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FERRET FOETAL INFECTION WITH INFLUENZA VIRUS AT EARLY GESTATION

M. H. COLLIE, D. I. RUSHTON*, C. SWEET, R. H. HUSSEINI AND H. SMITH

*From the Department of Microbiology and *Department of Pathology, University of Birmingham, Birmingham B15 2TT*

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Summary.—Ferret foetal infection at early gestation was ensured by direct intra-amniotic inoculation of influenza virus. Foetal infection was followed by death and resorption but was not associated with malformations.

THE ABILITY of influenza virus to cause widespread disease among susceptible populations has stimulated many investigations into the possible role of this virus in causing stillbirth, abortion or congenital malformations. The evidence, however, is conflicting: several studies have failed to demonstrate any correlation (Doll, Hill and Sakula, 1960; Leck, 1963; Widelock, Csizmas and Klein, 1963; Wilson and Stein, 1969), whereas in others influenza was implicated as being a harmful agent (Coffey and Jessop, 1959; Hardy *et al.*, 1961; Grigor'ev, 1973). The issue is complicated by the fact that influenza is a febrile illness which is often treated with aspirin: both pyrexia (Edwards, 1972) and aspirin (Saxen, 1975) have been cited as potential teratogens.

Few attempts have been made to quantify the incidence of foetal infection during influenza epidemics, though presence of anti-influenza IgM in cord sera has been used to indicate infection and immunological response of the foetus (Ruben, Winkelstein and Sabbagha, 1975). Reports of foetal infection itself are rare: in two fatal cases of influenza in pregnancy, virus was isolated from amniotic fluid and foetal myocardium of one (Yawn *et al.*, 1971), though only placenta and amniotic fluid (but not foetus) of the other (Jewett, 1974). However, in a further case of fatal influenza, the investigators failed to isolate virus from any foetal tissues, amnion or placenta (Ramphal, Donnelly and Small, 1980).

The paucity of convincing evidence suggests that any adverse effect on foetal well-being is not likely to be on the scale experienced with rubella. It is, however, important to define the risk associated with such a commonly encountered infectious agent. The ferret has been used as an animal model to study this problem. The adult ferret suffers a non-fatal, predominantly upper-respiratory-tract influenzal infection similar to that seen in human adults. However, intranasal inoculation does not lead to foetal infection and this may be connected to the unlikelihood of viraemia occurring in such infections of the adult (Sweet *et al.*, 1979). As a model to study the results of certain foetal infection, it is necessary to inoculate virus directly into the blood: this causes foetal infection at late gestation with consequent foetal death and resorption (Collie *et al.*, 1978). The formation of the ferret placenta probably prevents foetal infection occurring during the early gestational period (Beck *et al.*, 1976), when disruption of organogenesis could cause malformations. For this reason, experiments were undertaken to administer virus directly into the amniotic fluid during the critical period just following implantation of the embryo. Intra-amniotic inoculation of mumps and parainfluenza type 2 viruses caused hydrocephalus in foetal hamsters,

whereas foetal infection following viraemic spread was rare and did not result in congenital abnormalities (Kilham and Margolis, 1975; Margolis and Kilham, 1977). Thus it can be demonstrated that commonly encountered viruses possess teratogenic potential even though realization of this potential is probably very infrequent.

MATERIALS AND METHODS

Ferrets.—Ferrets obtained from A. S. Roe, Little Fakenham, Norfolk, were mated as described by Sweet, Toms and Smith (1977). Implantation of the embryo occurs 12 days after coitus; birth takes place 30 days later.

Influenza virus.—The recombinant virus A/PR8/34-A/England/939/69 clone 7a (H_3N_2) and the preparation of virus stocks were described by Sweet, Stephen and Smith (1974) and Gould *et al.* (1972). "Infected" animals were given live virus diluted in phosphate-buffered saline (PBS(A)) to produce an inoculum of 10^3 or 10^0 $EBID_{50}$ (50% egg-bit infectious doses) per 0·01 ml. Control animals received the same concentration of dead virus (heat-inactivaved at 56° for 2 h).

Isolation of virus.—The following tissues were taken and tested for presence of virus: uterus, placenta, pooled foetal membranes (amnion, chorion and cord), foetus, amniotic fluid, and maternal plasma and red blood cells (RBCS). Tissue homogenates and fluids were tested for infectivity as previously described (Sweet *et al.*, 1974; Collie *et al.*, 1978).

Intra-amniotic inoculation.—Ferrets, at 16 days of gestation, were continuously anaesthetized with a 50/50 mixture of halothane/oxygen during the inoculation procedure. The uterus was exposed through a small slit in the abdominal wall which had previously been shaved then swabbed with 70% ethanol. Each embryo was visible as a distinct bump in the uterus; each bump was inoculated with 0·01 ml of inoculum using a sterile 30-gauge needle. The uterus was then returned into the abdominal cavity and the opening sewn together. The wound was swabbed with 70% ethanol and sprayed with clear, plastic dressing (Hibispray, Avlex Ltd, Wigan, Lancs.). Few precautions were taken to maintain sterility. In all cases the wounds healed well and the outer stitches were shed within 2 weeks.

Analysis of serum antibody to influenza virus.—A haemagglutination-inhibition test was used as described by Basarab and Smith (1969). If haemagglutination was not inhibited with the highest concentration of serum used (1 in 10), the serum was scored negative for antibody.

Pathological analysis.—Seventy-five foetuses and placentae of animals receiving 10^3 $EBID_{50}$ influenza virus were examined. The uterine horns were opened and foetuses removed. The horns were then sectioned circumferentially to include the uterine wall and placenta. The foetuses were bisected longitudinally 1–2 mm on either side of the midline and the central block of tissue sectioned and stained with haematoxylin and eosin.

The distribution of foetuses inoculated with live virus (infected) or dead virus (control) was as follows: 3 days after inoculation—17 infected, 7 control; 4 days after inoculation—25 infected, 5 control; 5 days after inoculation—15 infected, 8 control.

RESULTS

Intra-amniotic inoculation with 10^3 $EBID_{50}$ influenza virus

Pregnancies continuing to term or beyond.—Foetuses infected with 10^3 $EBID_{50}$ of live virus failed to mature into viable offspring with 2 exceptions (Table 1, A and B), one of which was stillborn (B). This was in contrast to foetuses exposed to dead virus, the majority of which survived to be born without obvious defects. Ferrets which failed to give birth were killed on the 44th day of gestation and the contents of the uterus examined. In animals that had received live virus, the position of the resorbed foetus was indicated by presence of thick necrotic fluid. In the uterus of the control animal (F) that failed to give birth, foetal remnants were visible, suggesting that resorption had occurred later than for infected animals.

Pregnancies ended before term.—Foetuses were examined 1–6 days after exposure to 10^3 $EBID_{50}$ live virus. By the 4th day after inoculation foetuses had lost their translucent appearance; 1 day later their opacity was heightened and by 7 days after inoculation the foetuses had taken on a mottled appearance and the amniotic fluid was discoloured. This stage preceded foetal breakdown into the thick, necrotic fluid visible at the end of pregnancy. Virus titres equal to or in excess of the inoculum could be detected in the uterus and the foetus plus accompanying membranes at all the times tested after inoculation (Fig.

TABLE I.—*The effect of intra-amniotic inoculation with* 10^3 *EBID*$_{50}$ *influenza virus on offspring*

	Pregnant ferrets	No. foetuses inoculated	No. impressions in uterus after birth	No. foetuses born alive
Live virus	A*	7	7	1
	B*	7	8	1†
	C*	10	10	0
	D*	9	9	0
	E*	7	7	0
Dead virus	F*	5	5	0
	G	9	N.T.	7
	H	6	N.T.	6
	I	10	N.T.	7
	J	12	N.T.	9

* Evidence of resorptions found in these animals when examined on Day 42 of pregnancy.
† Neonate stillborn.
N.T. Not tested.

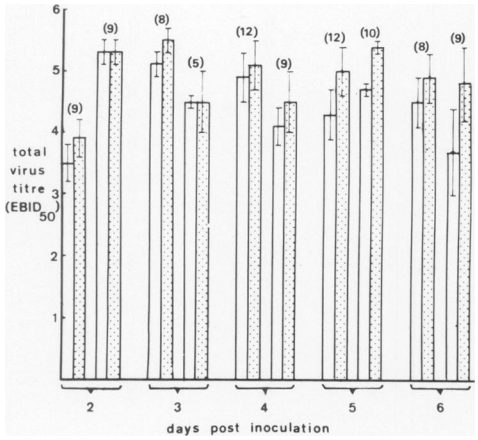

FIG. 1. Mean levels of virus ($EBID_{50}$) (±s.e.) in the foetus (□) and the uterus (▨) of ferrets following intra-amniotic inoculation at early gestation with 10^3 $EBID_{50}$ influenza virus. Each double block represents one ferret: the number of foetuses per ferret is given in parentheses.

1). Of 71 foetuses tested separately from the uterus, the titre of virus was higher in 13 foetuses (plus membranes) than in the uterus, but generally the reverse was true, which was probably a reflection of the tissue mass of the two samples.

Low, untitrable levels of virus could occasionally be isolated from maternal RBCS or serum.

No specific pathological changes were demonstrable in the foetuses, 3 of the infected conceptuses showing changes of maceration.

Lesions in the uterine horns included necrosis and degeneration of the trophoblast at the base of the haemophagocytic organ, focal necrosis of the haemophagocytic organ and thrombosis of the vessels within it. These lesions were more frequent in the infected animals but they were also found in the control specimens (Table II). It was not possible to distinguish between the two groups pathologically, suggesting that they may, in part, be related to the surgical procedures involved.

Intra-amniotic inoculation with 10^0 *EBID*$_{50}$ *influenza virus*

Foetuses exposed to a very low dose of either live (106 foetuses in 17 ferrets) or dead (56 foetuses in 10 ferrets) influenza virus were examined 3, 5, 7, 11, 14, 19 and 21 days after inoculation (Table II). Three malformed foetuses resulted from these inoculation procedures: 1 from live-virus inoculation was stunted (virus was isolated from the surrounding amniotic fluid), and the 2 from dead-virus inoculation showed microcephaly, anopthalmia and facial abnormalities. As these malformations were not confined to infected foetuses they were considered to have occurred by chance.

The number of resorptions were greater, and started earlier in gestation in animals given live virus (46%) than in those receiving dead virus (12·5%) (Table III).

A random check on foetal weights at

TABLE II.—*Number of foetuses and placentae showing pathological lesions after live (infected) or dead (control) virus inoculation containing* 10^3 $EBID_{50}$ *influenza virus*

Days after inoculation		Foetuses		Placentae	
		Normal	Degenerate	Normal	Abnormal
3	Control	7	0	4	3
	Infected	15	0	6	8*
4	Control	5	0	3	2
	Infected	23	2	8	13****
5	Control	8	0	1	6
	Infected	14	1	3	11*

* Indicates number of cases with unsuitable and inadequate histological preparation.

TABLE III.—*The effect of intra-amniotic inoculation with* 10^0 $EBID_{50}$ *influenza virus at early gestation on foetal viability*

Days after inoculation	No. foetuses exposed to live virus			No. foetuses exposed to dead virus		
	Normal	Abnormal	Resorbed	Normal	Abnormal	Resorbed
3	8	0	0	3	0	0
	7	0	0	7	0	0
	7	0	0			
5	7	0	0	5	0	0
7	5	0	0	2	0	0
11	3	0	6	2	0	0
	2	0	0			
14	5	0	1	5	0	0
	2	0	6			
	1	0	4			
19	2	1	5	6	0	3
	2	0	0	3	2	1
	2	0	4	6	0	1
	1	0	7			
	0	0	8			
	0	0	8			
21	2	0	2	8	0	2
	4	0	1			
Total No.	60	1	52	47	2	7
% resorption			46·0%			12·5%

Days 14 and 19 after inoculation did not reveal adverse effects accompanying live-virus inoculation: on Day 14, 2 animals containing 3 foetuses given live virus had a mean weight of 1·44 g compared with 1·42 g from 5 foetuses in 1 animal receiving dead virus; on Day 19 the corresponding figures were 4·62 g for live-virus inoculation (2 animals, 3 foetuses), and 3·54 g for dead-virus inoculation (2 animals, 11 foetuses). The apparently greater weight of infected foetuses examined at the later date is possibly due to the small number of foetuses left to compete for nutrients compared to controls.

In several cases virus could be isolated from the foetus and/or the tissues surrounding it in animals examined 3 days after inoculation with live virus. At 11 and 14 days after infection, virus was often associated with resorbed foetuses. Virus isolations from more advanced stages of resorption were less frequent, possibly due to inhibitory necrotic material. In some cases, however, virus was detected in the amniotic fluid of foetuses that were still viable.

DISCUSSION

The introduction of influenza virus into the amniotic fluid surrounding the ferret foetus at early gestation led to foetal death and consequent resorption. The result of foetal infection by this method of inocula-

tion is therefore the same as that effected at late gestation by intracardial inoculation (Collie *et al.*, 1978). The events leading to foetal death may, however, be different. Histopathological evidence suggests that, in the ferret, the route of bloodborne infection at late gestation is *via* the endometrium, decidua and haemophagocytic organ with later involvement of foetal liver and lung (unpublished observations). Haemophagocytic organ damage was discernible in the present experiments but, although there was a tendency for this to occur more frequently in infected animals, it was impossible to distinguish pathogically between infected and control groups. The unnatural introduction of virus directly into the foetal milieu in the early-gestation experiments was necessary in order to circumnavigate the ferret placenta which forms an effective barrier to bloodborne organisms up to Day 20 of gestation (Beck *et al.*, 1976). In normal circumstances this barrier would probably preclude a major teratogenic role for influenza virus in the ferret. However, even when this barrier is removed (as in intra-amniotic inoculation), malformations were not detected, which is not surprising in view of the relatively rapid onset of foetal death (visible by 4 days after inoculation). The balance between death and abnormality must necessarily be a fine one, and it is possible that in a polytocous species the balance may generally be tipped in favour of death rather than survival with deformities. Chemicals with teratogenic potential have, however, produced live, deformed neonates in ferrets (Beck *et al.*, 1976).

The relevance of ferret foetal deaths to reports of increased foetal wastage in human pregnancies during influenza epidemics must remain conjectural. It is not known whether influenza virus can cross the human placenta during the susceptible period (up to approximately 6 weeks' gestation). Human endometrium is susceptible to influenza *in vitro* (Rosztoczy, Toms and Smith, 1973) but for this to be significant the mother must either acquire infection venereally or suffer a sustained viraemia during her illness. Viraemia is probably rare during influenza but could perhaps occur during very serious infections which in themselves may lead to loss of pregnancy *via* nonspecific mechanisms, *e.g.* pyrexia, cardiorespiratory failure or shock. These factors may be instrumental in causing increased maternal mortality during some influenza epidemics (Greenberg *et al.*, 1958; Widelock *et al.*, 1963).

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