

Influenza infections during pregnancy in the mouse

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SUMMARY

The effect of a sublethal dose (0.5 LD₅₀) of influenza A/WSN (H0N1) administered intranasally in the 1st and 3rd weeks of pregnancy was studied in C3H inbred mice. Maternal and neonatal mortality rates were significantly increased by infections in the 3rd week of pregnancy, but not in the 1st week. Infections during the last part of the 1st week significantly depressed the growth rate of neonates. No evidence of viraemia, transplacental transmission or congenital malformations were observed from infections during either week. The results are discussed in terms of a possible model for human influenza infections during pregnancy to determine the potential risks to the mother, fetus and neonate.

INTRODUCTION

Influenza A virus infections in women during the 1st trimester of pregnancy have been associated with congenital malformations and stillbirths without malformation and, during the 2nd or 3rd trimesters, have been associated with abortion and an increased incidence of maternal mortality (reviewed by Mackenzie & Houghton, 1974). Transplacental transmission has been reported in a fatal case of influenza during the 3rd trimester with virus being isolated from fetal tissues and amniotic fluid (Yawn *et al.* 1971).

Congenital malformations have also been produced in Swiss albino mice inoculated intranasally with sublethal doses of influenza A/PR8(H0N1), either before or 4 days after mating, and although no effect was observed on litter sizes, pregnancy rates were decreased (Adams *et al.* 1956). In a further study from the same laboratory, congenital malformations were not observed in mice inoculated with high lethal doses of PR8 virus by intranasal, intraperitoneal or intravenous routes during the 2nd and 3rd weeks†. Transplacental transmission occurred in the 3rd week after inoculation by all three routes, but not in mice inoculated with influenza A/NWS(H0N1) by the intracerebral route (Seim, Ly, Imagawa & Adams, 1960).

This communication describes a preliminary study to find an animal model system in which to examine the risks that influenza virus infections during pregnancy might confer on both mother and fetus.

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† Each week in mice is approximately equivalent to a trimester in human pregnancy.

MATERIALS AND METHODS

Virus strain

Influenza A/WSN(H0N1) was used in all experiments. Virus stocks were prepared in 10- or 11-day-old embryonated chicken eggs. After 40 h incubation, infected allantoic fluids were removed, clarified by centrifugation at 800 g, and stored at -70°C .

Animals

C3H/HeJ inbred mice were obtained from the Perth Medical Centre Animal Breeding Unit. Before mating, 12- to 16-week-old male mice were isolated in separate cages for a minimum of 3 days. Two 12-week-old virgin female mice were then placed into each cage, and examined every 12 h thereafter for the presence of vaginal plugs as an indication that mating had taken place. The length of the gestational period of C3H mice was 20 days from the day of detection of a vaginal plug to the day of birth. Days 1-6, 7-13 and 14-20 post-conception were classified as 1st, 2nd and 3rd weeks respectively. Male mice were routinely removed before virus inoculation.

Mouse inoculation

Lightly etherized mice were inoculated by the intranasal route with $25\ \mu\text{l}$ of virus diluted in PBS to give a dose of 0.5 LD₅₀. Control mice were inoculated similarly with PBS.

Haemagglutination titrations

Serial twofold dilutions of virus in citrate buffer (0.85% sodium chloride, 2% sodium citrate) were made in disposable 'Microtitre' trays (Cooke Engineering Co., Virginia, U.S.A.), and a 0.5% suspension of chicken erythrocytes in citrate buffer was added to each well. After 35 min at 37°C , the end point was read as the highest dilution of virus causing haemagglutination.

Isolation of virus from fetuses

Pregnant mice were inoculated intranasally with 0.5 LD₅₀ of virus at various stages during the 1st and 3rd weeks. On days 4 and 8 after inoculation the mice were killed by the intraperitoneal administration of 0.2 ml Nembutal (Sodium pentobarbitone, 60 mg/ml, Abbot Laboratories). The uterine horns were removed and the fetuses separated and rinsed in sterile PBS before grinding in a pre-chilled mortar. Offspring born from mice inoculated in the 3rd week were killed and the head, limbs and skin removed before grinding. Fetuses and unweaned young were ground in 3 ml PBS, suspensions of three fetuses or two unweaned young pooled, and clarified by centrifugation at 800 g for 10 min. Supernatants were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs. After 3 days incubation at 37°C , allantoic fluids were harvested and tested for the growth of virus by haemagglutination.

Table 1. *Maternal mortality in mice following influenza infection during the 1st or 3rd weeks of pregnancy*

Gravid state	No. of mice	No. of deaths	Mortality (%)
Non-pregnant	24	6	25
1st week	64	17	26.6
3rd week	32	25	78.1*

* Significant, compared to non-pregnant mice, $\chi^2 = 15.66$, $P < 0.001$.

Blood collection for studies of viraemia

Blood was collected from the retro-orbital venous plexus in heparinized micro-capillary tubes (Gelman-Hawksley Ltd, England), and inoculated into embryonated chicken eggs for virus isolation, as described above.

RESULTS

Maternal mortality

Pregnant mice were inoculated with 0.5 LD₅₀ of WSN intranasally during the 1st or 3rd weeks, and their mortality rates compared with non-pregnant control mice. The results are shown in Table 1. No differences in mortality rates were observed between pregnant mice inoculated during the 1st week and non-pregnant mice, but mice inoculated during the 3rd week were significantly more susceptible, with deaths occurring 6–9 days after infection ($\chi^2 = 15.66$, $P < 0.001$).

Litter sizes, neonatal development and congenital malformations

The sizes of litters born to mice inoculated with WSN at various days after conception during the 1st or 3rd weeks (excluding mothers dying from the infection) were not significantly different from litter sizes from control mice (Table 2). The neonates were examined periodically for 8 weeks after birth to determine the effect that influenza infections during pregnancy might have exerted on their subsequent development. The results are shown in terms of infant mortality (Table 2) and infant growth rates (Fig. 1).

Offspring from mothers inoculated with WSN during the 3rd week were found to have significantly decreased survival rates compared with offspring from control mice ($\chi^2 = 8.61$, $P < 0.005$), and a similar trend was observed in offspring from mice inoculated during the 1st week ($P \geq 0.05$) (Table 2).

Litters from mice inoculated with WSN, and those from control mice, were weighed individually at birth and on every 2nd day over the following 8 weeks. From the 4th to the 8th week it was evident that the growth rates of males and females differed considerably. To eliminate inaccuracies due to variations in the distribution of the sexes among litters, separate growth rates were calculated for males and females from the 4th week onwards. The growth rates of mice born to mothers inoculated on days 1–4 after conception, or to mothers inoculated during the 3rd week, were not significantly different from mice born to uninfected control

Table 2. *Litter sizes and infant mortality in mice during the first 8 weeks of life after influenza infections in the 1st or 3rd weeks*

Week	Group	No. of litters	Mean litter size at birth	No. of young at birth	No. of survivors after 8 weeks
1	Virus-infected	17	7.53	128	106*
	Control	21	5.9	124	113*
3	Virus-infected	7	6.86	48	30†
	Control	9	5.56	50	44†

* Not significant: $\chi^2 = 3.83$, $P > 0.05$.

† Significant: $\chi^2 = 8.61$, $P < 0.005$.

mothers (Fig. 1A). When inoculation was carried out on days 5, 6 and 7 after conception, however, the offspring were found to grow more slowly than those born to control mothers (Fig. 1B–D). This difference was particularly significant for the offspring of mothers inoculated on day 5 after conception (Fig. 1B).

No congenital anomalies of any description were observed from 57 mice inoculated during the 1st week. Of the 57 mice, 23 produced litters, 26 were killed 8 days after infection, and 8 died from the infection.

Viraemia and transplacental transmission

Two groups of 11 mice, which had received 0.5 LD₅₀ or 5 LD₅₀ of WSN respectively during the 1st or 3rd weeks, were bled from the retro-orbital sinus at 12-hourly intervals for 7 days after infection, and the heparinized whole blood passaged twice through the allantoic cavity of 11-day-old embryonated eggs for virus isolation. After 3 days incubation, the allantoic fluids were harvested and assayed for virus by haemagglutination. All blood samples were negative, and no evidence of viraemia could be detected.

Attempts were also made to isolate virus from fetal or infant tissues as evidence for transplacental transmission. Uterine horns were removed 4 or 8 days after infection from 51 mice inoculated during the 1st week (7 mice each day after conception) and a further 12 litters were examined which had been born to mice inoculated in the 3rd week. Fetal or infant tissue homogenates were passaged twice through the allantoic cavity of 11-day-old embryonated eggs, and allantoic fluids assayed by haemagglutination for the growth of virus. No evidence was obtained for the occurrence of transplacental transmission during either week.

DISCUSSION

The evidence linking influenza infections during the 1st trimester with malformations of the central nervous and circulatory systems in the human fetus has been contradictory, both for (Coffey & Jessop, 1959; Hakosalo & Saxen, 1971) and against (Leck, 1963, 1971; Leck, Hay, Witte & Greene, 1969; Wilson & Stein, 1969), although stronger evidence has been presented for an association with cleft lip and

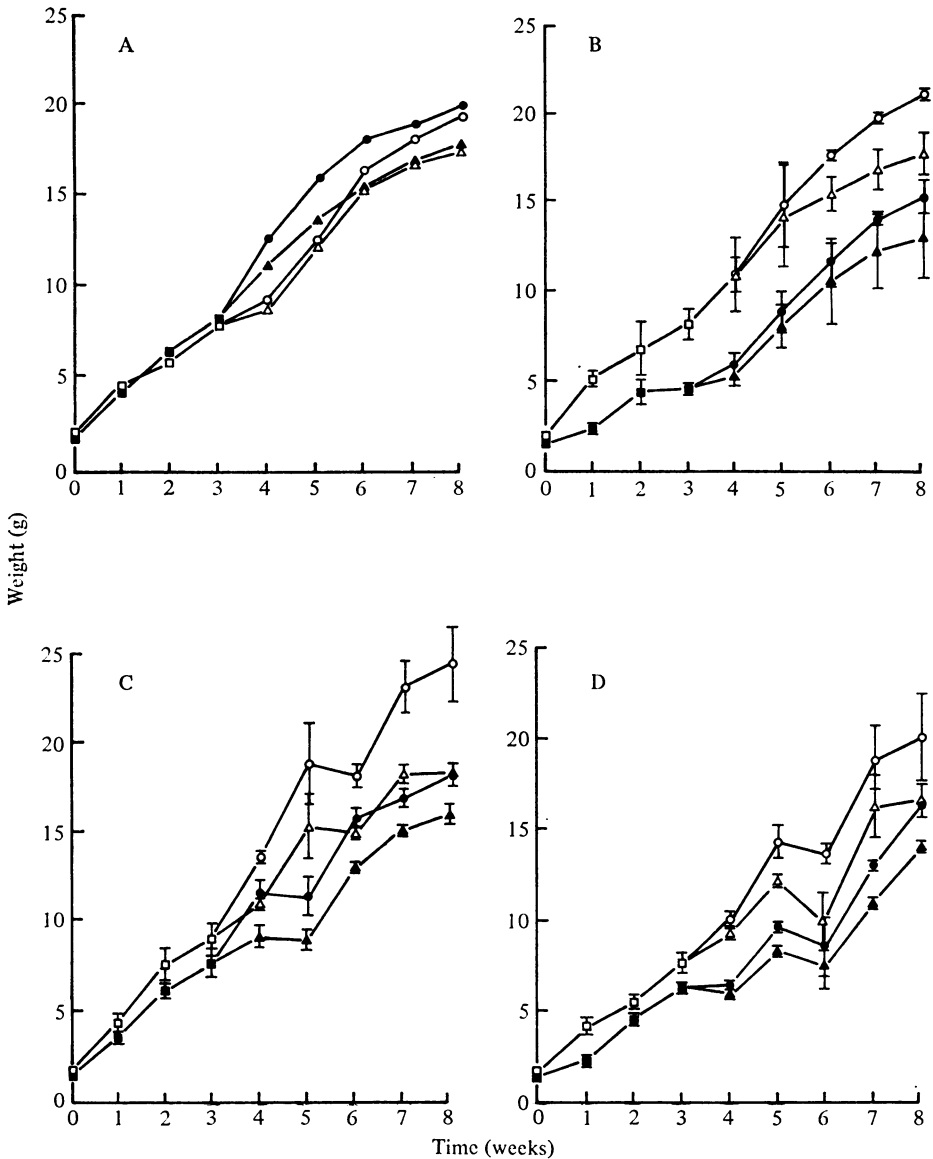


Fig. 1. The effect of maternal infection of mice with influenza on neonatal growth rates. Virus was administered intranasally on day 1 (A), day 5 (B), day 6 (C) and day 7 (D) post-conception. Offspring from infected mothers, \bullet — \bullet = males and \blacktriangle — \blacktriangle = females. Offspring from uninfected control mothers, \square — \square = males and females; \circ — \circ = males; \triangle — \triangle = females. Each point represents the mean of 18 mice, and vertical bars enclose 1 standard deviation.

reductive deformities (Leck, 1963, 1971; Leck *et al.* 1969). Chick embryos and rhesus monkeys have been used in addition to mice for assessing the potential teratogenicity of influenza infections. Direct inoculation of early chick embryos through the vitelline membrane with A/NWS (H0N1) and A/PR 8 (H0N1) strains of influenza produced a number of malformations including microencephaly, axial

flexion, retardation of the lens, and myeloschisis (Hamburger & Habel, 1947; Heath *et al.* 1956; Robertson, Williamson & Blattner, 1960). Hydrocephalus was observed in rhesus monkeys after direct inoculation of A/Aichi/2/68 (H3N2) into the brains of fetuses at the beginning of the 3rd trimester (London, Fuccillo, Sever & Kent, 1975). It should be noted, however, that in both animal systems virus was inoculated directly into embryonic or fetal tissues.

Sublethal doses of PR8 administered intranasally to Swiss albino mice in the 1st week produced malformations of the central nervous system (Adams *et al.* 1956), but malformations were not observed with high lethal doses of the same virus administered by intranasal, intraperitoneal or intravenous routes during the 2nd or 3rd weeks (Seim *et al.* 1960). In the present study, malformations were not detected in the litters or fetuses of 57 mice inoculated during the 1st week or in 24 litters of mice inoculated in the 3rd week. However, both virus and mouse strains differed from those used by Adams *et al.* (1956).

For congenital malformations to be produced, the virus must either be able to cross the placenta following viraemia, or must cause fetal damage through an indirect method such as toxæmia or by metabolites from damaged cells. Viraemia has been reported in severe human influenza infections (Naficy, 1963; Lehmann & Gust, 1971; Khakpour, Saidi & Naficy, 1969), but only a single case of transplacental transmission has been recorded (Yawn *et al.* 1971). This occurred in a fatal case of influenza during the 3rd trimester. Transplacental transmission has also been observed in the ferret after intracardial inoculation (Sweet, Toms, Kingsman & Smith, 1976). Viraemia has been reported in mice after aerosol infection with PR8 virus (Hamre, Appel & Loosli, 1955), and transplacental transmission has been observed in Swiss albino mice inoculated with PR8 by the intranasal, intraperitoneal and intravenous routes, but only during the 3rd week despite attempts to isolate virus from fetuses earlier in pregnancy (Seim *et al.* 1960). In this study viraemia could not be detected from 12 h to 7 days after infection in 22 mice inoculated during the 1st or 3rd weeks, nor was evidence obtained for transplacental transmission in 51 mice inoculated during the 1st week or in 12 litters born to mothers inoculated during the 3rd week. The lack of evidence to substantiate viraemia or transplacental transmission in this model may explain in part the absence of malformations but virus and mouse strains were different to those used in previous reports.

A human maternal mortality rate of 27% was recorded during the 1918-19 influenza pandemic which increased to 50% when complicated by pneumonia (Harris, 1919). Although such a high mortality rate has not been observed since, pregnant women were found to be at greater risk than non-pregnant women during the 1957 pandemic, particularly women in their 3rd trimester (Greenberg, Jacobziner, Pakter & Weisl, 1958; Freeman & Barno, 1959; Widelock, Csizmas & Klein, 1963). In this study, mice in the 3rd week also exhibited a highly significant increase in susceptibility to a lethal infection compared with mice in the 1st week or with non-pregnant mice. The reason for this increased susceptibility is unknown in mice, but is presently under investigation. In man, however, a depression in cell-mediated immunity has been observed during the 3rd trimester of pregnancy (Purtillo,

Hallgren & Yunis, 1972; Finn *et al.* 1972), which may be associated with increased susceptibility to viral infections.

Influenza infections during the 1st and 3rd weeks were found to exert a profound effect on neonatal development. Infections in the 3rd week resulted in a significantly increased infant mortality during the first 8 weeks of life, and a similar trend was observed in mice born to mothers infected in the 1st week (Table 2). Growth rates of mice born to mothers infected during the last 3 days of the 1st week were also significantly reduced (Fig. 1) for their first 8 weeks. Increased neonatal mortality and reduced infant growth rates following influenza infections during pregnancy have not been reported in other murine studies, nor have they been observed in human infections. Reduced growth rates in mice, however, have been described in neonates from mothers infected during pregnancy with reovirus but, if killed, no virus or morphological lesions could be detected (Hashimi, Carruthers, Wolf & Lerner, 1966). An increase in infant mortality was also described in the same report, but in all fatal cases reovirus was isolated from various infant tissues. Congenital infections with lymphocytic choriomeningitis virus may cause retarded growth rates, but the mice display a persistent tolerant infection throughout life (Mims, 1968). In the present study, the cause of infant mortality was not investigated, but in preliminary experiments, 6-week-old survivors from mothers infected with influenza during the 1st and 3rd weeks did not have antibody to influenza and were equally susceptible to a lethal challenge infection as were the offspring from uninfected mothers (J. S. Mackenzie & K. Williams, unpublished observations). Thus the mechanism by which influenza infections during pregnancy can influence neonatal development is unknown, but a persistent latent infection of the fetus without the induction of tolerance cannot be discounted, although evidence of viraemia and transplacental transmission was not obtained.

Finally, a murine system may provide a useful model for studying the potential risks which influenza infections during pregnancy may present to the mother, fetus and neonate. A number of parallels exist between the human situation and a murine model. They include the possession of three distinct tissue layers separating maternal and fetal blood; in other studies, the production of congenital malformations with influenza infections during the 1st week and transplacental transmission during the 3rd week; and an increased maternal mortality following infection in the 3rd week. In the latter instance, the pathological changes associated with fatal primary viral pneumonia in pregnant women in the 3rd trimester (Freeman & Barno, 1959) are similar to the lung lesions observed in murine infections (Straub, 1937; Hers & Mulder, 1961; G. Cambridge, J. S. Mackenzie, J.-M. Finlay-Jones, unpublished observations).

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