THE RELATION OF STREPTOCOCCI TO HERPES VIRUS ENCEPHALITIS.

By PETER K. OLITSKY, M.D., AND PERRIN H. LONG, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 2, 1928.)

It is generally believed at the present time that no convincing evidence has been brought forward in support of a definite bacterial incitant of epidemic and of herpes virus encephalitis.

Recently, Evans and Freeman¹ have described an amphophilic, aerobic, pleomorphic, green-producing streptococcus which they obtained from nasal washings, the heart blood, and the mesencephalon of a patient who died during an acute attack of epidemic encephalitis. This organism appeared in tubes of chopped meat medium inoculated with the washings, or a few drops of heart blood, or with small pieces of mesencephalon. It grew readily upon ordinary media in subplants and did not ferment salicin, raffinose, mannitol, or inulin. Litmus milk was curdled.

The microorganism grew at various times as a long chain streptococcus, a diplococcus, a spore-bearing rod, a giant coccus, or as a diphtheroid. Of these, the streptococcus was pathogenic in rabbits when inoculated into the brain, and produced a symptom-complex associated with pathological findings which were thought to resemble those of epidemic encephalitis in man.

In a continuation of these studies, Evans² reported that she was able to isolate the pleomorphic organism from rabbit brains containing one or another of six different strains of herpes virus. Brains carrying respectively Strains J. B.³ and H. F.,⁴ from The Rockefeller Institute, were among these specimens. Chopped meat medium inoculated with 1 to 2 cc. of a 10 per cent saline suspension of infected brain was used. In three instances the organism was procured directly from glycerolated brain material, while in the case of the other three specimens, it proved necessary for positive cultivation to secure fresh brain material by animal passage. The bacteria were thought to be identical with those isolated by Evans and Freeman from epidemic encephalitis in man.

¹ Evans, A. C., and Freeman, W., *Pub. Health Rep., U. S. P. H.*, 1926, xli, 1095.

² Evans, A. C., *Pub. Health Rep., U. S. P. H.*, 1927, xlii, 171.

³ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

⁴ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.

The ease with which the organisms were cultivated by the authors mentioned here led us to a study of their source and their relation to virus encephalitis in the rabbit. Cultivation experiments have been made with rabbit brains which contained either the J. B.,³ the H. F.,⁴ or the Levaditi^{3,5} strains of encephalitogenic virus.

EXPERIMENTAL.

Media.—The following media were used in the cultivation experiments.

Chopped meat medium, prepared according to the directions given by Evans.² "Ordinary beef infusion broth is prepared and the hydrogen ion concentration is adjusted to pH 8.0. Instead of discarding the meat from which the broth is made, the ground meat particles are placed in tubes to a depth of about 1 inch. Sterilization is at 15 pounds for 1½ hours. During sterilization the hydrogen ion concentration is reduced to about pH 6.8."

Dextrose beef infusion broth containing 1 per cent dextrose.

5 per cent rabbit's blood-beef infusion agar in Petri dishes.

Culture Technique.—Cultures were made not only of brains carrying respectively the three strains of encephalitogenic virus, but of normal brain tissue, of chopped meat particles, and of dextrose broth. In each test, 1 cc. of a 10 per cent saline suspension of a ground tissue or of broth was inoculated into three to six tubes of fresh medium. The broth, like the tissue, had been ground as such in a sterile mortar under a hood, with precautions for sterility.

Cultures from Normal and Virus Encephalitis Brain Material.

The results obtained with brain material are summarized in Table I. Cultures were made with herpes virus-infected rabbit brains placed for varying periods of time in sterile 50 per cent glycerol, fresh normal, and fresh virus-infected brains from rabbits and guinea pigs, and fresh brain material of guinea pigs inoculated intracerebrally with non-infective cerebral tissue.

From the table it will be noted that irregular results were obtained. A single culture of *Staphylococcus aureus* was obtained from one of the glycerolated specimens of virus-infected brains, and from the fresh virus-infected brains a streptococcus in one instance and a large coccus in another. Two strains of streptococci were isolated from normal guinea pig brain material and two from the brain tissue of guinea pigs which had been previously inoculated subdurally with

⁵ Levaditi, C., and Harvier, P., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 354; *Ann. Inst. Pasteur*, 1920, xxxiv, 911.

the Levaditi strain of virus. All positive cultures were procured from liquid media, the blood agar plates remaining sterile in all instances.

Cultures of Chopped Meat Medium and of Broth.

It was important to ascertain whether the meat particles used in the medium contained organisms which had resisted sterilization.

TABLE I.
Results of Cultures.

Animal	Virus strain	Status at death	Condition of brain	Growth in chopped meat medium	Growth on blood agar
Rabbit A	Levaditi	Typical virus encephalitis	Glycerolated 8 days	<i>Staphylococcus aureus</i> in 1 tube	Negative
" B	"	" "	Fresh	Negative	"
" C	"	" "	Glycerolated 11 days	"	"
" D	"	" "	Glycerolated 13 days	"	"
" E	H. F. 1	" "	Glycerolated 90 days	"	"
" F	J. B.	" "	Glycerolated 113 days	"	"
" G	Levaditi	" "	Fresh	Streptococci in 1 of 3 tubes	"
" H	"	" "	"	Large cocci in all tubes	"
Guinea Pig A	H. F. 1	" "	"	Negative	"
" " B	J. B.	" "	"	"	"
" " C	Levaditi	Normal	"	"	"
" " D	"	"	"	Streptococci in 1 of 3 tubes	"
" " E	"	"	"	" "	"
" " F	Control	"	"	Negative	"
" " G	"	"	"	"	"
" " H	"	"	"	Streptococci in 1 of 3 tubes	"
" " I	"	"	"	" "	"

Three uninoculated lots of meat medium, containing 260 tubes, prepared in the usual manner and at different times, were incubated from 3 days to 3 weeks at 37°C. The tubes were examined frequently for bacterial growth and were found to be sterile. As a further check, the meat particles from twelve tubes were smeared upon the surface of blood agar plates. These also remained sterile.

The next experiment concerned the possibility of obtaining organisms from ground meat particles and dextrose broth.

The meat particles from each of twenty-three tubes of uninoculated chopped meat medium were ground in a mortar with the usual precautions against contamination. 1 cc. of the resulting suspensions was inoculated into fresh tubes of meat medium and dextrose broth respectively, and upon blood agar plates. Six tubes of dextrose broth free from growth after 2 weeks incubation, were used to provide control material. From each of these, 4 cc. was removed and "ground" in a sterile mortar. This material was seeded into dextrose broth and upon blood agar plates.

Streptococci were obtained in culture from 6 of the 23 tubes from which the meat particles were removed and ground, diphtheroids from 6, spore-producing rods from 9 (together with streptococci in one case), giant cocci from 14, hay bacilli from 1, and *Staphylococcus albus* from 5. A streptococcus was isolated from one tube seeded with the "ground" dextrose broth. The blood agar plates were sterile.

Description of the Streptococci.

The streptococci procured in the way just mentioned grew readily in chopped meat medium, in dextrose broth, and upon rabbit blood agar plates. The growth was granular in the chopped meat medium and in dextrose broth. Small colonies having a greenish tinge and a small zone of hemolysis appeared on rabbit blood agar plates. Both short and long chain types developed in the liquid media, and often long chains of parallel rows of diplococci. Dextrose, saccharose, maltose, and lactose were fermented, while mannitol, xylose, salicin, inulin, and raffinose were not fermented. The reaction to Gram's stain was variable. The organisms showed no marked pleomorphism, and there was no suggestion of a change to a giant coccus, rod form, or diphtheroid.

Intracerebral Inoculation into Rabbits.

The virulence of eight strains of streptococci isolated as above described was tested by intracerebral injection into rabbits.⁶ Two

⁶ All experiments on animals were made with the aid of complete ether anesthesia.

rabbits were used for each strain. Four of these strains failed to cause death of the rabbits. Both the animals inoculated with a fifth died of a purulent meningoencephalitis, and so too did one rabbit out of each pair inoculated with the remaining three strains.

The following protocol is typical of the findings in infected animals.

Rabbit A was inoculated intracerebrally with 0.35 cc. of an 18 hour initial dextrose broth subplant of a streptococcus isolated from ground meat particles. 18 hours after injection, the rabbit's temperature rose to 105.2°F. It was weak, had frequent generalized involuntary muscular contractions, and a Parkinsonian type of head tremor. The pupils were widely dilated, the breathing slow and labored, and a marked urine retention was present. The animal became moribund within 24 hours and was etherized.

The brain, removed with sterile precautions, was markedly congested and soft. The cerebrospinal fluid was increased in amount and contained numerous fibrin flakes. A smear from the brain showed diplococci; and pure cultures of streptococci were isolated from it as also from the heart blood. Microscopically the brain showed diffuse polymorphonuclear infiltration of the meninges, large hemorrhages and small abscesses in the cortex, generalized edema, and a perivascular infiltration with polymorphonuclear neutrophils and lymphocytes. In addition, a generalized polymorphonuclear neutrophilic infiltration of the brain tissue was noted. No intranuclear inclusion bodies were found.

Diagnosis: Purulent meningoencephalitis.

Experiments on Immunity.

Rabbits which had proved resistant to intracranial inoculation of the streptococci were subsequently reinoculated subdurally with active herpes virus. All died from virus encephalitis.

Six rabbits were injected corneally with an initial culture of streptococci obtained from the brain of a rabbit dying from streptococcal meningoencephalitis. No keratitis developed, such as is produced by all three of the strains of encephalitogenic virus used in the experiments. 2 weeks later, these six animals were reinoculated in the cornea with the Levaditi strain of virus. All but one rabbit (an animal discarded from another experiment) developed a characteristic keratitis with fatal termination from virus encephalitis.

No cross-immunity between streptococci and encephalitogenic virus was demonstrated in these experiments.

Differential Glycerolation.

It is not known definitely how long bacteria will survive in glycerolated brain material, kept at $+4^{\circ}\text{C}$. Herpes virus survives indefinitely under such conditions. The brains of rabbits which had died of herpes virus encephalitis but which contained streptococci, as the cultures showed, were placed in 50 per cent glycerol for 120 and 133 days respectively. At the end of these periods it proved impossible to recover streptococci from the glycerolated brain tissue, in chopped meat medium, dextrose broth, or upon blood agar plates. Yet the same glycerolated brain material, inoculated intracerebrally, produced typical virus encephalitis in rabbits. In the brains of the animals that died no streptococci were found.

SUMMARY AND CONCLUSIONS.

Cultures of microorganisms similar to those described by Evans have been obtained in media inoculated with suspensions of herpes virus-infected brains prepared by grinding. But they have also been isolated from saline suspensions of uninoculated meat particles ground in a sterile mortar, and from dextrose broth treated in the same way. It is believed that these organisms are contaminants introduced during the process of grinding. Since they enter the material in no great number, one may suppose them to be suppressed by animals inoculated with the ground substance. In artificial media, on the other hand, they find favorable conditions for multiplication. In our experience, no growth of microorganisms is obtained in routine cultures of virus-infected brains, when fragments, instead of ground material, are used—a fact which may be taken to support the explanation just given.

The tests of the part played by streptococci in experimental virus encephalitis failed to disclose that the microorganisms have any etiological relationship to the affection. The intracerebral injection of rabbits with the cultures procured in the course of the experiments produces a purulent type of meningoencephalitis which does not resemble virus encephalitis either in its symptom-complex or in its pathology. The same type of meningitis follows the injection of streptococci derived from ground meat particles, from "ground" broth, from normal brains, and those infected with herpes virus. Some

rabbits manifested resistance to the streptococci, whereas all that have been inoculated intracerebrally with the three strains of herpes virus used in this study have proved susceptible thereto. Certain of the rabbits just mentioned which had proved resistant to streptococci inoculated into the brain or cornea were injected with herpes virus and reacted typically. Comparative tests have revealed that the streptococci are more sensitive to the destructive effect of 50 per cent glycerol than is herpes virus. From all this, it can be concluded that streptococci are not the visible form of herpes virus, nor do they produce in rabbits effects like those induced in the brain and cornea by the herpes virus.