PROPAGATION OF THE VIRUS OF HUMAN INFLUENZA IN THE GUINEA PIG FETUS*

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The reports of Woolpert and associates (1–4) have called attention to the use of the mammalian fetus as an experimental animal for the study of various infectious agents. It has been pointed out that the fetus is frequently more susceptible to a particular agent than the postnatal animal of the same species, and furthermore is originally free of bacteria. Since viruses especially are known to flourish in embryonic cells, it would be expected that fetal animals would constitute a good medium for the propagation of viruses. This has been found true for the vaccinia virus (3), the guinea pig submaxillary virus (4), and the herpes virus (5) in the fetal guinea pig, and the vaccinia virus (6) in the fetal rabbit.

For several reasons it seemed of interest to investigate the possibility of propagating the influenza virus in the fetal guinea pig. In the first place, guinea pigs as a species appear to be fairly resistant to this virus. Although McIntosh and Selbie (7) isolated a virus from the lung tissue of an influenza patient which could be transmitted through guinea pigs in series, causing fever and sometimes death, it is not clear that they were dealing with what we now recognize as the virus of human influenza. Stuart-Harris (8) was able to infect young guinea pigs in series by intranasal inoculation of a virulent ferret strain of the human influenza virus, but only subclinical infections were induced. The virus apparently proliferated and persisted in turbinate tissue only, since the lungs were shown to be free of virus. Our own experience and that of others indicate that the guinea pig is relatively insusceptible to this virus. The natural question arises: Does the fetus share this species resistance?

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Furthermore, assuming that the fetal guinea pig should prove susceptible to the influenza virus, it is of interest to inquire what the distribution of the virus would be in fetal tissues. In postnatal animals and man the virus is infectious principally, if not only, by way of the respiratory tract and is recoverable chiefly from tissues or secretions of the respiratory system. This holds true despite the findings of Smorodintseff and Ostrovskaya (9) that small amounts of virus can be detected by special means in other organs and fluids of inoculated mice. Will this evident pneumotropism be manifested also in the fetal animal, an animal in which the respiratory system is non-functioning and which, for practical reasons, must be inoculated by routes other than the intranasal?

Other considerations of interest in such work concern possible modification of the virus by passage through fetuses, and evaluation of the fetus as a potential experimental animal for the detection of small amounts of virus, or for the production of large quantities of virus free of bacteria.

Materials and Methods

Strain of Virus.—We employed the PR8 strain of human influenza virus isolated by Francis in 1934 (10) and kindly supplied us by him. It was in its 158th mouse passage when received.

Maintenance of Virus.—The stock virus has been maintained in our laboratory by passage through mice in the usual manner. For this purpose young Swiss mice are inoculated intranasally under light ether anesthesia and their lungs are harvested for virus 48 hours later. Formerly the whole lungs were stored in 50 per cent glycerine at ordinary refrigeration temperature and 10 per cent suspensions were made in physiological saline as needed for subinoculation. More recently we have made suspensions directly in saline and stored the suspensions at −78°C. in a thermos jug containing alcohol and solid CO_2 as suggested by Turner (11).

Preparation of Virus for Inoculation into Fetuses.—Since a bacteriologically sterile virus was required for fetal inoculation, the mouse lung tissue could not be used directly. We employed, therefore, either Berkefeld V filtrates of mouse lung virus, tissue culture virus derived from the stock strain, or mouse fetus virus (12) derived from the same source. There appeared to be no difference in the results obtained with virus from the various sources.

Guinea Pigs.—Pregnant guinea pigs of approximately 35 days gestation age were selected as needed from the breeding stock of the University Farms. Through

experience the fetal age was judged with a fair degree of accuracy by palpation of the mother, and was determined with precision by weighing the fetuses at the termination of the experiment. The guinea pigs were a hybrid assortment purchased by the Farms in small lots from various producers.

Technique of Inoculating the Fetuses and Recovering the Virus.—This has been previously described (1). It consists essentially in surgical exposure of the gravid uterus and inoculation of the fetus by needle puncture through the uterine wall and fetal membranes. In these experiments the inoculations were made intracerebrally in 0.05 to 0.10 cc. amounts. The fetuses were removed by cesarean section 2 to 7 days later, at which time gross examination was made and tissues were taken for virus determination and histologic preparation. Ether vapor anesthesia (13) was used for all operative work.

Tests for Virus.—Qualitative and quantitative tests for virus were made in young white Swiss mice, inoculated in the usual manner. Surviving test mice were sacrificed at the 10th day, or in some instances were used in cross-immunity studies. The sera for cross-neutralization tests were prepared by subcutaneous and intravenous immunization of rabbits and the tests were made in mice by intranasal inoculation of the serum-virus mixtures which had been allowed to stand for 30 to 60 minutes at 37°C., sometimes with an additional incubation for 12 hours at 4°C.

Transmission of the Influenza Virus to Fetal Guinea Pigs and Distribution of Virus in Fetal Tissues

In numerous instances an influenza virus infection was established in the guinea pig fetus by intracerebral administration of the virus. In the absence of occasional technical difficulties, transmission to the fetus was accomplished whenever a bacteriologically sterile virus of proven potency for mice was employed, regardless of the immediate source of the virus, whether mouse lung filtrate, tissue culture, mouse fetus, or guinea pig fetus. Table I shows the distribution and titer of virus in several tissues of a fetus 48 hours after the inoculum had been introduced intracranially. It is seen that the highest yield of virus appeared in lung tissue and that placenta and liver were also good sources of virus, whereas blood was of low titer, and no virus was demonstrable in the brain at the dilution (1–10) tested, even though it was the organ receiving the original inoculum. In general these results are typical of numerous similar determinations, except that the brain occasionally contained small amounts of virus.

Serial Passage of the Influenza Virus through Fetal Guinea Pigs

Several series of passages were accomplished by inoculating the fetuses intracerebrally and harvesting the fetal lungs for passage virus 48 hours later. The lung tissue was usually prepared for subinoculation as a 10 per cent suspension in saline. In one series the virus was carried through 10 transfers, in another through 16. Several shorter series were terminated because of the lack of suitable animals. The A series of 10 transfers, the first attempted, is shown in Table II. It was initiated with virus from the 2nd transfer in a mouse fetus series. The table indicates the distribution of virus in certain fetal tissues at progressive stages of serial passage and following different

TABLE I

*Distribution and Titer of Influenza Virus (Human) in Fetal Guinea Pigs as Determined by Intranasal Inoculation of Adult Mice**

Fetal tissue†	Dilution of tissue				
	Undiluted	1-10	1-100	1-1000	1-10,000
Lung	—	3/3	3/3	2/3	2/3
Placenta	—	3/3	2/3	1/3	0/3
Liver	—	2/3	0/3	0/2	0/3
Blood	2/3	0/3	0/3	0/3	0/3
Brain	—	0/3	0/3	0/3	0/3

* The figures show the number of mice dying of those inoculated.

† Tissues of a fetus of the 13th serial passage (K XIII).

incubation periods. It may be seen that, with rare exceptions, the lung tissue yielded virus of good titer throughout the series and after the several incubation periods. It is perhaps significant that virus was recovered from the brain at the first passage but could not usually be demonstrated in that organ in later transfers. Judging the potency of the virus from the survival time of test mice, it may be inferred that the conditions chosen for propagation of the virus through fetuses were favorable, and that the potency of the virus was maintained without significant alteration.

Table III depicts the K series of passages in which the original inoculum was a Berkefeld V filtrate of a suspension of infected lungs

of adult mice. This table records tests for virus in the fetal lungs throughout the series of 16 transfers, indicating the day of death for the test mice and the approximate percentage involvement of the

TABLE II

Distribution of Influenza Virus (Human) in Fetal Guinea Pigs at Progressive Stages of Serial Passage

Passage No.	Length of incubation in fetus	Tests in adult mice Tissue tested* and day of death of test mice			
		Lung	Brain	Liver	Placenta
	days				
A I	2	3, 3, 3	5, 6, 10		
	4	3, 3, 3	5, 6, 7		
	6	4, 5, 6			
A II	2	4, 4, 4		4, 5, 6	
	3	3, 6			
A III	2	4, 4, 5			
	4	S, S			
A IV	2	S, S, S, S	S, S, S	S, S	
A V	2	4, 5, 6	S, S, S		
	4	S, S	S, S		
A VI	2	3, 5, S	S, S	S, S	
A VII	2	4, 4, 6		7	
A VIII	2	4, 4			
A IX	2	4, 4, 5	7, S, S	4, 6, 6	4, 4, 4, 5
	4	4, 4, 5, 6		5	
A X	2	3, 4, 5, 5			

S = survived.

* In 10 per cent suspension.

mouse lungs. The table brings out a point not previously emphasized, namely, that the fetal guinea pig can be infected with amounts of virus which produce little if any gross change in test mice, and that

starting with such material one may obtain virus of good titer after passing it through one or two fetuses. This is illustrated by the falling off in titer, for one reason or another, at the 10th, 12th, and 15th passages, with a restoration to full potency in each case on further

TABLE III

Tests for the Influenza Virus (Human) in Lungs of Fetal Guinea Pigs Inoculated in Serial Passage

Date	Passage No.	Effects in test mice	
		Day of death	Lung consolidation Per cent involved in the gross
1937-38			
Oct. 20	K I	2, 3, 3, 3, 3, 3	60, 100, 80, 90, 90, 100
" 29	K II	3, 4, 4, 4, 4, 6	80, 90, 100, 100, 80, 80
Nov. 5	K III	3, 4, 4	90, 100, 100
" 11	K IV	3, 3, 4	90, 50, 100
" 17	K V	4, 4, 4	90, 100, 100
" 19	K VI	2, 3, 3	60, 90, 100
" 24	K VII	5, 5	80, 100
Dec. 1	K VIII	3, 3	80, 100
" 6	K IX	5, 6	100, 100
" 16	K X	Im,* Im, Im	—
" 22	K XI	7, 8, Im	80, 100, —
Jan. 9	K XII	3, 4, 4	95, 100, 100
Mar. 10	K XII (retested)	Im, Im, Im	—
" 11	K XIII	9, Im	100, —
" 13	K XIV	7, 7, 7, 8, 8	100, 90, 100, 100, 100
" 23	K XV	3, 4, 4	100, 95, 100
Apr. 15	K XV (retested)	Im, Im, Im	—
" 15	K XVI	3, 4, 5	100, 100, 80

* Im = not killed, but immunized to the virus, as shown by later tests with potent material.

passage of the virus through fetuses. For example, K XII virus was lethal for mice when it was first tested (January 9), but was capable only of immunizing mice when retested after 2 months storage (March 10). Each of the next 3 transfers enhanced its titer until it was again killing mice in 3 to 4 days.

Properties of the Passage Virus

It was of course pertinent to inquire whether the virus which had been carried through several transfers in fetal guinea pigs actually represented the influenza virus and whether its properties had undergone significant modification.

To determine the identity of the virus, cross-immunity (Table IV) and cross-neutralization tests (Tables V and VI) were carried out, using the mouse passage strain of virus as a basis of reference. For the cross-immunity tests, we used mice which had survived previous exposure to virus in other experiments. In some instances their resistance to the homologous virus had been confirmed and enhanced by reinoculation. Those mice which had survived inoculation with mouse passage virus were immune to the fetal guinea pig strain, and the converse was also true (Table IV).

In the cross-neutralization experiments, each of the two strains of virus was tested after mixture with saline, with normal rabbit serum, with the homologous rabbit antiserum, and with the heterologous antiserum, *i.e.*, serum of a rabbit immunized to the other strain. Since all tests were not carried out at one time, a duplication of the virus-saline control tests appears in both tables. Table V shows the results of testing the mouse passage strain against homologous and heterologous sera; a complete cross-neutralization was demonstrated. In Table VI are recorded the findings obtained in a comparable manner for the fetal strain. At first glance there appears to be an anomaly here, in that the fetal virus has been completely neutralized by heterologous serum but not by homologous serum. This was probably due to the fact that in the tests with homologous serum a stronger virus was employed, and accordingly neutralization by serum was somewhat masked. It may be seen that this virus when inoculated with saline or with normal serum killed within 3 days all of the mice tested, and that when it was given with homologous serum, death was deferred until the 6th, 7th, or 8th day. On the other hand, the lot of fetal virus used in tests against heterologous serum did not kill under 7 days and was evidently readily neutralized by the serum used at that time.

All of the fetal virus used in these cross-immunity and cross-neutralization experiments was from the K series of transfers (Table III)

TABLE IV

Cross-Immunity Tests in Mice: Mouse and Fetal Guinea Pig Strains of Influenza Virus (Human)

History of mice	Tested with	Mice dying of those inoculated
Survived inoculation with mouse virus	Fetal guinea pig virus	0/8
Normal controls	Fetal guinea pig virus	4/4
Survived inoculation with fetal guinea pig virus	Mouse virus	0/13
Normal controls	Mouse virus	5/5

TABLE V

Cross-Neutralization Tests of Mouse and Fetal Guinea Pig Strains of Influenza Virus (Human) in Adult Mice

I. Mouse Strain against Homologous and Heterologous Sera

Inoculum	Mice dying of those inoculated	Day of death
Mouse virus + saline	5/6	3, 4, 5, 7, 9
Mouse virus + normal rabbit serum	6/6	3, 5, 5, 6, 6, 6
Mouse virus + heterologous antiserum	0/6	—
Mouse virus + saline	3/3	4, 4, 4
Mouse virus + homologous antiserum	0/3	—

TABLE VI

Cross-Neutralization Tests of Mouse and Fetal Guinea Pig Strains of Influenza Virus (Human) in Adult Mice

II. Fetal Guinea Pig Strain against Homologous and Heterologous Sera

Inoculum	Mice dying of those inoculated	Day of death
Fetal virus + saline	3/3	3, 3, 3
Fetal virus + normal rabbit serum	6/6	3, 3, 3, 3, 3, 3
Fetal virus + homologous antiserum	6/6	6, 7, 7, 7, 8, 8
Fetal virus + saline	4/4	7, 7, 7, 8
Fetal virus + heterologous antiserum	0/5	—

and principally from the latter transfers in that series, that is, after a certain stabilization of the passage strain was assured. The results of these tests indicate therefore that the fetal passage strain was immunologically similar to or identical with the mouse strain from which it was derived.

As to other properties of the passage strain, we have studied only its behavior in routine manipulation in the laboratory. On this basis there was no evidence that changes in virulence or specificity of the virus had been induced. This was inferred from the observation of its pathogenicity for mice, the titers obtained, the incubation periods, the behavior in storage, etc.

Throughout serial passage the guinea pig fetal strain has remained bacteriologically sterile.

Effects of the Influenza Virus on the Guinea Pig Fetus

Although the influenza virus disseminated widely in the fetuses, there was little gross evidence of damage to fetal tissues. The fetuses, with rare exceptions, were living when examined 48 hours after inoculation. Death was occasionally observed after the longer incubation periods (4 to 6 days), and its occurrence was irregular. Details of the pathological changes encountered in these animals will be reported in a separate communication (14).

A particular study of the mother guinea pigs which carried the fetuses throughout the experimental period was not made, but no symptoms or lesions attributable to the virus infection of the fetuses were observed in the mothers.

DISCUSSION

It seems apparent, on the basis of the data recorded, that the influenza virus actually multiplied in the tissues of the fetal guinea pig. This may be inferred from a consideration of the quantitative relationship of virus inoculum to virus yield, especially in the serial passage experiments wherein there was no loss of titer throughout a number of transfers, although an enormous dilution of the original inoculum was effected. Multiplication of the virus is implied also by the distribution of virus in particular tissues following intracerebral inoculation. It seems probable that actual multiplication occurred

in those organs which were found to contain large quantities of virus, specifically, lung, liver, and placenta, and that these parts were seeded from the brain through the blood stream. However, it is evident that the virus did not circulate freely in blood channels, because it was actually detectable there only in small amount, and because its titer in the various organs differed widely and generally exceeded that in the blood. It is of interest that the brain which received the original inoculum appeared incapable of supporting growth of the virus, whereas the virus apparently found in the lung an environment favorable for proliferation. This is the more striking in that the lung is non-functioning in the fetal animal, and the invasion of this tissue reemphasizes the specific association of the influenza virus and the pulmonary tract. The high virus content here is coincident with the greatest histologic change in any fetal part (14). Evidently, the fetal lung of this relatively insusceptible species shows the same specific receptivity as the lung of susceptible animals.

It should be pointed out, however, that we may have encouraged the pneumotropic[1] proclivities of the virus by employing lung tissue for subinoculation in serial passage. It is consistent with this suggestion that a fair quantity of virus was found in brain tissue in the first passage of series A (Table II), and little or no virus in the same tissue in subsequent stages of the series. Detailed confirmation of this finding would be necessary before conclusions would be warranted. It would be of interest to determine whether the virus could be passed in series by using brain tissue for subinoculation, and if so, whether modification of the virus would be induced thereby.

Although no route of inoculation other than the intracerebral was used in these experiments, there is reason to suppose that infection through other channels could be accomplished. The conditions for serial passage of the virus, *viz.*, an incubation period of 48 hours and the use of lung tissue for subinoculation, were dictated by reasoning from analogy on the basis of the reactions of adult mice to the in-

[1] We have retained the term pneumotropic because of previous usage. We do not imply or believe that the virus is attracted to the lungs selectively; we wish to emphasize only that lung tissue constitutes a relatively favorable medium for growth of the virus.

fluenza virus. These conditions were found satisfactory at the outset and were consistently employed throughout the work. Presumably the series could have been continued indefinitely in this way, although it may be that modifications of this technique might have proved equally or more favorable.

It would be of interest to know whether passage of the influenza virus through the fetal guinea pig might adapt it to that species to the degree that it would produce in the postnatal animal a clinical infection. We have carried out a few experiments with this hypothesis in mind but the results have been inconclusive thus far.

SUMMARY

The PR8 strain of human influenza virus was found to proliferate and disseminate widely in the tissues of fetal guinea pigs inoculated *in utero*. Large quantities of virus free of bacteria were recovered from lung, liver, and placenta, and smaller quantities from blood and brain, after incubation periods ranging from 2 to 6 days. Although the fetuses proved to constitute an excellent medium for the propagation of influenza virus, they evinced little gross reaction to the infection.

Several series of passages from fetus to fetus were accomplished; one consisted of 10 transfers, another of 16. For serial passage the virus was inoculated intracerebrally into half-grown fetuses and the fetal lungs were harvested 48 hours later as a source of virus for subinoculation. It is concluded that multiplication of the virus occurred particularly in the lungs, which may be considered a significant reaffirmation of the pneumotropic tendencies of this virus.

Following passage in series the virus was found, on the basis of cross-immunity and cross-neutralization tests, to be immunologically identical with the mouse passage virus from which it was derived. Other properties also appeared to be unaltered by passage of the virus under these conditions.

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