PROPAGATION OF VACCINIA VIRUS IN THE RABBIT FETUS*

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PLATE 3

(Received for publication, May 16, 1940)

The student of viruses, being subject to the limitation that these agents are cultivable only in association with living cells, has sought continually to mitigate this restriction by extending the range of host tissues in various directions. Hence we find that viruses have been propagated and studied in many species of animals other than their natural hosts, and more recently much use has been made of the developing chick embryo and of tissues cultured *in vitro*. Several years ago it was shown by Woolpert and associates (1-4) that certain viruses could be cultivated in the mammalian fetus *in utero*, thus taking advantage of the inherent sterility of embryonic tissue while permitting a study of the reactions of the intact, though immature animal. In any of these techniques the use of new host-parasite combinations may be expected to enhance our knowledge of both host and parasite.

In this paper we are recording our experiences in the propagation of vaccinia virus in the fetus of the rabbit. Here we deal with a susceptible species, experimentally exposed to infection presumably at a most susceptible age. This presumption is based on the results of other studies indicating that often when a species of animal is susceptible to a particular virus its fetus is likely to be more so. The studies of Markham and Hudson (3) with the submaxillary gland virus of guinea pigs, of Stritar and Hudson (4) with the vaccinia virus in guinea pigs, and of Woolpert, Gallagher, Rubinstein, and Hudson (5) on the virus of epidemic influenza in guinea pigs support this generalization. Thus, in using the rabbit fetus for studies of vaccinia virus we are utilizing not only a new experimental host but also one which might be expected to prove much more susceptible than those commonly employed.

Materials and Methods

Strains of Virus.—Two strains of vaccinia virus have been used. For the preliminary experiments we employed the Levaditi, so called neurotesticular strain which had

* Aided by a grant from Eli Lilly and Company.

been maintained in our laboratory by passage through the testicles of rabbits. Most of the work, however, was carried out with a dermal strain, obtained through the kindness of Eli Lilly and Company, which had been recently passed through several transfers in the chorio-allantoic membranes of the chick. Both of these strains initially titered 10^{-5} to 10^{-6} by intradermal inoculation.

Animals.—Pregnant rabbits of appropriate gestation age were selected from the breeding stock of the University Farms. The mating date was known for each animal and pregnancy was confirmed by palpation prior to the inoculation procedure. Various breeds were used in these fetal studies. For skin titrations of the virus only white rabbits were employed.

Technique of Inoculating the Fetuses and Recovering the Virus.—This procedure has been described previously in detail (1). Briefly, the ventral surface of the mother animal was clipped and shaved over a wide area and then painted with disinfectant. Under ether anesthesia (6), and with the precautions of aseptic surgery, a mid-line incision 8 to 10 cm. long was made. The gravid uterus was brought out through this incision; the number and position of the fetuses in the two horns were noted; the individual fetuses were inoculated by needle puncture through the uterine wall and fetal membranes. After the uterus had been returned to the abdominal cavity, the peritoneum and muscle wall were closed in one layer with heavy silk or linen suture, and the skin was treated in similar fashion. A protective coating of flexible collodion was applied externally to the incision.

No special care of the mother was found necessary during the experimental period except for the removal of the skin stitches at about the 5th day in case the experiment was continued beyond that time. At the termination of the incubation period the fetuses were removed by cesarean section, placed in sterile petri dishes, and dissected for study.

Route of Inoculation.—Since the various parts of the fetal body could be recognized through the uterine wall, it was possible, within limits, to inject the inoculum into any desired part of the fetus, the particular site being governed by the purpose of the experiment. We have injected virus into the brain, thoracic cavity, peritoneal cavity, placenta, and amniotic fluid, as well as subcutaneously. In some of the later experiments, in an effort to obtain a better distribution of the virus, a combination of injections into the brain, thoracic cavity, and peritoneal cavity was used. In general it may be said that the intracranial route is most easily employed since the head is the most readily identified part of the fetal body. Occasionally we have added a little sterile India ink to the inoculum to mark the site of injection.

Age of Fetuses.—The normal gestation period of rabbits is about 31 days. From about the 20th day the individual fetuses are sufficiently large that they may be inoculated in the manner described. However, abortion is likely if the mother is too near term. For routine purposes we have found inoculation from the 22nd to the 25th day to be most practical.

Dosage of Virus and Incubation Period.—For the most part, the purpose of these experiments was to produce a severe generalized infection, but one not resulting fatally during the period of the experiment. It was early seen that the three factors of fetal age, virus dosage, and incubation period might be reciprocally effective toward this end. Accordingly, various quantitative combinations of these several factors were tried. For experiments using 24 day fetuses and with an incubation period of 4 days,

a virus dosage of 10,000 adult rabbit skin units appeared to be most satisfactory; whereas, with a 3 day incubation period 20,000 units could be used with advantage. It was found also that an inoculum distributed to several sites was more effective than the same total dose given in a single site.

Tests for Virus.—Tissues removed from infected fetuses were ground in sterile mortars, sometimes with the addition of a little sterile sand, and prepared as 20 per cent suspensions in neutral 50 per cent glycerine or in 0.85 per cent NaCl solution. As routine these suspensions were tested for virus content by the injection of 0.1 cc. amounts of serial tenfold dilutions into the skin of depilated white rabbits. A series of comparative tests was also made using the scarification technique of Leake and Force (7).

Preservation of Virus.—It was found advantageous to preserve virus suspensions in the frozen state according to the method of Turner (8). The material was distributed into small vials; these were sealed and immersed in a mixture of ethyl alcohol and solid CO₂ (dry ice) in a large thermos jug. In this way the gradual loss of titer, which accompanies preservation at 4°C., was avoided.

The Distribution of Vaccinia Virus in the Inoculated Rabbit Fetus

Infection of the rabbit fetus was readily accomplished by the technique described. This was true for both strains of vaccinia virus, but there was some variation in the results as the factors of fetal age, virus dosage, route of inoculation, and incubation period were varied.

That the virus disseminated freely to various organs was indicated both by the resultant gross lesions and by the virus titer of such organs. Representative data from some of the more successful experiments are recorded in Table I, showing the concentration of virus in certain fetal tissues from 3 to 5 days after inoculation by various routes. It will be seen that titers ranging from 10⁻⁴ to 10⁻⁶ were frequent, and that some virus was demonstrable in all of the tissues tested. In other experiments, not here recorded, in which the virus dosage was minimal, or other conditions were unfavorable, the virus yields were correspondingly reduced.

The Manifestations of Vaccinia in the Rabbit Fetus

Proliferation of the virus was found to be associated with characteristic tissue reactions and sometimes death of the fetus. The latter result was encountered more frequently in younger fetuses, or in those given the larger inocula followed by the longer incubation periods. If the fetus had died before the 3rd day following inoculation, gross lesions of vaccinia were absent or slight. In living fetuses, or in those that had survived the infection for 3 days or longer, numerous gross lesions were commonly demonstrable in various fetal organs. Although the distribution of gross lesions was widespread, considerable variation occurred in the degree of involvement of the several organs in different fetuses. Such variation was prob-

ably dependent in part on variations in the route of inoculation and also on fortuitous circumstances not subject to experimental control. Conceivably, such a circumstance might be the exact site of deposition of the inoculum, whether into or near a large blood vessel, which might markedly affect the distribution of the virus. In our experience the liver was most often affected; when lesions appeared anywhere in a particular fetus the liver was seldom spared. Skin and lungs were frequently involved, spleen and kidney

TABLE I
Distribution and Titer of Vaccinia Virus in the Rabbit Fetus Following Direct Inoculation

Rabbit No.	Age of fetus	Route of inoculation*	Incubation in fetus	Fetus No.†	Fetal organs tested and titers found‡					
					Brain	Kidney	Liver	Lung	Placenta	Skin
1	22	i.p.	3	l.v.2	10 ⁻⁴	10 ⁻⁵	10 ⁻⁴	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵
				r.v.1	10 ⁻²	10 ⁻⁴	10 ⁻²	10 ⁻⁵	10 ⁻⁴	10 ⁻⁴
4	23	i.t.	3	l.v.2	10 ⁻⁴	10 ⁻⁵	10 ⁻²	10 ⁻⁵	10 ⁻⁴	10 ⁻⁴
11	24	i.t.	4	l.v.2	10 ⁻⁴	10 ⁻⁶	10 ⁻⁵	10 ⁻⁶	10 ⁻⁵	10 ⁻⁵
19	22	i.t.	5	Pooled	10 ⁻⁵	10 ⁻⁴	10 ⁻⁶	10 ⁻⁶	10 ⁻⁵	10 ⁻⁵
37	23	i.c.	5	Pooled	10 ⁻⁶	n.t.	10 ⁻⁴	10 ⁻⁶	n.t.	10 ⁻⁶
70	26	i.c.	3	Pooled	10 ⁻⁵	n.t.	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵
		i.t.								
72	26	i.p.	3	Pooled	10 ⁻⁶	n.t.	10 ⁻⁵	10 ⁻⁶	10 ⁻⁴	10 ⁻⁵
		i.c.								
		i.t.								
		i.p.								

* i.p. = intraperitoneal; i.t. = intrathoracic; i.c. = intracerebral.

† Fetuses are designated as occupying the right or left uterine horn and numbered in serial order from the vaginal end of the horn, thus l.v.2 indicates the second fetus from the vaginal end in the left horn.

‡ Titers are expressed in terms of the greatest dilution of the tissue which produced a characteristic reaction when inoculated in 0.1 cc. amounts into the depilated skin of white adult rabbits; n.t. indicates the tissue was not tested.

less commonly. The brain rarely showed gross damage except when directly inoculated. Lesions were occasionally seen in the parietal pleura and peritoneum and were rarely noted in the placenta and other organs.

The gross appearance of individual lesions varied as the factors of necrosis, hyperemia, hemorrhage, and edema were less or more prominent. Pocks in the skin, ranging in number from 1 or 2 to 100 or more, appeared as white areas up to 5 mm. in diameter, sometimes umbilicated, and often surrounded by an erythematous areola (Figs. 1 and 2). Sometimes we noted a massive involvement of the tail or of one of the ears, reminiscent of

the observation of Camus (9) following intravenous inoculation of adult animals. Lesions on the visceral surfaces of internal organs more frequently manifested a hemorrhagic character; this was particularly true of kidneys and lungs. However, in the liver, where specific changes were most frequently noted, and in the spleen, necrosis was the outstanding component. Lesions induced in the brain by direct inoculation into that organ were diffuse and less characteristic. Edema, hemorrhage, and softening of the brain substance were often marked. Frequently there was enlargement of the lateral ventricles with relative collapse of the overlying tissue, an effect which we have seen also after the inoculation of agents other than vaccinia virus. Often it appeared that the virus inoculum had spread backward along the path of needle puncture and had permeated into the soft tissues overlying the calvarium, causing hemorrhage and necrosis in the scalp.

The microscopic pathology will be described in detail in a later communication.

Correlation of Virus Yield and Gross Lesions

It seemed likely that a fair degree of positive correlation would be found to exist between the virus content of particular tissues and the amount of gross change observable in them. However, it was learned that although there were never lesions without virus, the converse was not true: in other words, certain organs showing little or no gross pathology might contain virus in large amounts. This occurred frequently with brain, lung, kidney, and placenta. In skin and liver one could predict the virus titer with reasonable assurance on the basis of the number of discrete lesions discernible. In fact, by titrating separately the involved and uninvolved portions of skin dissected from the same fetus, we were able to show that most of the virus was associated with the lesions. Occasional tests of the blood disclosed that its virus content was consistently low, indicating therefore that the large amounts of virus found in various organs did not represent virus derived from the contained blood.

Cross Infection and Immunity Relationships of Fetus and Mother

On several occasions vaccinal lesions were observed in mother animals at the time of removal of their infected fetuses. These were seen particularly in the uterine wall, but sometimes also on the serous surface of the intestines. It was difficult to determine, however, whether the mother acquired vaccinia indirectly from the fetuses, or as a result of inadvertent contamination during the inoculation of the fetuses. The latter seems prob-

able, since the inoculating needle necessarily passed through uterine tissues before entering the fetuses. No direct evidence was obtained that the virus could pass from the fetus to the mother through the placenta, but in view of the fact that the placenta often contained virus in large amount this possibility must be considered. In all of our experiments it was clear that cross infection from inoculated to uninoculated (control) fetuses in the same litter did not occur.

In order to determine the effect of maternal immunity on fetal susceptibility we used several pregnant rabbits that had been passively immunized by the intravenous injection of hyperimmune rabbit serum just prior to the fetal inoculation procedure. The maternal immunity was checked, concurrently with the inoculation of the fetuses, by titrating in the mother's skin the same virus suspension which was used for the fetuses. The complete inhibition of the development of lesions within the experimental period at the sites of injection was taken as evidence of effective immunity of the mother animal. Under such conditions it was apparent that the fetuses were not adequately protected by the antibodies circulating in the maternal blood stream, for it was found possible to kill such fetuses by direct inoculation of the virus and to produce the gross lesions characteristic of vaccinia infection.

Serial Passage of the Vaccinia Virus through Fetal Rabbits

It was found that the vaccinia virus could be passed readily in series from fetus to fetus. The chief difficulty encountered was the determination of the proper relationship of the factors of fetal age, route of inoculation, virus dosage, and incubation period in order to insure a heavy infection at each passage without loss of the material due to premature death of the fetuses.

The principal series of transfers was initiated with the chick strain of virus. With the thought of maintaining the dermal properties of this strain, we used fetal skin as the tissue for subinoculation. In this way a series of 27 transfers was accomplished (Table II). Several variations in the route of inoculation were made during the course of transfer, in the interest of securing consistently satisfactory virus yields. The other factors mentioned also were varied from time to time. Although the virus titer of skin passage material ranged from 10^{-2} to 10^{-6} at different stages of transfer, the end product was of high potency and more consistently good titers were obtained in the latter half of the series.

Two separate series of passages were made using brain and liver respectively as the subinocula in an effort to determine whether modifications of

the passage strain could be induced thereby. These series were initiated with fetal skin of the 18th passage and were continued by intracerebral inoculation at each subsequent transfer. The titer of the brain substrain and its production of lesions in the fetus at different stages of passage are shown in Table III. During the 8 transfers through which this strain was carried no special affinity for nervous tissue was observed to develop. In general, this strain behaved in much the same manner as did the skin passage strain with respect to distribution in fetal organs. Whenever a good

TABLE II
Serial Passage and Titer of Fetal Skin Strain of Vaccinia Virus

Passage No.	Rabbit No.	Route of inoculation*	Titer of passage virus†	Passage No.	Rabbit No.	Route of inoculation*	Titer of passage virus†
1	A-4	i.t.	10 ⁻⁴	17	A-46	i.c.	10 ⁻⁶
2	A-6	i.t.	10 ⁻²	18	A-47	i.c.	10 ⁻⁶
3	A-8	i.t.	10 ⁻⁴	19	A-52	i.c.	n.t.
4	A-11	i.t.	10 ⁻⁵	20	A-54	i.c.	10 ⁻⁵
5	A-12	i.t.	10 ⁻²	21	A-58	i.c.	10 ⁻⁵
6	A-15	i.t.	n.t.	22	A-59	i.c.	n.t.
7	A-16	i.t.	10 ⁻⁵	23	A-60	i.c.	10 ⁻⁵
8	A-17	i.t.	10 ⁻⁶	24	A-61	i.c. }	10 ⁻⁵
9	A-18	i.t.	n.t.			i.p. }	
10	A-19	i.t.	10 ⁻⁵	25	A-63	i.c.	10 ⁻⁴
11	A-33	s.c.	10 ⁻²	26	A-68	i.c. }	10 ⁻⁵
12	A-34	s.c.	10 ⁻⁴			i.t. }	
13	A-37	s.c.	10 ⁻⁶			i.p. }	
14	A-39	i.c.	10 ⁻⁶	27	A-72	i.c. }	10 ⁻⁵
15	A-40	i.c.	n.t.			i.t. }	
16	A-42	i.c.	10 ⁻⁵			i.p. }	

* i.t. = intrathoracic; s.c. = subcutaneous; i.c. = intracerebral; i.p. = intraperitoneal.

† n.t. indicates tissue was not tested.

generalization was obtained in the fetus, the virus seemed to affect the skin quite as readily as it did the brain. Other organs were less regularly involved. The liver strain of virus was more difficult to maintain and was abandoned after the 4th passage but during that time no peculiar properties were noted.

During the course of transfer through fetal rabbits the several passage strains retained their identity as vaccinia virus. This was shown by their ability to produce typical vaccinal lesions both in fetuses and in the skins of adult rabbits, and by the production in the latter animals of an immunity to other known strains of the virus.

There was, however, evidence that certain qualitative changes had been induced in the skin strain of virus by passage through the fetus. While it continued to maintain its high titer, frequently inducing rabbit skin reactions at dilutions of 10^{-6} , the type of lesion resulting became distinctly milder. Whereas the original chick virus produced necrosis at dilutions of 10^{-3} and 10^{-4} , the fetal skin strain often caused little necrosis, if any, even in dilutions of 10^{-1} and 10^{-2} . The results of simultaneous titrations of the

TABLE III
Serial Passage and Titer of Fetal Brain Strain of Vaccinia Virus and Distribution of Gross Lesions in Certain Fetal Organs

Passage No.	Rabbit No.	Titer of passage virus	Fetus No.	Fetal organs and degree of involvement with gross lesions of vaccinia					
				Brain	Kidney	Liver	Lung	Skin	Spleen
1	A-47	10^{-5}	r.v.3	+++	+	++	++	++	-
			r.v.4	+++	++	+++	+++	++++	++
			l.v.6	+++	+	++++	+	++++	-
2	A-52	n.t.	l.v.5	+	-	+++	++	++	++
			l.v.6	+	-	++++	++	-	-
3	A-55	10^{-5}	r.v.1	++++	-	++	+	++	-
			r.v.2	+++	-	++	-	++	-
4	A-56	10^{-5}	r.v.1	+++	-	+++	-	++	++
			r.v.2	+++	++	++	++	++	-
			r.v.3	+++	++	+++	+++	++	++
5	A-58	10^{-5}	r.v.4	+++	-	+++	-	++++	-
			r.v.5	+++	-	+++	+++	+++	-
6	A-60	10^{-5}	l.v.2	++++	-	+	-	++	+
			l.v.3	+++	-	+++	++	+++	+++
			l.v.4	+++	-	++	++	++	++
7	A-62	n.t.	l.v.2	++	-	++	++	++	-
8	A-64	10^{-5}	l.v.4	+	-	-	-	+	-
			l.v.5	+	-	-	-	+	-

Plus marks indicate the amount of gross change, four plus implying maximal involvement.

two strains in the same rabbit are shown in Fig. 3. In Fig. 4 are shown similar titrations in which the Levaditi neurotesticular virus is compared with the fetal skin passage strain. In both instances the comparatively mild lesions were caused by the fetal product.

It was found also that the fetal skin strain, in contrast to the chick virus, gave poor takes when inoculated by the scarification method. Confluent lesions were obtained only with very low dilutions of the fetal virus, such as 10^{-1} , whereas the original chick strain gave confluent reactions at 10^{-3} or higher.

The fetal brain strain also produced mild lesions on intradermal inoculation and similarly had a low titer when tested by the scarification technique.

TABLE IV
Comparison of the Intradermal and Scarification Titers of Certain Strains of Vaccinia Virus

Strain of virus	Intradermal titer	Scarification titer*				
		Dilutions of virus				
		1/100	1/300	1/1,000	1/3,000	1/10,000
Original chick	10 ⁻⁶	++++	++++	++++	+++	5
Fetal skin—23rd passage	10 ⁻⁵	4	—	1	—	2
Fetal skin—24th passage	10 ⁻⁵	n.t.	25	1	1	—
Fetal skin—26th passage	10 ⁻⁵	13	—	—	—	—
Fetal brain—8th passage	10 ⁻⁶	35	—	—	—	—

* Plus marks indicate the degree of confluency of lesions in the scarified area. Figures indicate the number of individual lesions in the absence of confluent reaction.

TABLE V
Effects of Certain Strains of Vaccinia Virus on Adult Rabbits Intracerebrally Inoculated

Rabbit No.	Strain of virus inoculated	Dilution of virus inoculated	Reactions of the rabbit		
			Maximal temperature	Other symptoms	Outcome
			°F.		
6-30	Neurotesticular	1/100	105.4	Weakness	Recovery
6-44	Neurotesticular	1/5	105.5	Moribund	Sacrificed
6-65	Brain of No. 6-44	1/5	106.5	Moribund	Sacrificed
8-69	Brain of No. 6-65	1/5	106.4	Moribund	Died
9-43	Chick membranes	1/4	106.9	None	Recovery
6-28	Fetal skin—18th passage	1/5	106.3	None	Sacrificed
6-67	Brain of No. 6-28	1/5	103.2	None	Recovery
9-02	Fetal skin—22nd passage	1/10	106.0	None	Recovery
9-07	Fetal skin—22nd passage	1/10	106.4	None	Recovery
8-54	Fetal brain—4th passage	1/5	104.8	None	Sacrificed
9-22	Brain of No. 8-54	1/5	104.8	None	Recovery
9-05	Fetal brain—5th passage	1/5	104.3	None	Recovery
9-15	Fetal brain—5th passage	1/5	106.7	None	Recovery

It must be remembered in this connection that the brain substrain was split off from the fetal skin passage virus after the latter had already suffered attenuation by reason of 18 serial transfers in the fetus. The results of

comparative intradermal and scarification titrations of the original chick strain, the fetal skin passage strain harvested from some of the later transfers, and the fetal brain strain of the last transfer are shown in Table IV.

In order to determine whether the passage strains had acquired any neurotropic tendencies by reason of the intracerebral route of administration to fetuses, they were inoculated intracerebrally into adult rabbits. A similar study was made of the chick strain and the neurotesticular virus for comparison. Representative findings are set forth in Table V. In no case was the fetal skin strain found to be capable of eliciting other than a febrile response in these animals. The same may be said of the fetal brain strain and the parent chick virus. In contrast, the neurotesticular virus killed consistently on intracerebral administration, and this ability was maintained in brain-to-brain transfer through adult rabbits. These observations led to the conclusion that none of the fetal strains had acquired properties characteristic of the neurotesticular vaccinia virus.

DISCUSSION

The results of this study indicate clearly that the rabbit fetus may be used successfully as an experimental animal in work with the vaccinia virus. For certain purposes it offers advantages which are unique, specifically, its inherent sterility and high degree of susceptibility. Thus, by the use of the fetus, we were able to propagate and maintain free of bacteria a mammalian skin passage strain of the virus. There is no way in which this could be accomplished in the postnatal animal, except possibly by the use of the elaborate apparatus and technique developed by Reyniers (10).

It was of interest to follow the reactions of the very susceptible rabbit fetus to vaccinia virus. Although we do not ordinarily think of the dermal strain as being highly virulent, in the immature animal it often produced generalized lesions and death after periods of incubation as short as 2 or 3 days. Evidently the virus gained access to blood channels from the site of inoculation and was freely disseminated throughout the body. As a large vascular organ in which the white lesions of vaccinia stood out in marked contrast, the liver seemed to be most frequently and most severely affected. It was also the most dependable source of virus, a finding comparable to that of Buddingh (11) for the infected chick embryo. On the other hand, Stritar and Hudson (4) in a study of the guinea pig fetus infected with the Levaditi neurotesticular strain of vaccinia virus, found virus most consistently and in highest titer in fetal kidney. Although the dermal strain which we used appeared to have no exclusive affinity for the fetal skin, lesions were commonly found there and were easily discernible since the rabbit fetus is without hair.

It may be recalled that no adequate explanation for the relatively high susceptibility of embryonic tissues to virus infection has been forthcoming. Fellowes and Hudson (12) were unable to account for the susceptibility of the guinea pig fetus to vaccinia virus on the basis of the content of Duran-Reynals factor in fetal tissues, nor could they explain the distribution of lesions or virus on this basis. Stewart and Duran-Reynals (13) likewise failed to account for the distribution of vaccinia virus in the adult rabbit. In his study of the infected chick embryo Buddingh (11) reported vaccinia virus distributed throughout the entire organism. He concluded that mechanical or physical factors mainly determined the amount of virus distributed to the various organs.

Stewart and Duran-Reynals (13) found no correlation between virus yield and histologic change in most of the organs examined, a result in general agreement with our study of the rabbit fetus, and with the results of Stritar and Hudson (4) for the guinea pig fetus. We found repeatedly that certain organs showing little or no gross change might contain virus in large amount. However, unlike Stewart and Duran-Reynals (13) and Stritar and Hudson (4) we did not see lesions in organs that yielded no virus.

We wished particularly to learn whether serial passage of the virus through an unusually susceptible host would significantly alter its virulence. *A priori*, we had no notion whether to expect an enhancement or reduction of virulence under these conditions. Actually, the virulence of the skin passage strain was reduced for the passage host as judged by a falling off of its initial capacity to infect the adult rabbit when inoculated by the dermal scarification method; also, as judged by the mildness of the lesion produced by the passage strain when inoculated intradermally in adult rabbits, as contrasted to the much more widely hemorrhagic and necrotic lesion of the parent strain. These differences obtained despite the fact that there was no reduction of titer as determined by the intradermal route of inoculation.

The finding of a loss of virulence on passage of this virus through a medium highly favorable for growth is not a new experience. Commercial producers of vaccine have long known that continuous passage in the calf results in an attenuated virus. Rivers and associates (14-17) have encountered this difficulty in culture of the virus on a medium consisting of chick embryo tissue and Tyrode's solution. They found that both titer and virulence began to fall at about the 20th passage. A restoration of potency was accomplished several times by resorting to passage through the rabbit testis. Although their 2nd revived strain was maintained without much further change, it was definitely attenuated as compared with the original calf vaccine. Goodpasture and Buddingh (18) have emphasized the stability of vaccinia virus cultured for many generations in the chorio-allantoic

membranes of the chick; yet again, during the early passages, there was a reduction in pathogenicity as judged by dermal tests in various mammals.

In explanation of these observations one could hypothesize that a given "strain" of virus may consist of particles having different degrees of virulence, and that when cultivation is carried out in very susceptible tissues the particles of less virulence are permitted to proliferate freely, so that the harvested product appears attenuated. On the other hand, it may be supposed that in a more resistant host the elements of low pathogenicity are selectively suppressed, to the end that the initial virulence is maintained or enhanced.

SUMMARY

1. A method is described for propagation of bacteria-free vaccinia virus in the rabbit fetus.

2. For the production of severe generalized infection, affording a high yield of virus, the following conditions were found to be favorable: the use of 24 day fetuses, a virus dosage of 10,000 adult rabbit skin units, injection into multiple sites, and an incubation period of 4 days *in utero*.

3. Under these conditions the virus was found to disseminate widely in the infected fetus, being recoverable particularly from liver, lungs, brain, skin, placenta, and kidney.

4. Under varied conditions of virus dosage, fetal age, and incubation period, all grades of reaction were observed in the fetus, ranging from the mildest infection, with only an occasional small lesion demonstrable, to generalized vaccinia with pocks in nearly every organ of the body, and often death.

5. It was shown that the virus could be carried in serial passage through fetuses. A series of 27 such passages was made by using fetal skin for subinoculation at each transfer. Similarly, two other strains of the virus were evolved in shorter series of transfers by using respectively fetal brain and fetal liver as the subinocula.

6. The passage strains maintained their identity and titer, as judged by the intradermal inoculation of adult rabbits. However, these strains underwent a significant reduction in their capacity to infect by the scarification method, and the lesions induced by intradermal inoculation were much milder than those produced by the parent strain. None of the passage strains manifested properties characteristic of the so called neurovaccine strain, despite the frequent use of intracerebral inoculation in the fetuses.

7. In explanation of the reduction of virulence of the passage strains, it is suggested that a given suspension of virus may be considered as con-

sisting of particles of varying degrees of virulence, and that when cultivation is carried out in highly susceptible tissues a relative overgrowth of the less virulent elements is permitted, so that apparent attenuation results.

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EXPLANATION OF PLATE 3

FIGS. 1 and 2. Fetal rabbits infected with the fetal skin passage strain of vaccinia virus. About $\frac{1}{2}$ natural size.

FIG. 3. Intradermal titration of chick strain of vaccinia virus (upper row) and fetal skin passage strain (lower row) in the adult rabbit.

FIG. 4. Intradermal titration of Levaditi neurotesticular strain of vaccinia virus (upper row) and fetal skin passage strain (lower row) in the adult rabbit.

(Gallagher and Woolpert: Propagation of vaccinia virus in rabbit fetus)