CULTIVATION OF PSEUDORABIES VIRUS

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PLATE 40

(Received for publication, July 26, 1933)

All attempts at the cultivation of pseudorabies virus reported in the literature have been unsuccessful. Aujeszky (1) who first described the disease in 1902, Zwick and Zeller (2), Schmiedhoffer (3), Bertarelli and Melli (4), and others were unable to grow the causative agent on lifeless media aerobically or anaerobically. Sangiorgi (5) attempted to cultivate pseudorabies virus by the method devised by Flexner and Noguchi (6) for the cultivation of the globoid bodies in poliomyelitis. The first series of Sangiorgi's cultures was still infectious after 8 days' incubation at 37°C. The first series of subcultures, however, was inactive after 15 days' incubation.

Since pseudorabies virus in all its qualities is a typical representative of the filtrable viruses, some of which have been successfully cultivated in the presence of living cells, an attempt was made to grow it in this manner.

Pathogenic Properties of the Virus Studied

The Hungarian strain of pseudorabies virus was used in our experiments. In 1930 a sample of this strain had been sent to Dr. Shope by Prof. A. Aujeszky of Budapest. Since then the virus has been maintained by rabbit passage and storage in glycerol.

When injected subcutaneously into rabbits and guinea pigs pseudorabies virus causes a regularly fatal disease. Rabbits are considerably more susceptible than guinea pigs. In both species a characteristic symptom constantly present after subcutaneous infection is pruritus, which leads to repeated biting, scratching, and consequent mutilation of the skin at the site of inoculation. The incubation period is from 2 to 3 days and death usually occurs in from 12 to 24 hours after the onset of the first symptoms. More detailed descriptions of the clinical picture in pseudorabies are given by Aujeszky (1), Zwick and Zeller (2), Schmiedhoffer (3), and Shope (7).

While all authors have found both rabbits and guinea pigs susceptible to pseudorabies, their findings in regard to its pathogenicity for mice have been at variance.

Aujeszky (1) found mice to be less susceptible to the virus than rabbits, dogs, and guinea pigs. Schmiedhoffer (3) calls gray mice more susceptible than white ones. Von Rátz (8) could infect white mice by feeding the virus. The incubation period after this mode of infection was very long, 6 to 15 days. In Isobolinski and Patzewitsch's (9) experiments mice were susceptible to pseudorabies. The incubation period in white mice was 3 to 4 days. Zwick and Zeller (2) could not infect white mice by subcutaneous, intramuscular, or intraperitoneal injections of virus. In Shope's (7) experiments with the Iowa ("mad itch") strain of pseudorabies virus white mice developed fatal infections regularly from intracerebral inoculation, irregularly from intraperitoneal infection, and never from subcutaneous injections. Burggraaf and Lourens (10), using pseudorabies virus from an outbreak in Holland in 1932, could not infect mice by intraperitoneal or subcutaneous injection. Unless there are variations in the susceptibility of white mice used by different laboratories to pseudorabies virus the different results obtained by the authors mentioned above, who worked largely with the Hungarian strain, cannot be explained.

Since it was intended to use white mice for the titration of the virus, it was necessary to know whether our mice were regularly susceptible to the Hungarian strain of pseudorabies virus or not.

In a preliminary experiment it was found that white mice die regularly in 3 to 4 days after a sufficiently large dose of virus administered intraperitoneally. The intraperitoneal route of infection was preferred to the intracerebral one, since a greater amount of fluid could be injected in this way and therefore the dosage could be regulated more accurately.

The disease in mice was found to be as constantly fatal as in guinea pigs and rabbits. When approximately 9/10 of the inoculum was injected intraperitoneally and 1/10 subcutaneously at the same site, 70 to 80 per cent of the mice showed the symptoms characteristic in rabbits and guinea pigs after subcutaneous injection: active biting and scratching, leading to self-mutilation at the site of inoculation or some other place on the body surface. The mice which failed to develop pruritus showed a greatly accelerated respiratory rate, dyspnea, and salivation. After some experience with the disease in mice it is not difficult to establish the clinical diagnosis in every case.

In Table I the results of a titration of rabbit brain pseudorabies

virus in mice are given. For comparison, four guinea pigs were injected with decimal dilutions of the same virus. Both guinea pigs and mice were killed regularly.

TABLE I

Titration of Rabbit Brain Pseudorabies Virus

Dose* (virulent rabbit brain)	White mice (intraperitoneally)		Guinea pigs (subcutaneously)	
	No.	Result	No.	Result
mg.				
100	1	Died in 66 hrs.	778	Died in 73 hrs.
	2	" " 74 "		
50	3	" " 66 "		
	4	" " 114 "		
10	5	" " 66 "	777	Died in 82 hrs.
	6	" " 74 "		
	7	" " 68 "		
	8	" " 75 "		
5	9	" " 81 "		
	10	" " 125 "		
2.5	11	" " 73 "		
	12	" " 92 "		
1	13	No illness	776	Died in 91 hrs.
	14	" "		
0.1	15	" "	775	No illness
0.01	16	" "		

* Each dilution administered in 1 cc. of physiological saline.

Cultivation in Rabbit Testicle Media

Minced rabbit testicle was chosen as the tissue constituent of the culture media, since among animals the rabbit is most susceptible to pseudorabies virus and since the virus can apparently multiply in the testicle *in vivo*. Shope (7) found the testicles to be a dependable source of virus after intratesticular inoculation. Moreover, it is known that vaccinia virus (Maitland and Laing (11)), Virus III

(Andrewes (12, 13)), and herpes virus (Andrewes (14)) can be cultivated *in vitro* in series in minced rabbit testicle suspended in rabbit serum and Tyrode solution.

Preparation of Media Used

The testes of a healthy adult rabbit were removed aseptically, washed twice in physiological salt solution, and finely minced with long scissors on a watch-glass contained in a Petri dish. The tissue pulp in amounts of 100 to 150 mg. was distributed with a large loop or by wide mouth pipettes to 50 cc. Florence flasks. The flasks were closed with cotton plugs covered with a layer of tin-foil. As a rule, 2 cc. of rabbit serum (mixtures of sera from several normal rabbits were used) and 2 cc. of Tyrode solution prepared according to the formula given by Fischer (15) were added at once to the tissue pulp. The flasks were then slightly shaken to distribute the tissue fragments equally in the fluid. Sterilization of the serum and Tyrode solution had been effected by passage through Berkefeld N or W filters. The cultures of each serial passage were made up in duplicate or triplicate. From the testes of every rabbit killed for tissue, enough media for two or three culture series was usually made up. Uninoculated culture flasks were stored in the refrigerator, at approximately +4°C., until needed.

Course of the Serial Cultivation

Each flask of Culture Series I was inoculated with 0.3 cc. of a 10 per cent brain emulsion from a rabbit that had died of pseudorabies following intracerebral inoculation. Subcultures were inoculated with 0.4 cc. of the cultures of the preceding series. Thus the dilution factor in the serial culture passages was about 11. The inoculum usually contained a number of small tissue fragments which were drawn into the pipette with the fluid. After inoculation the flasks were either put into the refrigerator for 1 hour according to the method of Carrel (16), or directly into the incubator after it had been found that storage in the refrigerator prior to incubation was not necessary. This observation agrees with Andrewes' (13) findings concerning Virus III. Incubation was carried out at 37°C. for 2 days unless otherwise stated. After incubation the cultures were tested for sterility and contaminated cultures were discarded. The presence of the virus in cultures was determined by subcutaneous injection of 1 cc. of cell-containing culture fluid into a guinea pig or a rabbit.

The titer of the cultures was determined by injecting decimal dilutions of whole ground cultures or culture fractions into white mice. Before the titration of whole cultures the fluid and tissue were carefully separated. The tissue fragments were ground with a small amount of sand in a mortar and then the fluid portion slowly added. From the suspension thus obtained decimal dilutions were made with physiological salt solution. A fresh pipette was used for every dilution, 1 cc. of which was injected into one or two mice, 9/10 of the dose intraperitoneally, 1/10

subcutaneously at the same site. When, for comparison, the titer of the fluid and tissue portions of cultures was determined, they were centrifuged, following which the supernatant fluid was pipetted off and divided into two equal parts. The tissue was ground with sand and one-half of the supernatant fluid was added to obtain a suspension which could be injected. Decimal dilutions of this suspension and of the other half of the supernatant fluid were tested in mice. As a rule, several cultures of a serial passage were used for titration to lessen the effect of variations in virulence of single cultures. The results of the cultivations are given in Table II.

The virus was cultivated uninterruptedly for 12 serial passages. The cultures of Series XIII were inactive when tested in mice. In this series media were used which had been stored in the refrigerator for 6 days prior to inoculation. It is likely that the cells in the medium had died during this time and were no longer suitable for the multiplication of virus.

To determine whether the virus had actually disappeared from the cultures or whether its pathogenic properties for mice had been altered, subcultures were made and tested in guinea pigs and rabbits by subcutaneous and intracerebral inoculation. These animals developed no illness. The guinea pigs, when tested for immunity 3 weeks later, succumbed to the disease.

A new set of cultures (Xa) was inoculated from the cultures of Series IX which had been stored in the refrigerator for 21 days after incubation and proven to be still virulent for mice. The virus was then carried through 20 further serial culture passages. The cultures of Series XXXI were contaminated by bacteria and avirulent for mice. Therefore a new series of cultures (XXIXa) was started from Series XXVIII, the cultures of which had been kept in the refrigerator for 5 days. After the virus had been cultivated in 4 more serial passages the experiment was discontinued.

The titer of the serial cultures fluctuated considerably, as Table I shows. The reason for this fluctuation is not known. It is noteworthy that, while 1 cc. of the cultures of Series XXIX failed to infect a guinea pig by subcutaneous inoculation, 0.4 cc. of the same cultures contained enough virus to infect subcultures. The tissue portion of the cultures of Series XXV reached the titer of at least 1:1 million, which is almost incredibly high for pseudorabies. In all cultures fractionally titrated the tissue portion contained consid-

TABLE II

Cultivation of Pseudorabies Virus in Rabbit Testicle Medium

No. of serial culture passage	Date	Test for virulence by subcutaneous inoculation				Titer* of culture virus for mice			Length of time media was stored in refrigerator before inoculation
		Rabbits		Guinea pigs		Fluid portion of cultures	Tissue portion of cultures	Whole ground cultures	
		No.	Result	No.	Result				
	1933								*days*
I	Feb. 15	•†	•	•	•	1:10	•	•	0
III	" 19	574	+ 56 hrs.	•	•	•	•	•	0
V	" 28	652	+ 71 "	769	+ 55 hrs.	•	•	•	0
VIII	Mar. 6	•	•	768	+ 70 "	•	•	1:100	2
IX	" 8	•	•	789	+ 62 "	•	•	•	4
XII	" 14	•	•	805	+ 95 "	•	•	•	4
XIII	" 16	•	•	•	•	•	•	Avirulent	6
Xa (inoculated with 0.4 cc. of IX)	Apr. 1	•	•	821	+ 61 hrs.	•	•	•	0
XIIa	" 5	•	•	820	+ 64 "	•	•	1:1,000	4
XIIIa	" 7	•	•	828	+ 64 "	•	•	1:1,000	0
XIIIa	" 7	•	•	834	+ 67 "	•	•	1:1,000	0
XIV	" 9	•	•	822	+ 64 "	•	•	1:1,000	2
XIV	" 9	•	•	826	+ 67 "	•	•	1:1,000	2
XVI	" 13	•	•	855	+ 72 "	•	•	1:1,000	0
XX	" 21	•	•	841	+ 60 "	1:100	1:10,000	•	2
XXI	" 23	•	•	860	+ 92 "	•	•	Avirulent	4
XXIV	" 29	•	•	876	+ 46 "			1:10,000	0
XXV	May 1	•	•	839	+ 67 "	1:100	1:1 million	•	2
XXVII	" 5	•	•	881	+ 57 "	Avirulent	1:100	•	0
XXVIII	" 7	•	•	883	+ 66 "	•	•	•	2

XXIX	" 10	•	•	884	—	•	•	Avirulent	5
XXX	" 12	•	•	891	+ 99 hrs.	•	•	•	0
XXXI (contaminated)	" 14	•	•	•	•	•	•	Avirulent	2
XXIXa (inoculated with 0.4 cc. of XXVIII)	" 14	•	•	895	+ 70 hrs.	•	•	•	2
XXXa	" 16	•	•	889	+ 57 "	•	•	•	0
XXXIa	" 18	•	•	•	•	Avirulent	•	•	0
XXXII	" 20	•	•	•	•	•	•	1:10	4

* By titer in this table and the following ones is meant the highest decimal dilution, 1 cc. of which killed mice when inoculated 9/10 intraperitoneally and 1/10 subcutaneously.

† • = not tested; + = died after; − = no illness.

erably more virus than the fluid portion (see Series XX, XXV, XXVII, and XXXIa in Table II). The tissue could not be freed from virus by repeated washing.

In Table III the results of the titration of the cultures of the last series with rabbit testicle tissue are given as an example of the titration of cultivated virus.

Various factors governing the growth of the pseudorabies virus in cultures were studied in the following experiments.

TABLE III

Titration of Cultures of Series XXXII in Mice

Mouse No. (Series 33)	Dose (ground culture suspension)	Result
	cc.	
1	1.0	Died in 79 hrs.
2	0.1	" " 90 "
3	0.01	" " 104 "
4	0.01	No illness
5	0.001	" "
6	0.001	" "
7	0.0001	" "
8	0.0001	" "

Amount of Tissue Required

Rivers (17)[1] and Rivers and Ward (18) observed that too large amounts of tissue in cultures of vaccinia virus inhibited or prevented the multiplication of the virus. For pseudorabies virus the same seems to be true, although this virus requires a somewhat greater amount of tissue than does vaccinia virus. In Experiment 4 (Table IV) the virus did not multiply in Culture 1 containing 805 mg. tissue, whereas the titer of the control culture (135 mg. tissue) inoculated from the same source was at least 1:1,000. In cultures which contained from 12 to 270 mg. tissue, and which were inoculated with the usual amount of culture material, the virus readily multiplied. A

[1] According to a personal communication from Dr. Rivers, there is a misprint in his paper on "Cultivation of vaccine virus for Jennerian prophylaxis in man," *J. Exp. Med.*, 1931, **54**, 454, line 30: instead of reading "approximately 1 gm. of minced chick embryo tissue," the passage should read "approximately 0.1 gm. . . ."

relationship seems to exist between the amount of tissue in a culture and the amount of inoculum used in infecting it: the smaller the amount of inoculum, the greater the amount of tissue required in the culture to insure infection (Experiments 1 and 2, Table IV).

Amount of Serum Required

That rabbit testicle culture medium must contain serum in order to support growth of pseudorabies virus was shown by three unsuccess-

TABLE IV

Influence of the Amount of Tissue in Cultures on the Multiplication of Pseudorabies Virus

Experiment No.	Inoculum		Culture No.	Amount of tissue	Titer for mice
	cc.	Culture passage			
				mg.	
1	0.1	IX	1	130	1:100
			2	50	Avirulent
			3	10	"
2	0.1	XIIIa	1	270	<1:1,000
			2	140	1:100
			3	10	1:10
	0.4	XIIIa	4	210	1:1,000
			5	82	1:1,000
			6	12	1:1,000
3	0.4	XIa	1	180	1:1,000
			2	90	1:100
			3	10	1:100
4	0.4	XV	1	805	Avirulent
			2	135	<1:1,000

ful attempts to grow the virus in rabbit testicle tissue suspended in Tyrode solution without rabbit serum, according to the method of Li and Rivers (19).

In the first experiment a medium consisting of 100–150 mg. minced rabbit testicle suspended in 4 cc. Tyrode solution was inoculated with 0.5 cc. of a 10 per cent suspension of virulent rabbit brain. After the cultures had been incubated for 3 days at 37°C. they were avirulent for guinea pigs.

In the second experiment culture virus[2] was used as inoculum for the 1st series of cultures. The virus was still demonstrable in the 2nd series of subcultures. The 4th, 5th, and 6th series of subcultures were avirulent. The virus could apparently multiply in the cultures of the first 2 series because they contained traces of serum from the inoculum. When the amount of serum introduced in this way had reached a certain dilution, multiplication of the virus was no longer possible. In

TABLE V

Influence of the Ratio of Rabbit Serum to Tyrode Solution in Cultures on the Multiplication of Pseudorabies Virus

Culture series	Ratio of serum to Tyrode solution	Inoculum		Test for virulence and titer of serial passages			
		cc.	Culture passage	1st passage	2nd passage	3rd passage	4th passage
a	1:1	0.4	XX	•*	•	•	<1:1,000
b	1:3	0.4	XX	1 cc. s.c. Guinea Pig 870. Died in 58 hrs.	1 cc. i.p. in mouse. Died in 51 hrs.	•	<1:1,000
c	1:9	0.4	XX	•	•	•	1:100
d	1:49	0.4	XX	•	•	•	1:100
e	1:99	0.4	XXV	1 cc. i.p. in 2 mice. Died in 92 and 112 hrs.	•	1 cc. i.p. in 2 mice. No illness	
f	1:999	0.4	XXV	1 cc. i.p. in mouse. Died in 68 hrs.	•	1 cc. i.p. in 2 mice. No illness	
g (control for e and f)	1:3	0.4	XXV	•	•	1 cc. i.p. in mouse. Died in 93 hrs.	

* • = not tested; s.c. = subcutaneously; i.p. = intraperitoneally.

control cultures containing 2 cc. rabbit serum and 2 cc. Tyrode solution the virus readily multiplied.

[2] By "culture virus" is meant pseudorabies virus that has been grown *in vitro* by the methods of cultivation described. The term "culture virus" is used in distinction to the term "brain virus" by which is meant pseudorabies virus from the brain of an animal dead following cerebral infection.

The third experiment was carried out similarly. The 1st series of subcultures was still virulent; the 2nd series, however, was avirulent.

An attempt was then made to determine the ratio of serum to Tyrode solution most favorable to the multiplication of the virus. The results of these experiments are recorded in Table V.

The virus multiplied best in cultures in which the ratio of serum to Tyrode solution was 1:1 and 1:3. In cultures in which this ratio was 1:99 and 1:999, the virus could not be cultivated in series, al-

TABLE VI

Influence of Time of Incubation of Cultures on Multiplication of Pseudorabies Virus

Experiment No.	Inoculum cc.	Inoculum Culture passage	Length of time incubated	Titer for mice
			days	
1	0.4	XIIa	2	1:1,000
			3	1:100
			4	1:100
2	0.4	XV	2	1:1,000
			3	1:1,000
			4	Avirulent
			5	"
			6	"
3	0.4	XXIII	1	1:10
			2	1:100
			3	1:1,000
			4	Avirulent
			5	"
			6	"

though it multiplied in serial control cultures containing 1 part serum to 3 parts Tyrode solution inoculated from the same source. During the serial cultivation of pseudorabies virus the impression was gained that 1:1 represented a more favorable proportion of serum to Tyrode solution than 1:3.

Time of Incubation

A number of cultures containing equal amounts of fresh tissue, 2 cc. rabbit serum, and 2 cc. Tyrode solution were inoculated from the same source and titrated in mice after various periods of incubation at 37°C. The results are shown in Table VI.

In Experiments 1 and 2 (Table VI) the cultures had reached their maximum virus content after 2 days' incubation and in Experiment 3 after 3 days' incubation. Incubation of from 4 to 6 days inactivated the cultures in Experiments 2 and 3; this was probably due, as is shown later, to the necrosis of the tissue. For the cultivation of the virus in series 2 days' incubation proved to be sufficient.

Cultivation in Long Test-Tubes

Maitland, Laing, and Lyth (20) found that while vaccinia virus could be grown in Carrel flasks in which the depth of the medium was from 1 to 2 mm., it did not multiply in test-tubes when the height of the column of the fluid covering the tissue was 15 mm. Under such conditions the respiration of the tissue, as measured by the method of Warburg, ceased after 24 hours' incubation at 37°C. The authors believed that there was a correlation between the multiplication of the virus and the respiratory activity of the cells contained in the medium.

The growth requirements of pseudorabies virus differ from vaccinia virus since multiplication was demonstrated in 100–150 mg. minced rabbit testicle tissue suspended in 3 cc. Tyrode solution and 1 cc. rabbit serum in long test (Noguchi) tubes, in which the column of fluid overlying the tissue was from 5–6 cm. high.

Length of Time Media May Be Stored in Refrigerator before Inoculation

The question of how long minced rabbit testicle tissue suspended in 2 cc. rabbit serum and 2 cc. Tyrode solution could be stored in the refrigerator before inoculation was of importance in these experiments. In almost every case the tissue was still suitable for the multiplication of virus after it had been stored in the refrigerator for 4 days prior to inoculation. In one case (see Culture Series XXIX, Table I) the virus did not multiply in tissue which had been kept in the refrigerator for 5 days. In Culture Passage XIII a medium was used which had been stored in the refrigerator for 6 days. The virus became inactive in this medium after 2 days' incubation. In four other experiments media stored in the refrigerator for 6 days were tested. In two of these cases the virus was no longer demonstrable after 2 days' incubation, in the other two cases there was slight multiplication of the virus.

In culture media which had been in the refrigerator for 7, 8, 9, and 10 days prior to inoculation with virus, the virus did not multiply. Such cultures were inactive after 2 days' incubation. In all these experiments the virus multiplied in control media which were fresh or had been kept in the refrigerator for from 1 to 4 days. The failure to grow in stored media is probably due to death of the tissue.

Time of Survival of Culture Virus in the Refrigerator

Cultures of known virulence were stored in the refrigerator for various periods of time and then again tested for their virulence for mice. All cultures were still active after they had been kept in the refrigerator for from 1 to 15 days; after 19 days one culture out of two was avirulent; after 21 days three out of three cultures were still virulent. All cultures which had been in the refrigerator longer than 21 days were inactive. Subcultures could be made from cultures which had been stored in the refrigerator for 1, 2, 4, 5, 16, and 21 days.

Histological Examination of Culture Tissue

Freshly prepared culture media (100–150 mg. minced rabbit testicle suspended in 2 cc. rabbit serum and 2 cc. Tyrode solution) were inoculated with culture virus as usual and incubated for 1, 2, 3, or 4 days. As controls, uninoculated media from the same source were incubated simultaneously for the same period of time. After incubation, pieces of tissue from the culture and control flasks were fixed in Zenker's or Allen's fluid. Paraffin sections were made and stained with phloxin-methylene blue or hematoxylin-eosin. Mouse inoculations in every case showed that virus was present in those cultures from which pieces of tissue had been removed.

Sections from cultures incubated for 1 day showed slight necrosis of the epithelium and very little necrosis of the interstitium. In some interstitial cells the nuclear membranes were slightly hyperchromatic. No inclusions were seen.

In sections from cultures incubated for 2 days necrosis was more pronounced. The interstitium still looked fairly healthy, especially at the edge of the sections. Acidophilic intranuclear inclusions appeared in the interstitial cells of Leydig, in endothelial cells of capillaries, and in connective tissue cells of the lamellar membranes of the tubules (Fig. 1). These inclusions resembled those described by Hurst (21) in many different kinds of cells of rabbits infected with pseudorabies. They were irregular in size, and, sometimes, in shape. Some-

times they had well defined margins. Some nuclei contained several small inclusions, others a single large one. In inclusion-bearing cells and many others hyperchromatosis of the nuclear walls was constantly present; such hyperchromatosis appeared also in sections from uninoculated controls, but in them inclusions were never seen. When the inclusions were completely formed, there was a narrow free space between them and the nuclear membrane. In cells of the seminiferous epithelium inclusions were never found.

In cultures incubated for 3 days there was considerably more necrosis of the tissue. Inclusions were still present.

The tissue of cultures incubated for 4 days was generally necrotic. The nuclei of the cells in which inclusions usually appeared were karyorrhectic. Inclusions were no longer distinct (Fig. 2). In sections from uninoculated control media the interstitium, after 4 days' incubation, still looked surprisingly healthy (Fig. 3). The fact that tissues from cultures were always much more necrotic than those from uninoculated controls is attributed to the action of the virus.

Intranuclear inclusions appeared in only about 60 per cent of the pseudorabies cultures examined histologically. The reason for this irregularity may be that, in some cultures, not all of the many small pieces of tissue become infected by the virus, and that uninfected tissue particles were picked out in those cases in which inclusions were not found.

Inclusions of the same type and in the same kind of cells were also found *in vivo* in the testis of a rabbit that had been infected intratesticularly with virus from rabbit brain. The inclusions were completely formed 24 hours after inoculation, that is, during the incubation period.

Many of the inclusion bodies in pseudorabies cultures are somewhat similar to those found by Andrewes (12) in rabbit testicle cultures of Virus III. The Virus III inclusions, however, appeared in interstitial cells exclusively.

Attempts to Cultivate Pseudorabies Virus in Rabbit Kidney, Liver, and Blood

Since vaccinia virus was found by Maitland and Laing (11) and others to multiply in minced rabbit kidney tissue suspended in rabbit

serum and Tyrode solution, four attempts were made to grow pseudorabies virus under similar conditions. In two of these experiments, cell-free extracts of rabbit brain and rabbit testicle were added to the medium containing approximately 100 mg. minced rabbit kidney in 2 cc. rabbit serum and 2 cc. Tyrode solution. The same technique was followed as for the cultivation of the virus in testicular tissue. In none of the four experiments did pseudorabies virus multiply.

Two experiments with minced rabbit liver and one experiment with rabbit blood were equally unsuccessful.

It may be recalled at this time that Andrewes (13) was unable to grow Virus III in media with minced rabbit kidney and liver as tissue constituents.

Cultivation of Pseudorabies Virus in Guinea Pig Testicle Media

After several unsuccessful attempts pseudorabies virus was cultivated in series in minced testicle tissue taken from guinea pigs of about 500 gm. in weight and suspended in 1 cc. guinea pig serum + 3 cc. Tyrode solution. This ratio of serum to Tyrode solution appeared to be favorable to the multiplication of the virus. Virus cultivated in chick embryo media (see below) was used to infect the cultures of the first series. After the 6th serial passage of the virus through the guinea pig medium the experiment was discontinued. When rabbit serum was substituted for guinea pig serum in this medium, the virus failed to multiply. The pathogenic properties of pseudorabies virus cultivated in guinea pig testicle medium did not differ from that in rabbit testicle medium. The possibility of cultivating pseudorabies virus in guinea pig testicle tissue will be made use of later, when an attempt will be made to grow the virus in tissue from guinea pigs immune to the disease.

Cultivation of Pseudorabies Virus in Chick Embryo Media

Ten day chick embryos were finely minced with scissors, after their eyes had been removed, and 100–150 mg. of the tissue pulp was suspended in 3 cc. Tyrode solution and 1 cc. sheep serum. The same technique was followed as for the cultivation of the virus in rabbit testicle tissue. Virus from the 30th culture series in rabbit testicle medium was used to start the cultures, and the results of the experi-

ment, given in Table VII, show that pseudorabies virus multiplied in the chick embryo media.

From the 5th passage in chick embryo medium the virus was cultivated in both serum-containing (A) and in serum-free (B) media. The medium of Group A was the same as used in the previous culture series; while the medium of Group B, 100–150 mg. of minced chick embryo tissue suspended in 4 cc. Tyrode solution, was the same as used by Li and Rivers (19) for the cultivation of vaccinia virus.

TABLE VII

Cultivation of Pseudorabies Virus in Chick Embryo Tissue

No. of serial culture passage*	Test for virulence		Titer for mice
	Guinea pigs (subcutaneously)	Mice (intraperitoneally)	
Ch. E. I	•†	+	•
" II	+	•	•
" III	+	•	•
" IV	•	+	1:100
" V A	•	+	•
" V B	•	+	•
" VII A	+	•	•
" VII B	+	+	•
" X A	•	+	1:10
" X B	•	+	1:100
" XII A	+	•	•
" XII B	+	•	•
" XIII A	•	+	1:1
" XIII B	•	+	1:100
" XVI B‡	+	•	•
" XIX B	•	+	•

* Started from Transfer XXX in rabbit testicle medium.

† • = not tested; + = virulent.

‡ Series A discontinued.

In the rabbit and guinea pig testicle media a certain amount of serum had been necessary to insure multiplication of pseudorabies virus, but in chick embryo cultures serum was not necessary. The growth of the virus was even better in cultures which did not contain serum (Table VII). The titer of the chick embryo cultures, on the average, was considerably lower than that of the rabbit testicle cultures.

After Culture Series Ch. E. XIII the cultivation in media containing serum was discontinued. In serum-free media the virus was still under cultivation when this paper was completed.

Properties of Culture Virus

Pseudorabies virus did not change its pathogenic properties for mice, guinea pigs, and rabbits in the course of the cultivation and consistently induced a fatal disease, the salient feature of which was violent pruritus causing self-mutilation. The disease following the injection of cultivated virus had a shorter incubation period, a more rapid course, and the lesions at the site of inoculation were larger than in control animals receiving the brain passage virus.

Serum from guinea pigs immunized against either the Hungarian or Iowa strain of brain passage pseudorabies virus neutralized culture virus when the mixtures of serum and virus were tested in the usual way by subcutaneous inoculation into guinea pigs. The culture virus used in the neutralization tests had been under continuous cultivation for 36 serial passages, thus indicating that no immunological change had taken place; that is, it was still immunologically identical with the brain virus from which the cultures were originally made.

In like manner culture virus maintained its filtrability after prolonged cultivation outside the animal body, as was evidenced by the readiness with which culture virus of the 39th serial passage passed Berkefeld filters of N porosity.

Thus, in none of its salient properties did culture virus differ from the brain virus used in initiating the cultures; it was still filtrable, it was still neutralized by virucidal sera prepared against brain virus, and it still produced the typical clinical and pathological picture of pseudorabies in experimental animals.

DISCUSSION

Like many other filtrable viruses, pseudorabies virus can be cultivated in series in the presence of living tissue. The fact that the virus failed to multiply in tissue which had been stored in the refrigerator for more than 6 days prior to inoculation with virus, and in cell-free 20 per cent rabbit testicle extract suggests that living cells are necessary for its growth.

Growth has been obtained in media containing rabbit or guinea pig testicle or chick embryo tissues. Rabbit testicle tissue gave the best results both as to the regularity of the multiplication of the virus and the concentration the virus attained in the cultures. The chick em-

bryo medium, however, is the simplest, as it does not require serum, while the testicle media must contain a certain amount of homologous serum to insure multiplication of the virus. In rabbit kidney tissue, a medium suitable for the cultivation of vaccinia virus, pseudorabies could not be grown.

The pathogenic properties of pseudorabies virus were not altered during the 49 serial culture passages and there were merely quantitative differences between the cultivated virus and rabbit brain passage virus. This was to be expected from the experience of others, since, so far as we know, no filtrable virus has changed its pathogenic properties qualitatively during the course of its cultivation *in vitro*.

SUMMARY

Pseudorabies virus has been cultivated in series in rabbit testicle, guinea pig testicle, and chick embryo media, and its growth requirements have been studied. Intranuclear inclusions, similar to those produced by pseudorabies virus *in vivo*, have been found in rabbit testicle cultures. The virus has not changed its pathogenic properties for rabbits, guinea pigs, or mice during the course of cultivation.

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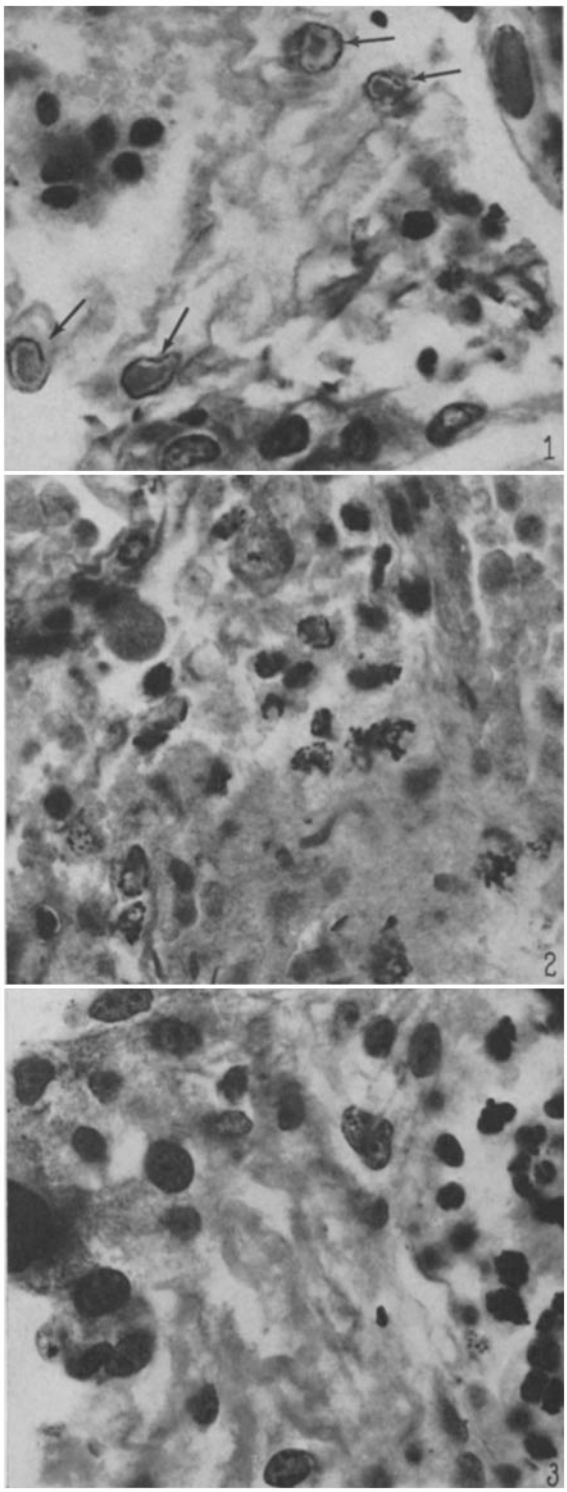
EXPLANATION OF PLATE 40

FIG. 1. Section through a piece of rabbit testicle tissue from a culture incubated for 2 days. Intranuclear inclusions appear in interstitial cells. Hematoxylin-eosin. ×1,033.

FIG. 2. Section through a piece of rabbit testicle tissue from a culture incubated for 4 days. General necrosis. Chromatin has disappeared from the nuclei in the seminiferous tubule (right side of picture). Nuclei of interstitial cells karryorrhectic. Inclusions no longer distinct. Hematoxylin-eosin. ×804.

FIG. 3. Section through a piece of rabbit testicle tissue from uninoculated control media incubated for 4 days. Necrosis much less advanced than in Fig. 2. Nuclei of seminiferous tubule (right side of picture) still contain chromatin. Interstitial cells fairly healthy. Nucleoli still visible. Hematoxylin-eosin. ×804.



(Traub: Cultivation of pseudorabies virus)