtrate from which it was made is still active. This difference is more likely to appear when glycerolated instead of fresh virus (brain) is employed for the inoculation. We have already pointed out that glycerolation may reduce the potency of the virus (number of viable germs) below the frankly infective, but not beyond the protective capability. Berkefeld filtration, on the other hand, diminishes the potency further below the latter threshold.[6]

Experiment 2. Paper Filtration.—Glycerolated virus (brain) is made into a 5 per cent suspension in salt solution by trituration with sterile sand, shaking for 30 minutes, centrifugalization at slow speed for 3 minutes, and filtration through triple folded chemical paper.

[6] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

Three rabbits were injected intracranially respectively with 0.01, 0.05, and 0.10 cc. of filtrate. All developed symptoms and all succumbed on the 3rd day.

The test was repeated in two rabbits with a specimen of virus which had been glycerolated for 6 months. Both animals showed characteristic symptoms and died on the 8th day.

Berkefeld Filtration.—Three separate tests of Berkefeld filtrates, prepared from glycerolated virus, failed to produce infection. A later test with such a filtrate of fresh virus (brain) gave an unequivocal positive result. From the brain of the animal thus infected a second Berkefeld filtrate was prepared and inoculated intracranially, likewise with a positive result.

Distribution of Virus in Central Nervous System.

The obvious lesions of experimental virus encephalitis, as far as they are known, arise in the forebrain, midbrain, and medulla; the spinal cord is visibly affected far less often. And yet the virus itself is detectable, by inoculation tests, throughout the central nervous system. It is of some interest, in view of the difficulty experienced in separating old or non-virus lesions of the brain from the true virus lesions[6] to find this lack of topographical relationship between site of lesions and presence of virus. The conditions in this repect simulate those of poliomyelitis in man and monkey, except that the predilection site of the lesions is reversed. While in poliomyelitis the spinal cord and medulla are visibly chiefly attacked, yet the virus is present in all parts of the central nervous organs.

Experiment 3.—May 26, 1923. A rabbit received intracranially 0.35 cc. of a 10 per cent suspension of glycerolated virus. Typical symptoms appeared and the animal was etherized on the 5th day. The brain was sterile for ordinary bacteria. June 2. Six rabbits were injected intracranially with a suspension from the brain of the preceding animal: two with tissue taken from the extreme forebrain, two from the medulla and cerebellum, and two from the dorsal and lumbar cord. All six rabbits showed the typical symptoms and all succumbed.

Corneal, Skin, and Nasal Inoculation.

Samples of herpes virus of diverse origin provoke, as a rule, fatal encephalitis when injected intracranially into rabbits. The case is different with inoculations into the surface structures of the body —the cornea, skin, and nasal mucosa. Hence, through the use of superficial (ectodermal) inoculations, distinctions of neural affinity may be detected.

The virus does not attack the unbroken skin of the rabbit, but it may be implanted on the cornea, or even on the nasal mucosa which has not been intentionally abraded. Probably, however, the procedures employed to bring the virus into the eye and nose suffice to produce microscopic injury to the delicate tissues.

The common experimental method is to smear the shaven and, often, injured (incised) skin surface with brain pulp carrying the virus, to instill a suspension of the pulp into the cocainized eye, in which superficial incisions have been made with the cataract knife, and to smear the pulp gently on the upper nasal mucosa, or to carry it there on a cotton plug. Ordinary inflammatory effects are thus produced, which, in respect to the skin and cornea, are open to inspection. Hence, beside suppuration, vesiculation is detectable. Probably vesiculation arises also in the nasal mucous membrane.

Besides these local effects, deeper and more severe ones often declare themselves. They consist precisely of those symptoms denoting an inflammation of the brain. It is with respect to this power, especially, to provoke virus encephalitis upon superficial inoculation that the various strains of herpes virus differ among themselves. Certain strains tend but little, other strains regularly, to this eventuality. With the former strains, also, the encephalitis induced inclines toward a milder clinical course from which recovery may ensue. With the latter recovery almost never occurs. The H. F. virus belongs in the latter class. Moreover, it is so potent that it may be said that superficial inoculation invariably leads to encephalitis followed by death. Hence it is appropriate to designate this strain as "encephalitogenic." The encephalitis-provoking propensity of the H. F. strain exceeds that of any of the so called epidemic encephalitis strains of virus (Levaditi, Doerr) at our disposal, is equalled perhaps by the J. B. strain,[6] isolated by us, and is approached, although probably not equalled, by the Goodpasture M herpes strain.[5]

Corneal Inoculation.—

Two rabbits were inoculated as follows: The left eye was cocainized for 20 minutes and then lightly scarified with a cataract knife dipped in a 10 per cent fresh brain (virus) suspension. The right eye was likewise cocainized and scarified, but not inoculated. Jan. 1, 1923. The left eye was closed by purulent inflammation. The right eye was uninflamed. Jan. 5 and 6. One animal

showing severe symptoms of virus encephalitis and turning to the left and falling over; the other also showing symptoms and drawing head backward and to the left side. Both rabbits succumbed.

Two additional rabbits were inoculated as above, in the left cornea. Suppuration and vesiculation followed and symptoms of virus encephalitis, including circling to the left and leading to death, resulted.

Glycerolation of the brain (virus) does not, as a rule, mitigate the effects, or change the result of the corneal inoculation. An occasional glycerolated specimen will, however, prove to have deteriorated and to have become wholly inactive. It has also been observed that the H. F. virus attacks the cornea vigorously merely after cocainization and without any scarification whatever.

May 19, 1923. The left eye of a rabbit was cocainized for 20 minutes, after which 1 drop of a 10 per cent suspension of fresh virus (brain) was lightly placed in the eye. Keratoconjunctivitis, followed by severe general symptoms and death on the 12th day, ensued. Among the symptoms was the characteristic circling to the side of the inoculated eye. With the brain of this rabbit a second animal was successfully inoculated into the cocainized eye, and with the brain of the second a third and a fourth animal were equally successfully inoculated. All the rabbits so treated developed first marked suppurative keratoconjunctivitis, then severe general symptoms of virus encephalitis, following which death ensued.

The strictly intact or normal eye does not respond to the instillation of the virus with keratoconjunctivitis. A fleeting redness (mild inflammation) alone results. Obviously, therefore, the cocaine solution induces changes in the cornea and conjunctiva of sufficient magnitude to enable the virus to become attached to and to multiply in those structures.

To recapitulate: Fifteen rabbits received instillation of H. F. virus into the cocainized eye, either with or without coincident scarification of the cornea. Eleven were inoculated with the fresh and four with the glycerolated virus. All the rabbits developed severe keratoconjunctivitis, succeeded by symptoms of virus encephalitis, and succumbed.

As will be apparent from the foregoing, the H. F. strain of virus is readily implanted upon the prepared eye of the rabbit and, without exception, when so implanted, invades the brain, bringing about a fatal issue. Since, recently, Levaditi has contended for a particular

encephalitogenic property of certain strains of herpes virus, derived especially from cases of epidemic encephalitis in man, it is fortunate that, through his kindness, we possess a sample of his variety (*souche*) C virus to which he ascribes in high measure this peculiar effect. In our hands the Levaditi virus has acted irregularly when inoculated into the eye after cocainization and scarification of the cornea. Of four rabbits, in which a keratoconjunctivitis of greater or less degree of severity was induced with this strain, in one only did symptoms of virus encephalitis and death supervene.

Skin Inoculation.—The abraded skin of the rabbit readily responds to herpes virus inoculation. Whether so slight a lesion as that produced in the eye by cocaine suffices to render implantation on the skin possible is not known. What is readily demonstrable is that the lightest shaving of the skin makes it vulnerable. After mere shaving, or shaving plus superficial incising, the smearing of brain pulp (virus) over the skin surface suffices to induce inflammation, vesiculation, and later desquamation. Not every rabbit so treated reacts typically, since a mild dermatitis sometimes results which, from subsequent immunity tests, appears not to be herpetic in nature.

The H. F. virus proved highly effective when inoculated into the skin. A certain difference was again noted between the effects of fresh and of glycerolated virus. The fresh virus may be regarded as producing almost without exception vesicular dermatitis, and almost invariably setting up, on the heels of this process, a fatal virus encephalitis. The glycerolated virus, on the other hand, behaves somewhat irregularly: either no dermatitis at all is produced, or a milder, specific inflammation arises, which does not lead to virus encephalitis. That this milder skin affection is really herpetic in nature is shown later by the immunity which has been excited by the process. The explanation of this somewhat anomalous effect is, we believe, to be found not in a mitigated H. F. virus as such, but merely in a numerical reduction in the implanted germs. In support of this belief is the oft observed fact that, when the same specimen of glycerolated virus is injected intracranially, it proves as virulent as ever, and the brain of the succumbing rabbits as active as ever upon skin implantation. Illustrative protocols follow.

Two rabbits inoculated with fresh virus, one upon the upper lip and gum, the other on the shaved and scarified skin of the abdomen. In both, pustulation and vesiculation occurred, and in both, on the 7th day, general symptoms, followed by death, ensued.

Four rabbits were inoculated with fresh virus over the shaved and scarified skin of the abdomen. All developed local and general symptoms and all died.

Four other rabbits were very lightly shaved with a particularly sharp razor over the abdomen and fresh virus was applied. Three developed mild, local, but severe, general symptoms and succumbed. The fourth animal showed neither local nor general effects and, on subsequent test, proved not to be immune to corneal inoculation of the virus.

Of thirteen rabbits inoculated on the skin surfaces with fresh brain (virus), eleven developed dermatitis, followed by virus encephalitis and death. Two rabbits did not respond at all and were susceptible to a second inoculation of the H. F. virus, to which they succumbed. In general it may be stated that the greater the local, traumatic injury, the severer the local inflammation. But the power of a strain of herpes virus to invade the body and set up encephalitis is independent of its ectotropic property. Not all strains of herpes virus which excite skin (or corneal) inflammation induce a coincident encephalitis. Hence, by the skin test also, the H. F. virus merits being considered encephalitogenic.

Intranasal Inoculation.—Cotton swabs carrying active virus were inserted into the nares and left in contact with the mucosa of four rabbits for 12 hours. All four animals developed symptoms of virus encephalitis and succumbed on the 6th to the 9th day following the inoculation.

In three other instances milder, less encephalitogenic strains of virus were introduced in the same way into the nares.[3] Symptoms of much less severity followed, from which the animals recovered. These rabbits, on being subsequently tested, proved to be immune.

Intravenous and Intratesticular Inoculation.

In view of the power displayed by the H. F. virus to penetrate from superficial structures into the body, and thus to provoke encephalitis, it was to be supposed that, when introduced directly into the blood, a similar effect would follow; and the same was predicated

for the injection when made into the testicle, in which organ it is known that the virus survives and probably multiplies. The accepted notion is that the herpes virus, like the virus of poliomyelitis, is capable of ascending to the central nervous organs by way of the peripheral nerves.[7] Probably also, when active and in adequate quantity, the virus passes the barrier of the choroid plexus, thus again acting in the manner of the virus of poliomyelitis. The matter of host susceptibility plays, however, an essential part in this regard. Not only does the rabbit respond to the intracranial injection of the herpes virus, but certain other rodents—guinea pig, rat, mouse—respond also. But no other rodent than the rabbit becomes infected when the herpes virus is introduced into the blood or the testicle.

As is to be expected, the symptoms and lesions arising from the intravenous and intratesticular injections of the virus in rabbits are identical with those contingent upon other modes of inoculation. In no instance did the fresh virus fail to induce characteristic symptoms and to bring about the death of the rabbits when injected either into the blood or the testicle.

Infection of Other Rodents.

Guinea pigs, rats, and mice were given intracranial injections of fresh virus (rabbit brain) with the effect of inducing severe symptoms of virus encephalitis terminating in death. When, however, identical samples of the virus were injected into the heart or the testicle, encephalitis did not follow and the animals showed no symptoms of note.

In general, it may be stated that the symptoms of virus encephalitis in the smaller rodents mentioned agree with those observed for the rabbit. Certain distinctions have, however, been noted: in the guinea pig, paralysis rather than excitation, and in the rat and mouse muscular excitement rather than depression and paralysis are the rule. It was not unusual for the guinea pigs to salivate, become tremulous, ataxic, convulsive, and then paralytic, while the rats and mice often executed complex, rapid, rhythmical movements, not

[7] Goodpasture, E. W., and Teague, O., *J. Med. Research*, 1923–24, xliv, 139.

observed at all in the guinea pig, and in a less degree of intensity in the rabbit. The circling movements of the rats and mice were sometimes bewilderingly rapid; they were, because of the intrinsic interest, recorded in moving picture films.

SUMMARY.

In this paper we have sought to show that unequivocal strains of herpes virus exist in man, which, in the rabbit, exhibit a degree of encephalitogenic power not exceeded, and perhaps rarely equalled, by any strain of the so called encephalitis virus.

The fact that such highly encephalitogenic strains of the herpes virus exist in nature has, at the moment, theoretical and practical importance. Until recently, the view has been accepted by certain workers in the field that two biologically distinct viruses of this class occur—one inducing epidemic encephalitis and the other febrile herpes in man.

This view, is, indeed, being supplanted at the present time by the notion, advocated by Levaditi, Nicolau, and Poincloux, of a group of closely related virus organisms for which the name "herpetico-encephalitic" is proposed. Within this group they distinguish strains of virus displaying special affinities for the central nervous organs and others exhibiting equal affinities for skin and membrane (cornea) structures. The first mentioned strains are responsible, under suitable circumstances, for epidemics of encephalitis in man; the others give rise to ordinary attacks of febrile herpes.

The H. F. virus described in this paper does not conform to the classification indicated. While being a true febrile herpes strain, it possesses, nevertheless, a high degree of power to attack the central nervous system as well as marked capacity to implant itself on the skin and the cornea of the rabbit. Not only does virus encephalitis follow invariably upon the intracranial injection of the H. F. virus, but as regularly upon corneal, skin, nasal, blood, and testicular modes of inoculation. The symptoms of virus encephalitis thus provoked and the character of the brain lesions induced are precisely those, in all their detail and variety, including the presence of intracellular inclusion bodies, which have been described for the so

called virus of encephalitis. Moreover, the H. F. virus is durably glycerol-resistant, is filterable through Berkefeld candles, and behaves immunologically as do the usual strains of herpes and of encephalitis virus.

On the basis of the experimental data presented, we conclude that any distinction made regarding, on the one hand, encephalitogenic power as a special property of a virus secured from cases of epidemic encephalitis, and, on the other hand, of ectotropic action as an equally special quality of a virus yielded by febrile herpes, is in its nature artificial and not in harmony with ascertained fact. What can, indeed, be distinguished are stronger and weaker strains of a virus, probably always herpetic in origin, as determined by the inoculation of rabbits. While a strong herpes virus is both dermatotropic and neurotropic, a weak virus tends, in its multiplication, to remain confined to the site of inoculation, to act chiefly on the tissues on which it is immediately implanted, and not to extend to distant parts. And this is equally true whether the strain of virus came originally from cases of epidemic encephalitis, or merely from cases of febrile herpes in man.

Hence direct comparison cannot be made between the stronger encephalitogenic and weaker non-encephalitogenic strains, according to any specific etiological property. The viruses we are discussing do, indeed, compose one group but it is the group of febrile herpes with which epidemic encephalitis is associated accidentally, if at all. It happens, indeed, that the Levaditi strain (*souche*) C and the Doerr Basel strain, both supposedly originating in cases of encephalitis in man, are less encephalitogenic for the rabbit than the true herpes strains, H. F. and Goodpasture M.