MULTIPLICATION IN VITRO OF PSEUDORABIES VIRUS IN THE TESTICLE TISSUE OF IMMUNIZED GUINEA PIGS

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It is known that certain animal tissues, when removed from the animal, minced, and suspended in a suitable fluid medium, will remain alive for some time and can be experimentally infected with virus. With some viruses the infection can be recognized histologically by characteristic tissue changes; for example, the formation of inclusion bodies, hyperplasia, or necrosis. The pseudorabies virus does not cause such changes regularly, and the infection of the tissue can be definitely determined only by the results of inoculation of susceptible animals. In cultures of the virus with rabbit testicle tissue intranuclear inclusions were found in interstitial cells in the majority of the sections (1). In cultures with normal guinea pig testicle tissue intranuclear inclusions have been found in only one out of four series of cultures. They appeared in interstitial cells and were acidophilic like the inclusions in rabbit testicle cultures, but they were much fewer, smaller, and often fragmented. It was evident that in this instance the search for inclusion bodies could not replace the animal test for the presence of the virus in the cultures.

Tissue from immune animals can be tested for susceptibility to viruses only *in vitro*. So long as the tissue is in its physiological environment humoral, and possibly other immunity factors, interfere with the results.

Steinhart and Lambert (2) in 1914 used the tissue culture technique in the study of immunity. They found that vaccinia virus did not multiply in immune rabbit cornea tissue grown in immune plasma, but they did not determine whether the plasma or the tissue was responsible for the failure to grow. In 1929, Andrewes (3, 4) and, simultaneously and independently, Rivers, Haagen, and Muckenfuss

(5) found that Virus III and vaccinia virus would form inclusion bodies in tissues *in vitro*. In Andrewes' experiments Virus III would readily grow and form inclusions in testicle tissue of immune rabbits in the presence of normal serum. In his later studies with herpes simplex (6), and the salivary gland virus of guinea pigs (7) similar results were obtained. With the salivary virus inclusions appeared in normal and immune tissue in the presence of normal serum, but the virus could not be subcultured. Rivers, Haagen, and Muckenfuss studied the formation of Guarnieri bodies in cultures of rabbit cornea in rabbit plasma. In normal corneas soaked in virus for 3 hours and then transferred to immune plasma, Guarnieri bodies developed. Immune corneas, however, even after washing, formed few or no inclusions when grown in normal plasma. Topacio and Hyde (8) in 1932 failed to confirm Andrewes' results with Virus III, although they used a similar technique. In their experiments immune rabbit testicle tissue in normal plasma could not be infected with virus even after washing with Tyrode solution. Immune plasma added to the cultures either before or after Virus III inhibited its growth in normal rabbit testis. The authors therefore tended to the conclusion that the immunity from Virus III infection was of both "the cellular and humoral types."

Because of the disagreement in the results obtained by different workers, an attempt was made to cultivate pseudorabies virus in tissue of immune guinea pigs. Testicle tissue was chosen because pseudorabies virus is known to multiply in this tissue from normal guinea pigs (1).

Methods

Immunization of Guinea Pigs

Immunization against pseudorabies is not a simple matter. Thus far it has failed with rabbits in this laboratory. Shope (personal communication) immunized guinea pigs against the Iowa (mad itch) strain of pseudorabies by repeated subcutaneous inoculations of sublethal doses of virus. He made the observation that this strain, when passed through a guinea pig (intracerebrally), became slightly attenuated for guinea pigs, and that subcutaneous inoculations of doses up to 800 mg. of the brain of such a guinea pig did not produce the disease in guinea pigs (9). When he gave guinea pigs four consecutive subcutaneous inoculations of 100 mg. infective guinea pig brain at 10 day intervals they resisted a subcutaneous inoculation of 100 mg. infective rabbit brain (approximately 100 M.L.D.) given 2 weeks after the last immunizing inoculation. Shope also found that, when an equally large dose of the more virulent Hungarian (Aujeszky) strain was used in the immunity test instead of the Iowa strain, not all of the treated guinea pigs would be immune.

Guinea pigs furnishing the testicle tissue used in the cultivation experiments were immunized according to Shope's method (see Table I). To increase the

degree of their immunity they were given additional inoculations of infective rabbit brain. The Iowa strain had been passed through a few more rabbits since Shope had performed his experiments and it had obviously increased in virulence for guinea pigs by such passages. Three guinea pigs died following the subcutane-

TABLE I

Immunization of the Guinea Pigs, the Testes of Which Were Used in the Cultivation Experiment

Guinea pig No.	Subcutaneous immunizing inoculations										Immunity test		
	Iowa strain virus								Aujeszky strain		Intracerebral inoculation Apr. 1		Inoculation into right testis Mar. 26
	Guinea pig brain					Rabbit brain			Rabbit brain				
	100 mg. Dec. 12, 1923	100 mg. Dec. 22	100 mg. Jan. 2, 1934	100 mg. Jan. 12	100 mg. Jan. 23	100 mg. Feb. 6	100 mg. Feb. 16	100 mg. Feb. 26	10 mg. Mar. 6	100 mg. Mar. 14	10 M.L.D.	100 M.L.D.	100–1000 M.L.D.
27-61	D												
26-77	D												
26-76	0	D											
28-66	0	Died of intercurrent disease											
27-63	0	0	0	0	0	0	0	0	0	D			
27-60	0	0	0	0	0	0	0	0	0	0	—	—	0
27-62	0	0	0	0	0	0	0	0	0	0	—	—	0
28-64	0	0	0	0	0	0	0	0	0	0	—	—	0
28-65	0	0	0	0	0	0	0	0	0	0	—	—	0
28-67	0	0	0	0	0	0	0	0	0	0	—	—	0
26-48	0	0	0	0	0	0	0	0	0	0	0	—	—
26-49	0	0	0	0	0	0	0	0	0	0	—	D	—
36-83													D
38-29													D
38-48											D		
36-19											D		
35-86												D	

D = died.
0 = No illness.

ous inoculation of a dose of infective guinea pig brain which previously was found to be non-fatal. Five guinea pigs were tested for immunity by the inoculation into the right testis of between 100 and 1000 M.L.D. of highly virulent pseudorabies virus cultivated in chicken embryo tissue (36th culture passage). All five guinea pigs resisted this inoculation. Two other guinea pigs were tested for immunity

by intracerebral inoculation of Hungarian virus passed through the brain of a guinea pig. Guinea Pig 26-48 (Table I) resisted the inoculation of 10 M.L.D. (0.1 mg. virulent guinea pig brain), whereas Guinea Pig 26-49 succumbed to the inoculation of 100 M.L.D. (1 mg.) after a prolonged incubation period.

Preparation of Cultures

The testicle tissue of Guinea Pigs 28-64 and 28-65 used in the cultivation experiment was removed approximately 1 month after the intratesticular test for immunity. At that time the sera had strong neutralizing power, and the testicles did not contain virus demonstrable by the intracerebral inoculation of an emulsion of the carefully washed testicle tissue into mice. Control cultures were made with normal guinea pig testicle tissue.

The animals were killed and the testes were removed aseptically through the peritoneal cavity, finely minced with scissors in Petri dishes, and the tissue pulp was transferred with pipettes to large test tubes each containing 20 cc. Tyrode solution. After the tissue had been soaking in the solution for 10 minutes—during which time the tubes were occasionally shaken—the Tyrode solution was pipetted off, and replaced by an equal amount of fresh solution. This procedure was repeated twice. The tissue pulp was then transferred to 50 cc. Florence flasks (50 to 100 mg. per flask) containing mixtures of 3.2 cc. Tyrode solution + 0.8 cc. normal guinea pig serum. In a preliminary experiment, test tubes had been used as containers for the media, but the virus would not grow in them. The cultures of each group registered in Table II were made in triplicate.

Growth was initiated in each culture with 0.1 cc. of a Berkefeld V filtrate of ten ground cultures of pseudorabies virus in chicken embryo tissue (51st culture passage). The titer[1] of this filtrate was 1:1000. An amount of virus corresponding to approximately 100 M.L.D. for mice was thus added to each culture. According to a comparative titration experiment, this amount when inoculated intratesticularly into guinea pigs would correspond to about 1000 to 10,000 M.L.D. It would have been preferable not to inoculate more than 100 M.L.D. for guinea pigs (intratesticularly) into each culture, since the immunity of the guinea pigs used in this experiment was probably not an absolute one, and might have been overwhelmed by too large doses of virus (*vide* Guinea Pig 26-49, Table I). As will be seen from Table II, however, the titer of the first culture passage of group A, the most important one in the experiment, was low enough so that only an amount of virus corresponding roughly to from 4 to 40 M.L.D. (guinea pigs, intratesticularly) was transferred to the cultures of the second passage, and these became infected nevertheless as evidenced by the multiplication of the virus in them.

All cultures were incubated at 37.5°C. for 48 hours. The dilution factor between consecutive culture passages was 10. The titer of the cultures was de-

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[1] By "titer" is meant the highest decimal dilution, 1 cc. of which killed mice when inoculated intraperitoneally.

termined by intraperitoneal inoculations of undiluted ground cultures and decimal dilutions into white mice.

The titration results registered in Table II indicate that multiplication of the virus took place in all three groups of cultures. In normal guinea pig testis the virus always reached the highest concentration. In the culture with right immune testis the rate of multiplication was lower. In the group with left immune testis the virus was lost for an unknown reason after the fourth culture passage. Multiplication of the virus seems to have occurred in the cultures of this group, since there was no gradual decline of the titer from the first to the fourth passage but an equally high titer in the first, second, and third passages.

TABLE II

Cultivation of Pseudorabies Virus in Testicle Tissue from Immune Guinea Pigs

Culture passage No.	Group A		Group B		Group C	
	Cultures with right immune testis		Cultures with left immune testis		Cultures with normal testis	
	Tissue from guinea pig No.	Titer	Tissue from guinea pig No.	Titer	Tissue from guinea pig No.	Titer
1	28-64	1:1	28-64	1:10	40-73	At least 1:100
2	28-64	1:10	28-64	1:10	40-73	At least 1:1000
3	28-64	1:10	28-64	1:10	40-73	At least 1:1000
4	28-65	1:100	28-65	1:1	41-28	At least 1:1000
5	28-65	1:100	28-65	Avirulent	41-28	At least 1:1000
6	28-65	1:10	28-65	Avirulent	41-28	At least 1:1000

SUMMARY

Pseudorabies virus was cultivated *in vitro* in washed testicle tissue from immune guinea pigs, and evidence was thus procured which indicated that the testicle cells themselves had not become immune to pseudorabies. The rate of multiplication of the virus was considerably greater in control cultures with normal guinea pig testis than in cultures with immune testis. The reason for this fact may be that even by repeated washing the immune tissue could not be completely freed from fluid antibodies, and that such antibodies somewhat inhibited the multiplication of the virus.

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