THE EFFECT OF TESTICULAR EXTRACT ON FILTERABLE VIRUSES

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Duran-Reynals (1, 2) has shown that the extract from normal testicles of animals can enhance remarkably the lesions produced in the skin by vaccine virus. In view of the fact that Pijoan (3) has found that the extract also promotes the pathogenic action of 20 kinds of ordinary bacteria, an attempt has been made to study the possible enhancing effect of the agent, called by Ledingham and Barratt (4) the Reynals factor, on the action of four different filterable viruses, namely, those of herpes, vesicular stomatitis, Borna disease, and vaccinia.

Herpes virus was chosen because two strains were available: one (H. F.) designated by Flexner "strong" (5), and the other (F.) "weak" (6). In the rabbit the strong virus has high and the weak low neurotropic activity; each strain has a moderate dermatropic action. A special object, therefore, in using this material was to note whether, by the aid of the Reynals factor, a weak strain could be made strong and a strong virus more active.

In employing the virus of vesicular stomatitis of horses, we depended on the facts that this agent fails to infect any other part of the skin of guinea pigs than the hairless pads, and that the rabbit is generally much less susceptible than the guinea pig (7). Hence it was also attempted, in this instance, to determine whether the Reynals factor could so increase the action of the virus as to infect (a) ordinarily insusceptible tissue, *e.g.*, the abdominal skin of the guinea pig, and (b) a relatively resistant animal, the rabbit.

In the case of Borna disease, the virus of which was selected because of the unusually long incubation period of the affection—3 weeks or even much longer (8)—the study concerned the possibility of increas-

ing the invasiveness of the virus and diminishing the period of incubation.

With vaccine virus, it was desired to find out (a) if it is possible to reproduce in rabbits the equivalent of postvaccinal encephalitis as it occurs in man (which Turnbull and McIntosh (9) believe they have accomplished even without the aid of the Reynals factor), and (b) in what manner the virus reaches the central nervous system after peripheral inoculation.

Methods and Materials[1]

The viruses and the testicular extract were prepared under sterile conditions and were implanted in the tissues of rabbits or guinea pigs as follows:

Herpes Virus.—Fresh rabbit brain virus in 1:5 to 1:10 suspensions was used. 0.5 cc. of the suspension and 0.5 cc. of saline solution were inoculated endermically in one area of the shaved abdominal skin of rabbits and similar amounts of virus and testicular extract in another area. In guinea pigs, only 0.3 cc. of each of the materials was injected.

Vesicular Stomatitis Virus.—With all animals a 1:20 suspension of glycerolated, guinea pig pad virus was employed and 0.4 cc. of each of the materials was injected in a manner similar to that used with herpes virus.

Borna Disease Virus.—The type of virus inoculated varied in different experiments: 0.25 cc. of a 1:5 suspension of fresh rabbit brain virus, or 0.4 cc. of 40 per cent suspension of glycerolated brain, or 0.001 to 0.004 gm. of dried cerebral tissue suspended in 0.4 cc. Ringer's solution was injected intracerebrally in rabbits as controls, while 0.25 cc. of testicular extract plus an equal amount of virus suspension was inoculated into the test animals.

Vaccine Virus.—The virus employed was the neurovaccine strain obtained several years ago from Professor Levaditi and at intervals propagated in rabbits' testicles or brain, or preserved in glycerol. 0.4 cc. of a 1:5 to 1:10 suspension of fresh brain virus and 0.4 cc. of a 1:5 dilution of testicular extract were injected. One group of rabbits were inoculated in the sciatic nerve, and another in the skin. Of the first group, the nerve of some animals was severed, a 2 cm. section resected and inoculation made about 4 cm. below the cut end. Control tests consisted of substituting broth or saline solution for the extract, or of inoculating the Reynals factor alone.

The results of the experiments may be summarized as follows:

[1] All operations were done under full ether anesthesia.

Herpes Virus

Nine rabbits and five guinea pigs were inoculated endermically with the strong (H.F.) strain of herpes virus and eleven with the weak (F.) strain. In each of the nine rabbits the area containing the herpes agent mixed with the Reynals factor showed, usually after 48 hours, an extensive hyperemia and edema which was promptly followed by well-defined vesiculation. Thereafter considerable induration occurred with drying and scaling of the vesicles. The lesions endured until the death of the animal, usually about 10 days after inoculation. In the area containing the virus alone, simple vesiculation was noted which soon became nodular and showed signs of early healing; the extent of the dermatitis was only one-fourth to one-half that caused by virus mixed with testicular extract. Moreover all nine rabbits exhibited on about the fourth, and occasionally as late as the seventh day, signs of encephalitis. Death ensued from the fifth to the eighteenth day (average 10.3 days) after inoculation.

In the guinea pig still more striking differences were discernible in the lesions induced by the H. F. virus alone and by this material mixed with the Reynals factor. Clinically, the changes were respectively similar to those noted in the rabbit; quantitatively, the dermatitis caused by the virus in association with the Reynals factor covered an area 4 to 10 times larger than that produced by the virus alone.

Of eleven rabbits inoculated endermically with the weak (F.) strain of herpes virus, three revealed, in the areas containing virus alone, no lesions and eight small nodules or single vesicles, 0.5 to 1 cm. in diameter, which rapidly subsided. On the other hand, all eleven animals exhibited at the site of inoculation of virus mixed with the Reynals factor, reactions of hyperemia, vesiculation, induration, and in some instances marked ulceration. Indeed, the lesions covered a space from 6 to 20 times larger than the area of dermatitis induced by the virus itself. Of the eleven rabbits, ten developed encephalitis after an average incubation period of about 6 days and nine of the ten died 11 days after inoculation. These results are in striking contrast to those obtained from the action of the F. strain alone, as described by Flexner (10) and Gay and Holden (11), who found that the F. virus produces in rabbits only a non-fatal vesicular dermatitis.

To summarize the experiments with the two strains of herpes virus: the effect of the Reynals factor on both was to intensify their pathogenicity in the skin and brain. In other words, a strong strain has been made still more active, and a weak, dermatropic strain has acquired the properties of a strong virus, as characterized by its more intensive action in the skin and by its marked and usually fatal effect in the brain.

Vesicular Stomatitis Virus

As a preliminary experiment, two guinea pigs were each inoculated, in one posterior pad with vesicular stomatitis virus, and in the other with virus mixed with testicular extract. The pad first mentioned showed, after 48 hours, moderate vesicular dermatitis, the vesicles of which yielded a small amount of clear exudate. The other exhibited, after 24 hours, edema and, a day later, a prodigious vesicle covering the entire pad; ultimately the skin was shed in a complete, massive cast. The Reynals factor is, therefore, capable of enhancing the effects of the virus in guinea pig pads, an ordinarily sensitive tissue.

Twenty-eight guinea pigs were injected intracutaneously in the shaved abdominal skin, in one area with the virus itself, in another with virus mixed with testicular extract. In agreement with the known relative insensitiveness of the abdominal skin, the areas containing virus alone showed, from 48 to 72 hours after injection, no reaction in five animals, and a pin-head sized nodule in the remaining 23. Of these last, only seven animals yielded a drop of exudate on pricking the small nodules, which after a day or two rapidly subsided. On the other hand, those skin areas harboring virus and testicular extract all exhibited, after 24 hours, extensive vesicular dermatitis, yielding a plentiful supply of clear, serous exudate which in turn proved active in the pads of normal guinea pigs. The site of inflammation, characterized by hyperemia, edema, vesiculation, and induration, extended to an area of 3 cm. in diameter, and did not begin to show signs of healing until after 5 to 7 days. Microscopic study of the tissues removed from 14 of the animals revealed no specific changes in the sites inoculated with virus alone, but a varying degree of edema, cellular exudation, vesicle formation, with characteristic intranuclear inclusion bodies in the epidermis and subcutaneous tissue in the areas containing virus mixed with the Reynals factor.

Eight rabbits received endermically in the shaved abdominal skin virus alone. Four were unaffected and four showed only a minute, firm nodule at the site of inoculation. By way of contrast, an area of skin into which was injected a mixture of virus and extract exhibited, in seven of the eight animals, well-defined vesicular dermatitis. The clear fluid obtained from the vesicles was specifically active in guinea pig pads, and sections of the involved skin revealed lesions similar to those observed in guinea pigs.

In another experiment, Berkefeld V filtered testicular extract was used instead of the unfiltered material. In 13 rabbits and guinea pigs enhancing effects were observed which were not different from those of the unfiltered Reynals factor. An extract of guinea pig pads, prepared after the manner of testicular material (1) was substituted for the latter in another series of six animals: only a slight exaltation of the virus effects was noted in guinea pigs and practically none in rabbits.

The filtered or unfiltered Reynals factor promotes the action of vesicular stomatitis virus. Furthermore, by means of testicular ex-

tract, the virus can be activated not only in relatively resistant tissues of a susceptible host, *e.g.*, in the abdominal skin of guinea pigs, but also in similar tissues of a relatively insusceptible animal, the rabbit.

Borna Disease Virus

In view of the fact that the onset of infection by Borna disease virus is not clearly defined in rabbits, the interval of time between implantation of the virus and death from its specific effects was accepted as a measure of its activity.

Twelve rabbits were inoculated intracerebrally with 0.4 cc. of material containing the virus. The average time between inoculation and death from specific infection was 53.7 days. Eleven animals were similarly injected with only 0.25 cc. of virus suspension and with a similar amount of the Reynals factor. In this case the average period of survival was 32 days.

In this experiment, the pathogenic action of the virus was markedly increased by the Reynals factor.

Vaccine Virus

The first experiments related to the inoculation of neurovaccine virus into the sciatic nerve of rabbits.

Intraneural Inoculation.—Of seven animals, as controls, which were injected intraneurally with neurovaccine virus, five showed from the second to the seventh day after inoculation varying degrees of clinical reactions indicative of encephalitis. They developed fever (40.1 to 41°C.), hypersensitiveness, ataxia, and, in one case, complete paralysis of the posterior extremities, gnashing, salivation, urine retention, and opisthotonos. Three of the animals died on the third to fifth day as a result of vaccinal infection. The others were etherized on the seventh and eighth days for microscopic study of the nervous system.

The histopathological changes were, first, those of meningo-encephalitis. Edema of the pia-arachnoid with infiltration of monocytes and of relatively fewer polymorphonuclear leucocytes was observed. In most instances the meningitis was localized to certain areas. The cortex occasionally showed edema, with diffuse infiltration of monocytes, of varying degree in the different animals. Perivascular infiltration with similar cells was also present. In one rabbit the severity of inflammation was paralleled by that following the inoculation of vaccine virus directly into the brain. Secondly, a myelitis was present. The changes in the spinal cord of two rabbits consisted, in the membranes, of thickening by edema and cellular infiltration and, in the white matter, of areas of localized necrosis, perivascular infiltration, and occasional small hemorrhages. The infiltrating cells here were monocytes and a few polymorphonuclear cells. Thirdly, a neuritis occurred: the sheath of the sciatic nerve was thickened by edema and cellular infiltration. The nerve tissue was hyperemic; localized hemorrhages and cellular infiltration

also occurred. In one rabbit the changes described could be followed throughout the sciatic nerve to the spinal cord and thence to the brain. Furthermore, the brains of five and the spinal cords of two rabbits were examined and appreciable amounts of virus were found in all.

Fifteen rabbits were submitted to the influence of testicular extract injected into the brain or spinal cord either at the time of intraneural inoculation of vaccine virus, or 2 days later. Thirteen of the animals revealed clinical reactions indicating encephalitis; seven yielded active virus from the brain or cord or both and, of twelve rabbits examined, eleven showed definite microscopic evidence of encephalitis. Comparing the results of this series with those already given, it may be stated that the animals treated with the Reynals factor exhibit more pronounced vaccinal lesions than those not so treated. The differences are, however, in degree rather than in kind.[2]

In another series of tests in which seven rabbits were employed, the virus either alone or mixed with the Reynals factor was injected into the cut and resected sciatic nerve, as already indicated. It was found that blocking the path in the nerve obstructs the passage of the virus and diverts it from nerve extension to blood distribution.

The next experiments with neurovaccine virus concerned its endermic inoculation in rabbits.

Intracutaneous Inoculation.—It is known that the injection of neurovaccine virus into the skin of rabbits induces no outward signs of cerebral involvement, nor any distinct histopathological changes of encephalitis. Virus, however, can be recovered from the brain (12). Under the influence of the Reynals factor, on the other hand, a definite neurotropism is demonstrable after endermic inoculation of the vaccine virus, as is disclosed by the results on 16 rabbits. In six of these animals testicular extract was injected admixed with the virus, and in the remainder it was injected independently into the brain, in two cases at the time of endermic inoculation of the virus, and in eight after a lapse of 2 days. Seven of the 16 rabbits exhibited clinical signs of cerebral involvement, and seven died from vaccinal infection. Virus was recovered from the brain of 13 animals—all that were examined; the spinal cord of seven of these was tested, and in five cases was found to contain the vaccinal incitant. The central nervous system of twelve rabbits was studied microscopically. Of these animals, eleven revealed characteristic lesions of vaccinal encephalitis, together with, in four instances, changes in the spinal cord also. The lesions in the nervous system were similar to but milder than those occurring after intraneural inoculation. Such effects as necrosis and destruction of neurones were not seen and the polymorphonuclear cell exudation was relatively less marked.

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[2] We are indebted for some of the observations recorded to the kindness of Dr. C. P. Rhoads.

To summarize the results of experiments with neurovaccine virus, it appears that after peripheral inoculation the virus may extend by way of the peripheral nerve to the spinal cord and thence to the brain. It can therefore evoke definite disturbances of the nervous system, neuritis, myelitis, and encephalitis, which are detectable clinically and pathologically.[3] Moreover, virus can be recovered in appreciable amounts from the nervous tissues. While all these effects arise from vaccine virus alone, they are particularly noticeable when the virulence of the incitant is promoted by means of the conjoint action of the Reynals factor. A clinical and pathological process reproducing postvaccinal encephalitis as it occurs in man was not secured by these experiments.

CONCLUSIONS

The Reynals factor promotes the pathogenic action of the viruses of herpes, vesicular stomatitis of horses, Borna disease, and vaccinia. The heightening of virulence is revealed in various ways. The effects of the viruses may be accentuated; or a weak strain converted into a strong one, as in the case of the F. strain of herpes virus; or the power acquired to infect resistant species or tissues, as, *e.g.*, rabbits and the abdominal skin of guinea pigs, with acute vesicular stomatitis.

The Reynals factor should serve as an important agent in the study of filterable viruses.

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[3] The lesions induced by neurovaccine virus are distinguishable from those of spontaneous encephalitis and myelitis in rabbits. The reactions to the vaccinal incitant are primarily of an acute inflammatory type, involving, first, the coverings of the brain or cord, then the perivascular spaces, and finally the substance of the cortex or of the cord. The characteristic changes include edema and a varying degree of polymorphonuclear cell exudation.

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