EXPERIMENTAL INFECTION OF THE CHICK EMBRYO WITH THE VIRUS OF PSEUDORABIES

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PLATES 15 AND 16

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Experimental infection of the chick embryo with certain bacteria (1–6) and viruses (7–11) has yielded new information on the host-parasite relationship of these agents. For example, when inoculated on the chorioallantoic membrane, vaccinia virus spreads to the embryo and produces a generalized pock disease (7). Following intra-amniotic injection the virus of human influenza causes a destruction of the embryo lungs (8). Swine influenza virus does the same following membranal inoculation (9), and rabies virus produces extensive destruction of the embryo central nervous system (10). In this last, the general pattern of infection simulates that of the natural disease: many Negri bodies are found in all types of nerve cells; neuronophagia and perivascular infiltration are not observed.

The virus of pseudorabies readily infects the chorioallantoic membrane of chick embryos and produces pocks which may coalesce to form large ulcers (12, 13). Since pseudorabies causes a rapidly destructive encephalitis in a wide variety of animals (14, 15), a selective effect on the central nervous system of the chick embryo is to be expected. In contrast to the virus of herpes which has no apparent neurotropic tendencies in the chick embryo (11), all four of the strains of pseudorabies here studied have a marked neurotropism.

EXPERIMENTAL

The following four strains of virus were kindly furnished by Dr. R. E. Shope. (1) A Hungarian strain sent to Dr. Shope by Dr. Aujeszky in 1931. (2) The Iowa A strain isolated in 1930 by Shope (15) from cattle with "mad itch." Both have since been put through over 50 serial rabbit brain passages and are here arbitrarily considered as "fixed" strains. (3) The Iowa B strain isolated by Shope (16) from a pooled sample of two cow brains from Johnson County, Iowa. (4) The swine strain isolated by Dr. J. D. Ray (17) from naturally infected pigs in Nebraska. The last two had had, respectively, 15 and 5 intracerebral passages. All were again identified after chick embryo passage by one or more of the following characteristics: ability to produce a rapidly fatal (24 hours) encephalitis in the rabbit when inoculated intracerebrally; typical pruritus in rabbits and guinea pigs following subcutaneous inocu-

lation; neutralization of the pock lesions on the membrane by specific hyperimmune swine serum; and constant intranuclear inclusions in the infected chick embryo.

The chorioallantoic membrane was infected by dropping a suspension of infected rabbit brain on the previously prepared membrane (18), 12 day embryos being used as a standard. Multiplication of the virus on the membrane caused scattered pocks when the inoculum was dilute, large ulcers when more concentrated. The infection could be transferred at 4 or 5 day intervals by grinding the membrane in a glass grinder and reinoculating it in saline suspension, or simply by transferring bits of infected membrane.

Neutralization tests were carried out by mixing serial tenfold dilutions of virus tissue with undiluted swine hyperimmune serum and control serum[1] (19), then immediately inoculating control and experimental embryos. Tests were read at 2 days when the pocks were well developed but had not yet grown together.

In studying the pathogenesis of the infection, the embryos were fixed in Zenker's 10 per cent acetic acid fixative 4 or 5 days after infection of the membrane, and paraffin sections were stained with hematoxylin and eosin.

RESULTS

Pseudorabies produces in the embryo a relatively constant, easily recognizable intranuclear inclusion, indistinguishable from that of herpes (11). Thus the course and progress of the infection can be followed, and the types of tissue affected can be determined.

Following the first passage of the Aujeszky strain a hemorrhagic bulging of the embryo cranium (Fig. 1) appeared about the 4th day after inoculation on the membrane. In later passages this bulging was fairly constant by the 4th day. Sections of such embryos show that almost all of the nerve cells are involved, for inclusions are present everywhere. Not only purely neural elements, but the meninges (Fig. 4), the choroid plexus, and the endothelium of blood vessels may be infected. Even the cells in the retina sometimes show the typical inclusions, and virus has been recovered from the retina and choroid of other experimentally infected animals (20). Thus the embryo infection follows the usual pattern of the disease (21).

Despite this widespread destruction of many cells, several titrations showed no large amount of virus in the central nervous system (Table I).

It is important to note here that leucocytes do not penetrate the central nervous system, even when the virus has destroyed the majority of the cells. Hence there is no neuronophagia. But the virus may evoke a leucocytic response elsewhere, as examination of infected membranes has shown. This was particularly clear in one embryo which had a marked neuritis (Fig. 2) with polymorphonuclear leucocytes throughout the nerve. The leucocytic reaction ceased before the nerve entered the central nervous system (Fig. 3).

[1] Kindly furnished by Dr. Shope.

The marked destruction of the central nervous system of chick embryos by pseudorabies has not previously been reported, although the virus has been successfully cultivated on the membrane (12, 13) and in tissue culture (22). This suggested a difference in our strain, and for this reason three more strains were studied.

Inoculation of the 12 day membrane with a suspension of rabbit brain infected with the Iowa A strain also produced a hemorrhagic destruction of the brain in four of five cases. Gross examination showed that the hemorrhage was particularly marked in the cerebellum, in contrast with the diffuse destruction produced by the Hungarian strain. Microscopic examination showed the rest of the brain to be infected with virus (frequent inclusions), but vascular destruction was limited to the cerebellum.

TABLE I

Titration of Brain and Membrane from Infected Embryos

Passage	Material	Days after infection	Titer on chorioallantoic membrane of 12 day embryos
6	Brain	4	10^{-4}
8	"	4	10^{-4}
10	"	4	10^{-5}
11	Membrane	2	10^{-5}
12	"	3	$10^{-5}+$*
12	Brain	4	10^{-5}*
14	Membrane	4	10^{-3}

* Titrated by intracerebral inoculation of 2 guinea pigs at each dilution.

The virus of pseudorabies is probably transmitted in nature as a respiratory infection from swine to swine. The infection of the central nervous system of cattle, "mad itch," is a blind alley in the usual progress of the infection (23). Thus in nature there is no tendency to accentuate the neurotropic qualities of the virus, as there is on serial intracerebral passage in the laboratory. This makes it possible that more recently isolated strains of virus might fail to destroy nerve tissue selectively.

The Ray strain isolated from swine (17) and the Iowa B isolated from cattle grew on the chorioallantoic membrane and were carried for eight serial membrane passages. There was marked destruction of the central nervous system in the embryos studied, but the hemorrhagic features of the two fixed strains were entirely lacking.

Both strains were then put through eight intracerebral passages in rabbits, reinoculated on embryos, and their neurotropic tendencies studied again. Both could still infect the embryo brain, but there was no apparent increase in neurotropism or hemorrhagic destruction. Comparative titrations on the

embryos and in guinea pigs by intracerebral inoculation also failed to show any relative increase in the tendency to destroy nervous tissue over the tendency to produce lesions on the membrane (Table II).

The fixed strains of pseudorabies are peculiar among chick embryo-cultivated viruses in that the hemorrhagic tendencies are produced by a virus recognizable by its intranuclear inclusions. It is therefore possible to tell whether the endothelium of the destroyed blood vessels is actually infected with virus. Brains infected with the fixed strains frequently showed such inclusions in the endothelium, while brains of embryos infected with recently isolated strains did not.

TABLE II

Effect of Intracerebral Passage of Iowa B Strain on Neurotropism

Virus	Dilution	Embryo, Average No. of pocks	Result in guinea pigs
After 5 membrane passages	10^{-3}	3.2	2 of 2 killed
	10^{-4}	0.4	2 " 2 "
	10^{-5}	0	0 " 2 "
After 8 intracerebral rabbit passages	10^{-4}	24	3 " 3 "
	10^{-5}	4	2 " 3 "

TABLE III

Neutralization of Aujeszky Strain by Hyperimmune Serum

Dilution of virus	Virus + normal pig serum	Virus + hyperimmune serum
10^{-2}	Large central ulcers with scattered pocks	0,* 0, 0, 0
10^{-3}	6, 0, 34	0, 0, 0, 0

* Figures represent number of pocks on chorioallantoic membrane 2 days after inoculation.

In order to identify the virus, neutralization tests were carried out several times, and it was possible to produce a consistent decrease in the pock count when the virus suspensions were combined with hyperimmune serum (Table III).

Embryos which failed to develop pocks also failed to show the usual hemorrhagic hydrocephalus.

Chick embryos of varying ages differ in their reaction to a number of viruses (7, 11, 24). Infection by pseudorabies is no exception to this rule. This was shown by the simultaneous inoculation of 13, 15, and 18 day embryos on the chorioallantoic membrane, and the subcutaneous injection of virus into 2 day chicks (Table IV). A 1 per cent infected chick embryo suspension was used.

Adult chickens are resistant to subcutaneous inoculation of the virus (15). This increase of resistance with age may be related to the increase in the temperature of chick embryos after the 14th day; for in two experiments, we have found that incubation at 40°C. of 12 day embryos inoculated with pseudorabies produces fewer and more discrete lesions than at 37°. It is interesting to compare the results of pseudorabies in the 18 day embryo with those produced by rabies virus, as reported by Dawson (10). He finds that inoculation of chick-adapted virus on the membrane fails to produce a lesion on the membrane, but will destroy the central nervous system.

Cellular reaction in the brain itself differs markedly with the different age groups, as shown in Figs. 5 to 7. In 12 day embryos (16 day when fixed), the nerve tissue is extensively involved and hemorrhage is prominent. In 15 day embryos (20 day when fixed), although many nerve cells are destroyed, hemorrhage has ceased; a few leucocytes penetrate the brain tissue. In

TABLE IV

Effect of Age of Embryo on Type of Lesions

Age	No. inoculated	Reaction
days		
13	6	Large ulcers and confluent pocks
15	4	5 to 10 scattered pocks
18	7	No visible lesions on membrane; subsequent encephalitis
2 day chick	4	2 developed encephalitis in 6 days

contrast to the embryo, there is no hemorrhage in the hatched chick and only slight destruction of nerve tissue. There is perivascular polymorphonuclear and mononuclear infiltration, with neuronophagia. For the first time the nerve tissue itself seems to react, for glial nodules are common.

DISCUSSION

Study of pseudorabies in the chick embryo has emphasized the neurotropic qualities of this virus. It is a unique infection, in that a reaction occurs both on the membrane and within the central nervous system. This is analogous to the natural disease in which a primary lesion develops at the point of inoculation and the virus subsequently infects the brain. Since there is no portal of entry to the embryo proper other than the blood stream, virus spreads to the embryo by this route and may be recovered from the embryo blood. This contrasts with the frequent neural spread of the natural disease, but recalls the humoral spread of certain strains (19) in the rabbit.

Some strains of influenza virus may simulate this disease pattern, but only after prolonged cultivation on the membrane (25, 26). Small pocklike lesions

develop on the membrane, and the influenza virus subsequently spreads to the embryo and produces a hemorrhagic destruction in the brain. This acquired neurotropism is confirmed by the ability of the virus to multiply in the brains of mice (26).

SUMMARY

The chick embryo responds to experimental infection with the virus of pseudorabies with a disease pattern simulating the natural infection. Virus lesions of the membrane are followed by infection of all tissues of the central nervous system.

Fixed strains produce a hemorrhagic destruction of the central nervous system of the embryo, which is referable to destruction of blood vessel endothelium. Field strains lack the hemorrhagic tendency, but infect the brain when inoculated on the membrane.

Neutralization of the virus by specific hyperimmune serum can be demonstrated by inoculation on the membrane.

The reaction of the embryo to the virus varies with the age of the embryo. This is reflected both in the membranal lesion and in the subsequent encephalitis.

BIBLIOGRAPHY

1. Gallavan, M., and Goodpasture, E. W., *Am. J. Path.*, 1937, **13,** 927.
2. Buddingh, G. J., and Polk, A. D., *J. Exp. Med.*, 1939, **70,** 485.
3. Cromartie, W. J., *Am. J. Path.*, 1941, **17,** 411.
4. Goodpasture, E. W., *Am. J. Path.*, 1937, **13,** 175.
5. Buddingh, G. J., and Womack, F. C., Jr., *J. Exp. Med.*, 1941, **74,** 213.
6. Bang, F., *J. Exp. Med.*, 1941, **74,** 387.
7. Buddingh, G. J., *J. Exp. Med.*, 1936, **63,** 227.
8. Burnet, F. M., *Brit. J. Exp. Path.*, 1940, **21,** 147.
9. Bang, F. B., unpublished data.
10. Dawson, J. R., Jr., *Am. J. Path.*, 1941, **17,** 177.
11. Anderson, K., *Am. J. Path.*, 1940, **16,** 137.
12. Burnet, F. M., Lush, D., and Jackson, A. V., *Australian J. Exp. Biol. and Med. Sc.*, 1939, **17,** 35.
13. Glover, R. E., *Brit. J. Exp. Path.*, 1939, **20,** 150.
14. Gerlach, F., and Schweinburg, F., *Z. Infectionskrankh.....Haustiere*, 1935, **48,** 270.
15. Shope, R. E., *J. Exp. Med.*, 1931, **54,** 233.
16. Shope, R. E., personal communication.
17. Ray, J. D., personal communication to Dr. Shope.
18. Goodpasture, E. W., and Buddingh, G. J., *Am. J. Hyg.*, 1935, **21,** 319.
19. Shope, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1932–33, **30,** 308.
20. Remlinger, P., and Bailly, J., *Ann. Inst. Pasteur*, 1940, **64,** 40.

21. Hurst, E. W., *J. Exp. Med.*, 1934, **59**, 729.
22. Traub, E., *J. Exp. Med.*, 1933, **58**, 663.
23. Shope, R. E., *J. Exp. Med.*, 1935, **62**, 85.
24. Enders, J. F., and Pearson, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 143.
25. Burnet, F. M., *Brit. J. Exp. Path.*, 1936, **17**, 282.
26. Stuart-Harris, C. H., *Lancet*, 1939, **1**, 497.

EXPLANATION OF PLATES

Sections stained with hematoxylin and eosin.

These photographs were made by Mr. Julian A. Carlile.

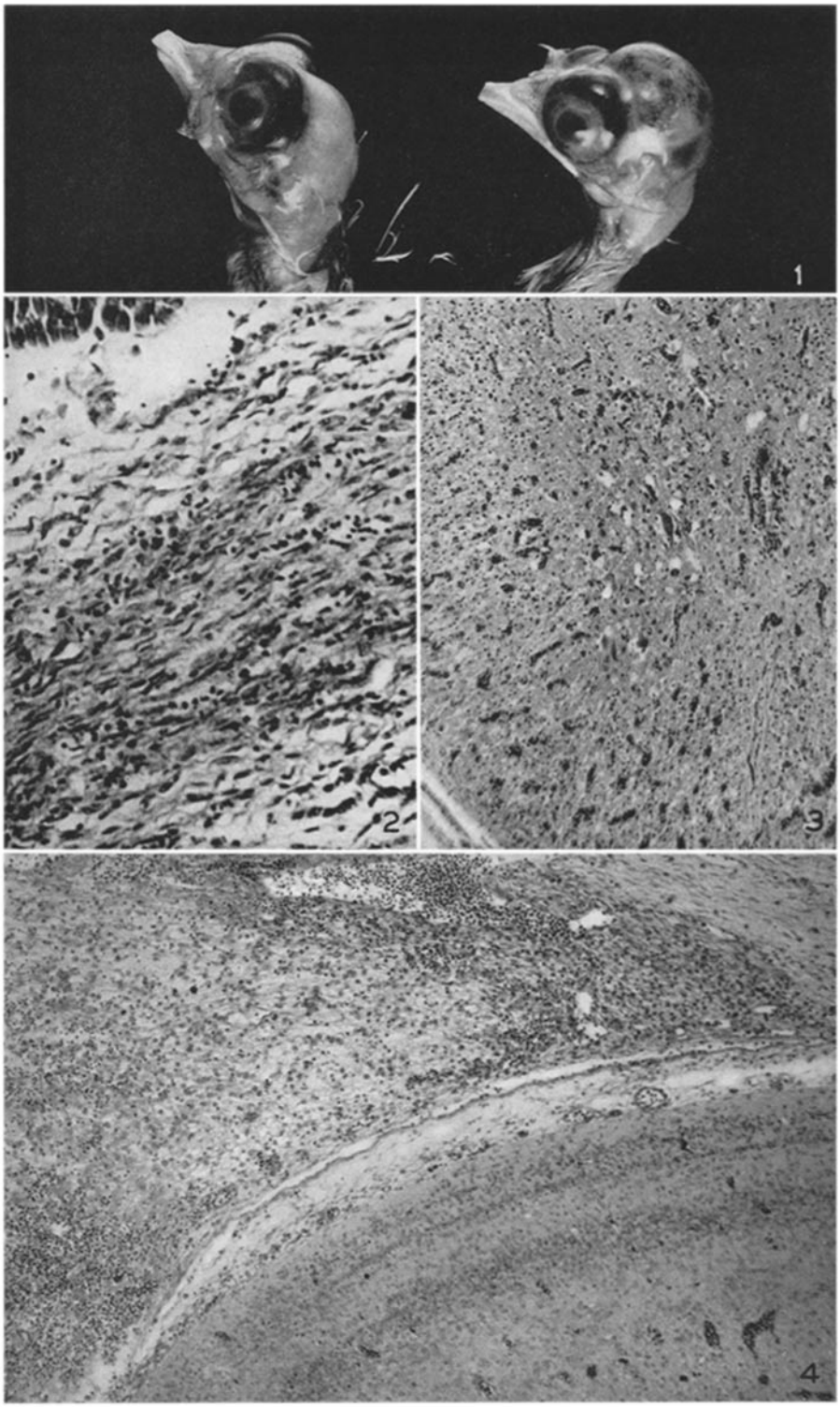
PLATE 15

FIG. 1. Normal 16 day chick embryo on left. Embryo on right shows hemorrhagic bulging of cranium as a result of infection by Aujeszky strain of virus. Slightly enlarged.

FIG. 2. Neuritis in embryo infected with Aujeszky strain. Darkly staining cells are polymorphonuclears. × 304.

FIG. 3. Spinal cord of same chick showing destruction of nerve tissue. × 111.

FIG. 4. Meningitis in 16 day embryo following membranal inoculation. × 100.



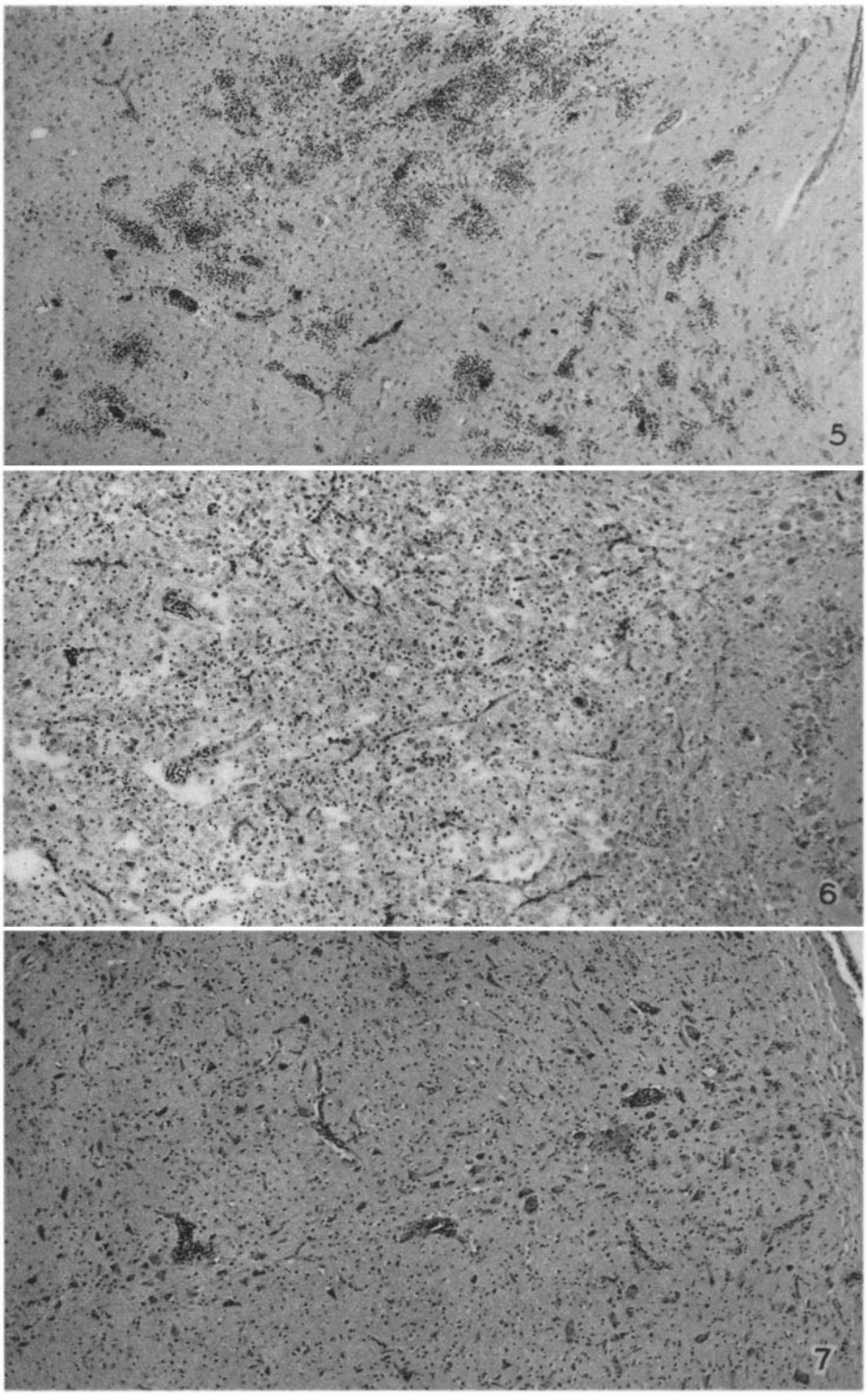
(Bang: Infection of chick embryo with pseudorabies virus)

PLATE 16

FIG. 5. Destruction and hemorrhage in 12 day embryo following membranal inoculation. If the infection had been allowed to develop, almost all nerve cells would have been destroyed. × 100.

FIG. 6. Destruction of nerve cells in 15 day embryo following membranal inoculation. Note lack of hemorrhage and relative immunity of nerve cells at right. × 100.

FIG. 7. Reaction in newly hatched chick following subcutaneous inoculation. Fewer cells are destroyed. × 100.



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