THE COMPARATIVE SUSCEPTIBILITY OF FETAL AND POSTNATAL GUINEA PIGS TO THE VIRUS OF EPIDEMIC INFLUENZA*

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Studies designed to investigate the relationship between age of the host, and susceptibility to particular infectious agents, have usually compared the reactions of newborn or young animals to those of the adult. However, it is apparent that by the time of birth the mammalian animal is already well developed, structurally and functionally. It is pertinent then to project such studies into the period of prenatal life to envisage the whole course of development and to learn whether the reactive capacities that prove to be characteristic of the species are acquired gradually during intrauterine growth, or evolve after the incident of birth.

It occurred to us that a comparison of the reactions of fetal and postnatal guinea pigs of various ages to the virus of epidemic influenza might contribute significant information in this connection. Studies in our laboratory (Woolpert, Gallagher, Rubinstein, and Hudson (1)) have shown that the immature fetal guinea pig is demonstrably susceptible to this virus, and on the other hand, the work of Stuart-Harris (2) had indicated that young adult animals are relatively resistant. We determined, therefore, to extend these studies to include intermediate age groups represented by the mature fetal guinea pig and the newborn, to learn, if possible, when and how this increased resistance develops.

Materials and Methods

Strain of Virus.—The PR8 strain of epidemic influenza virus isolated by Francis in 1934 (3) was used.

Maintenance of Virus.—The stock virus is maintained in our laboratory by the usual method of propagation through mice. For this work, mouse lung filtrates were prepared as follows: A number of young white Swiss mice were inoculated intranasally from the stock material while under light ether anesthesia. As soon as deaths appeared in the group of mice, generally about 4 days after inoculation, the lungs of the entire lot were

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harvested. These were ground with sand and diluted approximately 1 part to 7 with sterile broth, and then the suspension was passed through a Berkefeld V or N candle. The filtrate was divided into small amounts and stored in vials at −78°C. in a large thermos jar containing alcohol and solid CO_2. The strength of each lot of virus suspension was determined by intranasal inoculation of young mice and appropriate cultures were made to insure bacterial sterility.

At the termination of experimentation we confirmed the identity of the virus which had been used throughout the work by testing it against a specific neutralizing serum kindly supplied by Dr. Francis, and also against an antiserum previously prepared in our own laboratory. At the same time the histopathology induced by the stock virus in the lungs of infected mice was examined and found to be that which is characteristic for the virus of epidemic influenza.

Guinea Pigs.—The guinea pigs used were a hybrid assortment obtained as needed from various sources. For studies on the mature fetus we secured pregnant animals near term. Although the exact age of the fetuses was not known, it was found that their age could be judged with fair accuracy by consideration of the size of the fetal heads (taking into account the number of fetuses present), and the spread between the maternal pelvic bones at the symphysis pubis. The pubic bones begin to separate about 5 to 6 days before term and the space gradually widens. Just previous to delivery there is a separation of approximately a finger's width. The gestation period of the guinea pig is 60 to 65 days and we used fetuses 45 or more days old.

All newborn guinea pigs were delivered in our own laboratory and were inoculated within 10 hours after birth. Guinea pigs of approximately 600 gm. were employed as adults.

Technique of Inoculation.—The newborn and adult guinea pigs were inoculated either intranasally or intracranially, under ether anesthesia, in accordance with the usual methods. The mature fetal guinea pigs were inoculated by a method previously used by Woolpert (4), as follows: Hair was removed from the abdomen of the pregnant animal by clipping and shaving over a wide area. The guinea pig was then anesthetized with ether and secured to the table by the forelegs only. This provided a relaxed muscle wall and allowed freedom of movement for the identification and manipulation of the fetuses. Under these conditions the heads of the fetuses were readily palpated through the abdominal wall of the mother. They were held securely between the thumb and fingers of the left hand and approximated to the abdominal wall. The overlying skin surface was then painted with disinfectant and the inoculum was introduced by means of a fine gauge needle passed directly through abdominal wall, uterine wall, and fetal membranes into the cranium of the fetus. By this method 0.2 cc. of virus suspension was regularly injected without any apparent damage attributable to the manipulation. It was found that as many as 3 fetuses in one litter could be inoculated easily in this way, but the identification and inoculation of a greater number involved excessive manipulation which occasionally resulted in premature delivery.

Recovery of the Experimental Material.—Following suitable incubation periods *in utero*, the fetuses were usually removed aseptically by cesarean section. In certain instances such fetuses were allowed to live as newborns under the care of foster mothers. Occasionally, pregnant animals were permitted to deliver naturally. If the fetus was to be sacrificed for lung examination, care was taken to prevent the animal's breathing and it was killed by exsanguination through the umbilical vessels.

Tests for Virus.—Routine tests for virus in the organs of experimental animals were made by preparing suspensions in broth in suitable dilutions and inoculating young white mice intranasally, under ether anesthesia, in groups of 3 for each material tested. The results were judged on the basis of lethality of the material and the degree of lung involvement in those test mice surviving to the 10th day. In certain studies the more delicate method of Smorodintsev and Ostrovskaya (5) was employed. In accordance with their procedure, if none of the 3 test mice died within 6 days, all were sacrificed, their lung lesions were noted, and a 1:10 suspension of their lungs was inoculated into 3 other mice. The subinoculated mice were observed for a maximal period of 10 days and the survivors sacrificed for examination at the end of that time.

TABLE I

Distribution of the Virus of Epidemic Influenza in Mature Fetal Guinea Pigs after Various Periods of Incubation

(Intracerebral Inoculation)

Incubation period	Relative amounts* of virus demonstrated in the following fetal organs				
	Brain	Lung	Liver	Kidney	Placenta
days					
1	++	++	±	n.t.	−
2	−	+++	−	n.t.	−
4	+	++++	+	+	−
6	+	++++	+++	+++	n.t.
10	−	−	−	−	−
10	−	−	−	−	−

* Relative amounts of virus were estimated on the basis of the reactions of the 3 test mice inoculated for each fetal tissue: ++++ indicates all 3 of the test mice died with typical findings and within the observation period of 10 days, implying maximal amount of virus; +++ = 2 mice died; ++ = 1 mouse died; + = extensive lung lesions but no deaths; ± = a few lung lesions; − = no lung lesions; n.t. = not tested.

Susceptibility of the Mature Fetal Guinea Pig

Varying Incubation Periods.—It was found that the mature fetal guinea pigs could be infected readily by intracerebral inoculation of the virus, as previously reported (1) for the immature fetus. In the study cited, incubation periods of 2 to 6 days were used successfully. In order to determine the optimal incubation period for producing a large yield of virus in the mature guinea pig fetus, we tried periods from 1 to 10 days, as indicated in Table I. The yield of virus from brain, lung, liver, kidney, and placenta, as determined by the mouse test, is indicated for the several incubation periods. To make the results more clear graphically, and in the interest of brevity, we have recorded our estimates of the relative amounts of virus in terms of plus marks, rather than in the usual form expressing directly

the reactions of test mice. It will be seen that virus was recovered from all the organs tested except the placenta; that dissemination and proliferation of the virus apparently continued through the 6th day; but that virus was not found in any of the tissues examined at the 10th day. It appears also that the lungs were the most consistently favorable site for proliferation of the virus, as we have found to be the case in younger fetuses.

None of the fetuses died as a result of the virus infection nor were gross lesions seen in any of the organs, although an inoculum equivalent to 4,000 minimal lethal intranasal doses in mice was used. It is evident, therefore, that the mature guinea pig fetus serves as a good culture medium for influenza virus, permitting spread and multiplication of the virus, but the animal cannot be said to be susceptible in the sense of the white mouse intranasally inoculated.

The question naturally arose, why virus was not demonstrable at the 10th day of incubation. We made a few tests to investigate the possibility that specific neutralizing antibodies might account for the disappearance or masking of the virus, and these preliminary studies showed that antibodies in high titer actually were present in fetal blood at the 10th day. This finding may be sufficient to account for the apparent disappearance of virus, but until this aspect of the matter is pursued further we cannot say definitely whether these antibodies were produced actively by the fetus or were derived from the mother. Our data suggest that the former explanation is probably the correct one, since the antibody titer was generally higher in the fetus.

Serial Passage of the Virus.—From the results recorded in Table I it appeared that it should be possible to pass the influenza virus in serial transfer through mature guinea pig fetuses, as had been reported for the immature fetus (1). Obviously, serial passage constitutes a more rigorous test of susceptibility and is necessary to prove conclusively that multiplication of the virus occurs in these animals.

For passage we used cerebral inoculation of lung tissues at each transfer, and a 48 hour incubation period. In this way 8 serial passages of the virus were accomplished, as shown in Table II. The virus flourished during the first 6 transfers, as evidenced by high yields in the lung tissue, and by an increased dissemination to other organs. All test mice died that were inoculated with 6th passage material, whether from brain, lung, liver, kidney, placenta, or fetal blood. However, in the 7th and 8th passages there was a reduction in potency and spread of the virus, so that only the lung yielded significant quantities. In several repetitions of the latter passages the same results were obtained.

At this time we have no satisfactory explanation for the apparent diminution in the invasive powers of the virus. Possible explanations which suggest themselves are: modification of the virus by passage, the use of unusually resistant guinea pigs, and seasonal variation in resistance of the experimental animal, as encountered by Lillie and associates (6) in working with the virus of St. Louis encephalitis in mice and endemic typhus in guinea pigs, and by Shope (7) in studies on the virus of swine influenza. In our study, the later passages of the virus were made in the warmer weather.

TABLE II

Distribution of the Virus of Epidemic Influenza in Mature Fetal Guinea Pigs during Serial Passage Utilizing a 48 Hour Incubation Period

(Intracerebral Inoculation)

Passage No.	Relative amounts of virus demonstrated in the following fetal organs					
	Brain	Lung	Liver	Kidney	Placenta	Blood
I	−	+++	−	n.t.	−	n.t.
II	n.t.	++++	n.t.	n.t.	n.t.	n.t.
III	n.t.	++++	n.t.	n.t.	n.t.	n.t.
IV	−	++++	±	n.t.	+	n.t.
V	±	++++	+	n.t.	−	−
VI_a	++++	++++	+++	++++	++++	++++
VI_b*	−	±	−	−	−	±
VI_c*	±	++++	−	−	−	−
VII_a	−	+++	−	−	−	−
VII_b	−	+++	−	−	−	−
$VIII_a$	−	±	±	−	−	−
$VIII_b$	−	++++	−	−	−	−

* These passages were made after passage $VIII_a$ had been completed.

In any case, the results of serial passage add further evidence that the mature guinea pig fetus is moderately susceptible to this virus and show that actual multiplication of the virus took place, even though, as in the single inoculations, there was no gross damage to the tissues.

Findings with the Newborn Guinea Pig

Intracerebral Inoculation.—In order to afford a basis of comparison with the results obtained in fetal guinea pigs, the first series of newborn animals was inoculated intracerebrally within a few hours after birth. As indicated in Table III, 8 animals were used in this experiment. The inoculum was the same as for the fetuses, *viz.*, 4,000 mouse units, and the incubation periods ranged from ½ day to 10 days. Routine tests for the virus in brain, lung,

liver, kidney, and blood showed that there was little or no virus in any of these organs. Although gross lesions were noted in the lungs of a few of the test mice they were for the most part slight and of irregular occurrence. That these changes were not caused by influenza virus was confirmed by the more delicate method (5) of subinoculation to other mice. In no instance did any of the mice die, whether receiving an inoculum directly from the guinea pigs, or secondarily from the 1st passage test mice. There were no symptoms of infection in the guinea pigs throughout the observation period,

TABLE III

Distribution of the Virus of Epidemic Influenza in Newborn Guinea Pigs after Various Periods of Incubation

(*Intracerebral Inoculation*)

Incubation period	Relative amounts* of virus demonstrated in the following organs									
	Brain		Lung		Liver		Kidney		Blood	
	A	B	A	B	A	B	A	B	A	B
days										
½	−	−	−	−	−	−	n.t.	n.t.	−	n.t.
1	−	−	−	−	−	−	n.t.	n.t.	n.t.	n.t.
2	−	−	±	−	−	−	−	−	−	−
3	−	−	−	−	−	−	n.t.	n.t.	n.t.	n.t.
4	−	−	−	±	−	−	−	±	−	−
6	±	−	−	−	−	−	±	−	−	−
8	±	−	−	−	−	−	−	−	−	−
10	−	−	+	−	−	−	−	−	−	−

* In column A, in each case, are listed the estimates based on the primary inoculation of test mice; in column B are recorded the more delicately accurate estimates derived from the results of subinoculation to other mice.

nor were there detectable pathologic changes in any of the organs at termination of the experiments.

We concluded then that following intracerebral inoculation in newborn guinea pigs the virus of epidemic influenza is unable to set up a generalized or focal infection. However, tests for neutralizing antibodies in the sera of animals carried for the 8 and 10 day periods were strongly positive, indicating that there was a specific serologic response, perhaps accounting for the failure to recover the virus. Control tests for similar antibodies in the blood of normal newborn guinea pigs showed that the content of such substances was very low.

Intranasal Inoculation.—A second series of newborn animals was inoculated intranasally as shown in Table IV. The same virus dosage and in-

cubation periods were used as in the previous series, and tests for virus were carried out in a comparable manner, the method of mouse passage being employed to detect any small quantities of virus not found on primary inoculation of test mice. It will be seen that virus was recoverable in considerable quantity, especially in the lungs, and in certain instances in other organs, after incubation periods ranging from ½ to 6 days. At the 8 and 10 day periods virus was not demonstrable by the methods employed, and again, neutralizing antibodies were shown to be present. No symp-

TABLE IV

Distribution of the Virus of Epidemic Influenza in Newborn Guinea Pigs after Various Periods of Incubation

(*Intranasal Inoculation*)

Animal No.	Incubation period	Relative amounts of virus demonstrated in the following organs									
		Brain		Lung		Liver		Kidney		Blood	
		A	B	A	B	A	B	A	B	A	B
	days										
1	½	±	−	++++	n.t.	−	−	−	−	−	−
2	1	−	−	++++	n.t.	+	++++	−	−	+	++++
3	2	−	−	−	−	+	−	−	−	−	++++
4	2	−	n.t.	++	n.t.	−	n.t.	−	n.t.	n.t.	n.t.
5	2	−	n.t.	++++	n.t.	−	n.t.	−	n.t.	n.t.	n.t.
6	4	±	±	±	−	−	±	−	−	−	−
7	4	−	n.t.	++++	n.t.	−	n.t.	−	n.t.	n.t.	n.t.
8	4	n.t.	n.t.	++++	n.t.	−	n.t.	n.t.	n.t.	n.t.	n.t.
9	6	−	++++	+	++++	−	±	−	±	−	−
10	6	−	n.t.	++	n.t.	−	n.t.	−	n.t.	−	n.t.
11	8	−	−	−	−	−	−	−	−	−	−
12	10	−	−	−	−	−	−	−	−	−	−

toms, deaths, or gross lesions, attributable to the action of the virus, resulted.

To determine whether virus actually multiplied in these young animals after nasal inoculation, we made 5 serial transfers from guinea pig to guinea pig using a 24 hour incubation period and lung tissue for the subinoculum. Virus was found in the lungs in high titer at each transfer, indicating that proliferation of the virus must have taken place. This is compatible with the findings of Stuart-Harris (2), who was able to pass the virus serially through somewhat older, recently weaned guinea pigs. In that study turbinate and turbinate-lung suspensions were used as passage material, the virus being detectable most consistently in the turbinates.

A Study of Fetal Infections Extended into Neonatal Life

From the foregoing results it appeared that with the incident of birth the guinea pig suddenly becomes refractory to systemic (intracerebral) infection with influenza virus, although it still remains moderately susceptible when the virus is given intranasally. It became of interest then to investigate more closely the effects of this transition from fetal to postnatal life. We were curious to learn whether animals inoculated intracerebrally only a few hours before birth, and allowed to live after they were born, would respond in a manner comparable to the mature fetus, or behave as animals initially inoculated after birth.

In order to obtain information on this point we inoculated a number of fetuses intracerebrally shortly before the natural time for their birth and either delivered them by cesarean section at definite intervals or permitted spontaneous birth when this occurred at a time favorable for the experiment. The details of this experiment are recorded in Table V. Altogether, 34 animals in 13 litters were used. The incubation period *in utero* ranged from 6 hours to $3\frac{1}{3}$ days and the total incubation period from 12 hours to 13 days. At the end of the combined intrauterine and extrauterine incubation period the animals were sacrificed and certain organs were examined for virus in the usual manner.

The results of this study may be summarized as follows: Virus could not be demonstrated if the total incubation period had been 18 hours or less, or if it exceeded 6 days. Regardless of the total incubation period virus could not be found in significant amount unless there had been an intrauterine period of at least 12 hours. An intrauterine period of 36 hours seemed necessary to insure a good growth of virus in the lungs, and for generalization of the virus a period of 48 hours before birth seemed requisite.

It appeared then that mere inoculation of the animal prior to its birth was not sufficient to permit the establishment of an infection which would progress in postnatal life. There was necessary a minimal incubation period *in utero* during which time it may be presumed the virus was becoming established and spreading from the cerebral site of inoculation. If this process was interrupted the infection was aborted. But if the suitable intrauterine period of 24 to 36 hours was provided the infection proceeded in postnatal life just as it would have done in the fetus.

Studies of the Adult Guinea Pig

Although Stuart-Harris (2) had shown that inapparent infection can be produced in adult guinea pigs by intranasal instillation of the influenza virus, we wished to confirm these reports in our own laboratory and to obtain

data on the adult under conditions comparable to those used for the younger animals.

TABLE V

Distribution of the Virus of Epidemic Influenza in Guinea Pigs Inoculated Intracerebrally in Utero and Permitted to Live after Birth

Incubation period		Total hrs.	Relative amounts of virus demonstrated in the following organs				
Hrs. before birth	Hrs. after birth		Brain	Lung	Liver	Kidney	Blood
6	42	48	−	−	−	−	−
	90	96	−	±	−	−	−
12	0	12	−	−	−	−	−
	36	48	−	++	−	−	−
	84	96	−	±	−	−	−
18	0	18	−	−	−	−	−
	30	48	−	+	−	−	−
	30	48	−	+++	−	−	−
	77	95	−	−	−	−	−
	78	96	−	±	−	−	−
	222	240	−	−	−	−	−
36	9	45	−	++++	−	−	−
	12	48	−	++++	+	±	−
	36	72	−	±	−	−	−
	60	96	−	−	−	−	−
	80	116	−	±	−	−	−
	106	142	−	−	−	−	−
	158	194	−	±	−	−	−
48	0	48	±	++++	+	−	−
	0	48	−	++++	−	−	−
	3	51	±	+++	+	+	
	6	54	−	++++	±	±	
	12	60	±	±	+	±	
	24	72	−	++++	++++	+++	−
	48	96	±	±	−	±	±
	48	96	−	−	−	−	−
	48	96	−	±	−	−	±
72	24	96	++++	++++	+	++++	−
	96	168	−	±	−	−	−
	168	240	−	−	−	−	−
80	0	80	±	+++	±	++	
	40	120	−	++++	−	±	−
	64	144	−	++++	−	−	−
	232	312	−	±	−	−	−

In this study 10 adult guinea pigs were inoculated intranasally with approximately 10,000 minimal lethal mouse doses each. This is a somewhat larger dosage than was used for the young animals, but of course the inoculum is spread over a much larger area in the respiratory passages of

the larger animals. Dosage by intranasal administration must in any case be considered rather inexact. After incubation periods of 1, 3, 6, and 10 days the animals were sacrificed and tests for virus were made on various organs.

As with the younger animals intranasally inoculated, virus was recoverable up to and including the 6th day but not at the 10th day (Table VI). In line with the results obtained by Stuart-Harris (2), the turbinates proved to be the most reliable source of virus. A moderate amount of virus was often recovered from the lungs; other organs were occasionally positive, so that a certain degree of spread of the virus beyond the respiratory system was indicated.

TABLE VI

Distribution of the Virus of Epidemic Influenza in Mature Guinea Pigs after Various Periods of Incubation

(Intranasal Inoculation)

Animal No.	Incubation period	Relative amounts of virus demonstrated in the following organs											
		Brain		Turbinate		Lung		Liver		Kidney		Blood	
		A	B	A	B	A	B	A	B	A	B	A	B
	days												
1	1	−	+	±	++++	+	++++	−	−	−	−	−	−
2	1	−	−	++++	n.t.	++	++++	−	++++	−	−	±	++++
3	3	++++	n.t.	++++	n.t.	+	++++	+	++++	+	++++	−	++++
4	3	−	n.t.	++++	n.t.	++++	n.t.	−	−	±	−	−	−
5	6	++++	n.t.	++++	n.t.	++++	n.t.	±	++	±	+++	−	++++
6	6	++	++	++++	n.t.	++++	n.t.	−	−	−	++++	−	++++
7	6	−	−	++++	n.t.	−	−	−	−	−	++++	±	++++
8	6	−	−	++++	n.t.	++++	n.t.	±	n.t.	−	−	−	−
9	10	−	−	−	n.t.	−	−	−	−	−	−	−	−
10	10	−	−	−	n.t.	±	−	−	−	−	−	−	−

Again there were no evident clinical symptoms; temperature readings on the animals were within normal limits. Gross changes were not seen in any of the tissues examined.

From this study we concluded that the susceptibility of the adult guinea pig to the virus of epidemic influenza is of approximately the same order as that of the newborn.

DISCUSSION

On the basis of these experiments, and other studies which have been cited, it seems clear that the age of the host is a significant factor in relation to susceptibility of the guinea pig species to the virus of epidemic influenza. Two points of particular interest in this connection may be noted. In the first place there is evidently a gradual reduction of susceptibility during the

stage of intrauterine development. The young fetus may be readily infected and the virus proliferates in various tissues, inducing pathologic reactions and sometimes death. In the mature fetus the virus again finds a medium favorable for its growth, but in this instance the tissues present much less evidence of pathologic change and the disease seems to be self-limited; the virus either disappears or is masked by antibodies after the 6th day. Postnatal animals (if inoculated intranasally) are only slightly more resistant than the mature fetus, nor is there much further increase in resistance with increasing age of the extrauterine animal. Indeed, if one confined his observations to the postnatal animal, he would be forced to conclude that the guinea pig species is fairly resistant to the influenza virus and that this resistance does not vary with the age of the host.

The second noteworthy point is that at the time of birth of the animal there is a sudden alteration of reactivity. Although previously, that is as a fetus, the guinea pig may be effectively inoculated intracerebrally and presumably by any other systemic route, infection after birth can be accomplished only by introduction of the virus into the respiratory tract. Experiments directed to this point indicated that there was no sharp difference in tissue susceptibility between the mature fetus and the newborn animal, for if animals were inoculated intracerebrally sufficiently long before birth for the virus to become established, the infection progressed after the animal was delivered into the outer world.

Of course at the time of an animal's birth it undergoes many profound physiological changes, particularly circulatory. Some of these may be related to this fact of altered susceptibility. Since the virus proliferates best in the lung tissue of these young animals, and since the lungs first begin to function as respiratory organs at the time of birth, we thought that a causal relationship might be involved. It seemed possible that the postnatal animal might be infectible intracerebrally, as was the fetus, if one of its lungs could be collapsed to simulate the condition of the lungs in the fetus. A few experiments were done to follow out this hypothesis but the results were inconclusive, principally because of technical difficulties in effecting and maintaining adequate collapse of the lung.

It should be pointed out that the experiments dealing with the mature fetus, intracerebrally inoculated, again emphasize the striking pneumotropism of this virus, in confirmation of our previously reported studies on the younger fetal guinea pig.

From the standpoint of its usefulness as a culture medium for the propagation of influenza virus, the mature guinea pig fetus might prove more valuable than the younger fetus because the technique for its inoculation is simpler and the yield is higher.

SUMMARY

Experiments were carried out to determine the relative susceptibility of guinea pigs at different ages to the virus of epidemic influenza.

From a correlation of these studies on the mature fetus, the newborn, and the adult animal, with previously reported findings on the immature fetus, we draw two conclusions: first, that there is a gradually increasing resistance to infection with this virus during intrauterine development, with but little change thereafter; and second, that at the time of birth there is a sudden loss of infectibility by routes other than the intranasal.

These results illustrate then the benefits which may accrue if one projects into the period of antenatal life studies dealing with the age factor in relation to susceptibility to infection. It is implied that data collected from observations of the postnatal animal alone are of necessity incomplete and may be misleading.

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