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Influenza virus infection in the second and third trimesters of pregnancy: a clinical and seroepidemiological study

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Objective To determine whether maternal influenza virus infection in the second and third trimesters of pregnancy results in transplacental transmission of infection, maternal auto-antibody production or an increase in complications of pregnancy.

Design Case–control cohort study.

Population Study and control cohorts were derived from 3975 women who were consecutively delivered at two Nottingham teaching hospitals between May 1993 and July 1994. A complete set of three sera was available for 1659 women.

Methods Paired maternal ante- and postnatal sera were screened for a rise in anti-influenza virus antibody titre by single radial haemolysis and haemagglutination inhibition. Routine obstetric data collected during and after pregnancy were retrieved from the Nottingham obstetric database. Cord samples were tested for the presence of IgM anti-influenza antibodies, and postnatal infant sera were tested for the persistence of influenza-virus specific IgG. Paired antenatal and postnatal sera were tested against a standard range of auto-antigens by immunofluorescence.

Main outcome measures Classification of women as having definite serological evidence of an influenza virus infection in pregnancy (cases) or as controls.

Results Intercurrent influenza virus infections were identified in 182/1659 (11·0%) pregnancies. None of 138 cord sera from maternal influenza cases was positive for influenza A virus specific IgM. IgG anti-influenza antibodies did not persist in any of 12 infant sera taken at age 6–12 months. Six of 172 postnatal maternal sera from cases of influenza were positive for auto-antibodies. In all cases the corresponding antenatal serum was also positive for the same auto-antibody. There were no significant differences in pregnancy outcome measures between cases and controls. Overall, there were significantly more complications of pregnancy in the cases *versus* the controls, but no single type of complication achieved statistical significance.

Conclusions Influenza infection in the second and third trimesters of pregnancy is a relatively common event. We found no evidence for transplacental transmission of influenza virus or auto-antibody production in pregnancies complicated by influenza infections. There was an increase in the complications of pregnancy in our influenza cohort.

INTRODUCTION

Influenza virus infection in pregnancy has been implicated in causing various adverse events including congenital malformations (e.g. neural tube defects, limb deficiency, congenital heart defects), abortion or stillbirth, low birthweight, the subsequent development of malignancies of the reticuloendothelial system in childhood and of Parkinson's disease in adulthood[1–6]. Recently, a number of studies from different laboratories and countries have demonstrated that maternal exposure to pandemic and interpandemic influenza is a significant risk

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factor for the development of schizophrenia in later life[7,8]. Most of these studies have been epidemiological in nature, relying either on a clinical diagnosis of maternal influenza or on the observation of a rise in incidence of the abnormality in question, following influenza epidemics or pandemics in specific geographical areas. Despite the possible importance of these studies, there are difficulties with their interpretation, particularly with regard to the accuracy of diagnosis of influenza. Mild or asymptomatic infections with influenza viruses may not have been detected, and infections with other respiratory viruses may have been erroneously attributed to influenza. Such studies also fail to address the possible pathogenic mechanisms whereby maternal influenza infection may result in damage to the fetus. Indeed, there is a dearth of accurate information relating to the biological consequences of maternal influenza virus infection during pregnancy. In the ferret model of influenza, virus infection in the female results in transplacental transmission of infection to the fetus[9], although evidence that such a phenomenon occurs in humans is lacking.

This report describes a case–control cohort study designed to identify a group of pregnant women with unambiguous serological evidence of influenza virus infection during the second and third trimesters of pregnancy and to examine whether:

1. Influenza virus may cross the placenta and infect the fetus.
2. Maternal influenza virus infection may induce autoantibody production, which may cross the placenta and mediate damage to the fetus.
3. Maternal influenza virus infection in pregnancy is associated with an increased risk of complications of pregnancy.

METHODS

The strategy adopted for identification of maternal influenza virus infection was to demonstrate a significant increase in influenza-specific antibodies in paired maternal sera, comprising an antenatal sample taken for routine rubella serology, and a postnatal sample, taken in the delivery or postnatal wards. As antenatal samples are not taken until around 12 weeks of pregnancy, this strategy would only succeed in identifying intercurrent maternal infections in the second and third trimesters of pregnancy.

The study and control cohorts were derived from 3975 women giving birth at the University and City Hospitals, Nottingham, between May 1993 and July 1994, from whom either a postnatal or cord blood sample was obtained, with informed consent. These samples were taken by the midwifery staff in the delivery and postnatal wards. Antenatal sera were sought and retrieved from those sent to the local Public Health Virology Laboratory for rubella and syphilis screening. These sera had been stored at −20°C. Only 1659 women from whom a complete set of three sera (comprising antenatal, postnatal, and cord serum samples) were available were investigated further. Parental consent was given for a small number (n = 12) of heel-prick blood samples from infants born to cases of influenza at age 6–12 months. Ethical permission for the study was obtained from the appropriate hospital ethical committees.

Routine obstetric data collected during and after pregnancy were retrieved from the Nottingham Obstetric database.

Paired maternal ante- and postnatal sera were tested in parallel for anti-influenza virus antibodies by single radial haemolysis, using A/Taiwan/1/86/H1 N1, A/Beijing/32/92/H3 N2, and B/Panama/45/90 antigens and chromium chloride treated sheep (for A H1 N1 and B viruses) and goose (for A H3 N2 virus) red cells (Bradshaw Biologicals, Leicestershire, England)[10]. Results were defined as positive if the diameter of the haemolysis zone produced by the postnatal serum was at least 3 mm greater than that produced by the antenatal serum, indeterminate if the postnatal zone was 1–3 mm greater than the antenatal zone, or negative if the postnatal zone was less than or equal to the antenatal zone.

Pairs of sera positive or indeterminate in the single radial haemolysis assay were further tested in a haemagglutination inhibition assay[11]. Women whose paired sera showed a fourfold or greater rise in haemagglutination inhibition assay titre were considered to have unambiguous serological evidence of recent infection and constituted the cases of influenza infection in pregnancy.

A control for each case was selected from women whose paired sera were negative by single radial haemolysis and also showed no rise in haemagglutination inhibition assay titre. Controls were matched to cases using four criteria: maternal age (< 16 years, 16–20, 21–25, 26–30, 31–35, 36–40, > 40); gestational age of offspring (< 35 weeks, 35–36, 37–42, > 42); parity (primigravid or multigravid); and calendar month of delivery. For one case, a suitable control could not be identified, and for one control detailed casenotes covering the pregnancy could not be located.

Paired cord and postnatal maternal serum samples were tested using an indirect IgM enzyme linked immunosorbent assay for influenza A. Sucrose density gradient purified influenza H3 N2 (A/Johannesburg/34/94 and A/Beijing/32/92) was used to coat 96-well plates. After 16 hours at 4°C, the plate was washed with PBS/Tween 20 (0·05%). A 1:100 dilution of serum (100 μL) in PBS/0·05% Tween 20/5%

skimmed milk (reagent diluent) was incubated with antigen for 2 hours at 37°C. Human serum IgM was detected using HRP-conjugated mouse anti-human IgM monoclonal antibody (Dako, Cambridge, England). TMB was added and absorbance read at 620 nm. Cord blood samples from six influenza cases were also tested for the presence of IgM anti-influenza by haemagglutination inhibition assay following sucrose density gradient fractionation[12-13].

The postnatal maternal samples from the influenza cases were screened by indirect immunofluorescence at a dilution of 1 in 20 using human thyroid, rat stomach, liver, kidney and oesophagus as substrate to enable identification of antibodies to thyroid microsomal antigens, nuclear antigens, smooth muscle, mitochondria, gastric parietal cells and reticulin.

Statistical analysis

Nonparametric methods (χ^2 with Yates' correction for comparison of frequencies and Mann-Whitney U for comparison of medians) and parametric methods for comparison of means (Student's t test) were used to analyse the data.

RESULTS

Overall, 3975 women were enrolled into the study between 10 May 1993 and 19 June 1994, representing 37% of all pregnancies at the two hospitals during this time. A full set of sera (antenatal, postnatal and cord) was available from 1659 pregnancies. Only these pregnancies were studied further (Fig. 1).

Of the 1659 ante- and postnatal paired maternal sera, 1295 (78%) showed no rise in single radial haemolysis titre to influenza A (H1 N1 and H3 N2) and B viruses, 145 pairs (8·7%) gave indeterminate single radial haemolysis results, and 219 pairs (13·2%) were positive, two of which showed a rise to two viruses. On retesting of 116 of the indeterminate pairs, 34 gave positive results, giving a total number of 253 pairs with an single radial haemolysis rise in antibody titre.

For four pairs of single radial haemolysis positive sera, there was not enough antenatal sample to perform haemagglutination inhibition assay testing, so only 249 of the positive paired sera were further tested by haemagglutination inhibition assay. Paired sera from 182 women (11% of all women tested) showed a four-fold or greater rise in haemagglutination inhibition assay titre to the same virus type/subtype as generated a

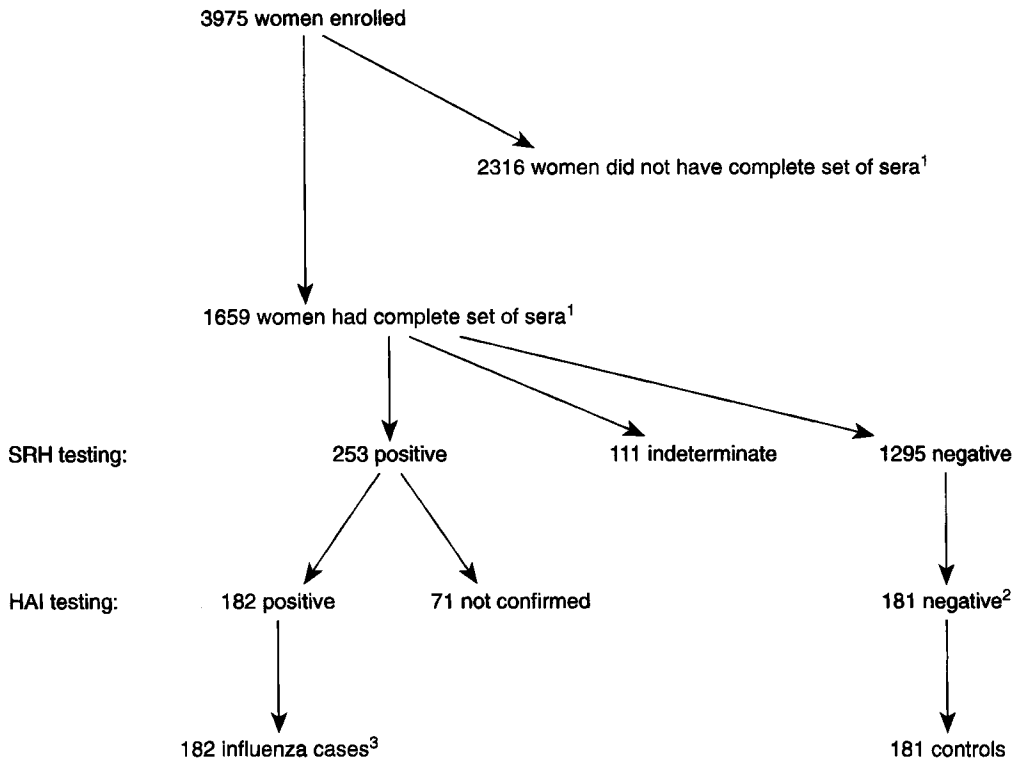

Fig. 1. Numbers and progress of women enrolled in study. SHR = single radial haemolysis; HAI = haemagglutination inhibition. [1]Complete set of sera = ante- and postnatal maternal sera plus cord serum; [2]Remaining 1114 paired sera not tested, [3]Influenza case defined as positive single radial haemolysis and ≥ 4-fold rise in haemagglutination inhibition assay titre in paired maternal ante- and postnatal sera.

positive single radial haemolysis test, and were therefore classified as influenza cases (Fig. 1). Of these 182 women, 137 had serological evidence of influenza A/H3 N2 infection, 20 had A/H1 N1, and 27 had B (in two women, antibody rises were recorded to both A/H1 N1 and A/H3 N2 viruses).

For each case of influenza (except one, for whom a suitable control could not be identified), a control was selected, matched for maternal age, gestational age, parity, and month of birth, whose paired ante- and postnatal sera showed no rise in anti-influenza titres by either single radial haemolysis or haemagglutination inhibition assay. Subsequent analysis showed that, in addition to the matching factors, the cases and controls were not significantly different for ethnic group, rhesus group, maternal height and weight, and number of multiple pregnancies (Table 1).

Effect of influenza virus on pregnancy

The frequency of complications of pregnancy in the cases and controls is shown in Table 2. The incidence of any single complication was not significantly different between cases and controls, but when taken overall, the cases suffered a significantly greater number of fetal, medical and obstetric complications than did the controls (106/181 *versus* 73/180, $P < 0{\cdot}001$) (Table 2).

There were no significant differences between the cases and controls in pregnancy outcome measures (e.g. mode of delivery, percentage of live-born babies, gender, birthweight, head circumference, Apgar score at 5 minutes, necessity for admission to neonatal intensive care, and congenital anomalies) (Table 3).

Table 1. Comparability of influenza cases and controls. Values are given as %, *n* (%) or mean [SD].

	Cases	Controls
Matching criteria		
Age (years)	26·5 [5·5]	27·0 [5·7]
Primigravid	74/180 (41·1)	75/176 (42·6)
Gestation at delivery (weeks)	39·4 [1·6]	39·4 [1·9]
Other criteria		
Ethnic group		
Caucasian	85·6	88·4
Afro-Caribbean	0·6	0·0
Asian	6·6	8·3
Other	7·2	3·3
Rhesus positivity	136/182 (74·7)	135/175 (77·1)
Maternal weight at booking (kg)	66·5 [13·0]	64·8 [11·2]
Maternal height (m)	1·62 [0·25]	1·62 [0·22]
Singleton pregnancy	180/181 (99·5)	176/180 (97·8)

Table 2. Complications in pregnancy. Values are given as *n*. PET = pre-eclampsia; IUGR = intrauterine growth restriction; UTI = urinary infection.

	Cases ($n = 181$)	Controls ($n = 180$)
Fetal		
IUGR	5	7
Reduced fetal movements	2	3
Preterm	0	3
Medical		
Hypertension/PET	15	12
Abdominal pain	8	2
Anaemia	3	1
UTI	1	1
Chest pain	1	0
Viral illness	1	0
Pneumonia	1	0
Pulmonary embolus	1	0
Atrial fibrillation	1	0
Gestational diabetes	1	0
Medication in pregnancy	35	33
Obstetric		
Bleeding	15	7
Post dates induction	8	2
Breech presentation at delivery	6	2
Unstable lie	2	0
TOTAL	106	73

Transplacental spread of influenza virus

Influenza virus-specific IgM was sought in cord blood samples from 138 influenza cases and matched controls by enzyme linked immunosorbent assay. None of the cases or controls had detectable influenza A specific IgM. A further six cord sera from cases with an haemagglutination inhibition assay titre > 80 to H3 N2 were assayed following sucrose density gradient centrifugation to fractionate IgM. No influenza specific IgM was detected. Twelve heel-prick samples obtained at age 6–12 months from the offspring of influenza cases were tested in parallel with the matching cord sera for the persistence of influenza specific antibody. Haemagglutination inhibition assay antibody was not detected in any of the infant sera (Table 4).

Titres of haemagglutination inhibition assay antibody in cord sera from 138 cases and their matched controls were compared with the titres present in their respective postnatal maternal sera. Thirty-seven of 138 (27%) cord sera from the cases had haemagglutination inhibition assay titres fourfold or greater than maternal postnatal sera, while this occurred in only 9/138 (7%) of the control cord sera ($P < 0{\cdot}0001$). However, the haemagglutination inhibition assay titres in all postnatal sera from the cases were at least 40 (this being the minimum titre at which a fourfold rise between a pair of ante- and postnatal sera could be demonstrated),

Table 3. Pregnancy outcome measures. Values are given as *n*, *n* (%) or mean [SD], unless otherwise indicated. ICU = intensive care unit.

	Cases (n = 181)	Controls (n = 180)
Labour/delivery		
Onset		
Spontaneous	141 (77·9)	147 (81·7)
Induced	35 (19·3)	26 (14·4)
Elective caesarean section	5 (2·8)	7 (3·9)
Mode of delivery		
Spontaneous vaginal	117 (64·6)	123 (68·3)
Assisted vaginal	32 (17·7)	30 (16·7)
Breech vaginal	3 (1·7)	2 (1·1)
Caesarean section (elective and emergency)	29 (16·0)	25 (13·9)
Blood loss at delivery (mL)	274·5 [182·1]	282·9 [243·6]
Babies		
Liveborn	179/180 (99·4)	180 (100)
Ratio male:female	94:87 (1·08:1)	99:81 (1·2:1)
Birthweight (kg)	3·40 [0·54]	3·33 [0·58]
Head circumference (cm)	34·6 [1·7]	34·4 [1·7]
Apgar score 7 or less at 5 min	3 (1·7)	3/179 (1·7)
Admission to neonatal ICU	1/137 (0·7)	6/138 (4·3)
Babies with congenital anomalies		
Talipes	3	3
Hypospadias	0	2
Sacral dimple	0	4
Mongolian spot	3	0
Pre-auricular skin tag	0	1
Undescended testis	0	1
Inturned fifth digit	0	1
Facial palsy	1	0
Port wine stain	1	0
TOTAL	8 (4·4)	12 (6·7)

while haemagglutination inhibition assay antibody was < 10, 10, 20, and > 40 in 117 (85%), 7 (5%), 7 (5%) and 7 (5%) of the control postnatal sera, respectively.

Auto-antibody studies

Maternal postnatal sera from 172 women in the influenza cohort were tested for a range of auto-antibodies. Six sera (3·5%) were positive: two with anti-reticulin antibodies; two with gastric parietal cell antibodies; and two with anti-nuclear antibodies. In all cases the corresponding antenatal serum was also positive for the same auto-antibody, in the same titre.

DISCUSSION

Influenza infection, rigorously defined by a fourfold rise in specific antibody titre, was clearly a common phenomenon in pregnancy in the present study. Eleven percent of 1659 pregnancies fulfilled a serological definition of infection during pregnancy. This is clearly a minimum estimate, as women whose sera were indeterminate by single radial haemolysis or unconfirmed by haemagglutination inhibition assay testing were excluded, and women with influenza infections in the first trimester of pregnancy (i.e. prior to the antenatal sample) were not identified. In comparison, a study of laboratory-proven influenza virus infections in pregnancies selected to span the winter seasons of 1975–1976, 1976–1977 and 1977–1978 in London reported infection rates of 22%, 1·3%, and 1·9%, respectively, with an overall rate of 5%[14].

The type and subtype of virus infection in affected pregnancies accurately reflected the national epidemic curves of influenza-like illnesses reported to the Royal College of General Practitioners Weekly Returns Service[15,16] and the pattern of influenza virus isolates reported by the Virus Reference Division of the Public Health Laboratory Service at Colindale during the period of this study (data available from the authors).

Our study design might have led to a selection bias of pregnancies, given that the collection of maternal and cord blood samples was the responsibility of midwives, and as a result samples from complicated pregnancies or difficult deliveries might have been under-represented. However, this did not occur in practice. The incidence of complications and profile of delivery patterns in our study cohorts were comparable

Table 4. Haemagglutination inhibition (HAI) titres in paired cord and infant sera. A/N = antenatal maternal serum; P/N = postnatal maternal serum; Infant = serum taken at 6–12 months of age; < = Titre < 20.

Study No.	Month of birth	Virus infection of mother	HAI titres			
			A/N	P/N	Cord	Infant
1	May 1993	A H1 N1	<	80	320	<
181	May 1993	A H1 N1	<	40	320	<
283	Jun 1993	B	<	80	20	<
608	Jul 1993	A H1 N1	<	40	320	<
2122	Nov 1993	A H3 N2	<	40	10	<
2172	Nov 1993	A H3 N2	<	40	<	<
2972	Jan 1994	A H3 N2	<	160	320	<
3084	Feb 1994	A H3 N2	20	80	320	<
3144	Feb 1994	A H3 N2	<	40	20	<
3386	Mar 1994	A H3 N2	<	160	640	<
3889	Jun 1994	A H3 N2	<	40	160	<
3963	Jun 1994	A H3 N2	<	40	160	<

to the hospitals' annual statistics (data not shown). It would appear that the impact of influenza infection after the first trimester on the mother and fetus in terms of individual complications is small. This conclusion is at variance with other reports. Griffiths *et al.*[14] found a significant excess of male births in an influenza cohort which we did not, although those authors could not find an obvious biological explanation for their finding and admitted that it might have been a chance observation. We found no effect of intercurrent influenza on birthweight, in contrast to an earlier study which reported lower birthweights in influenza-affected pregnancies[4]. We did find an overall excess of complications of pregnancy in our influenza cohort (Table 2), but given the wide variety of complications assessed, and the lack of a plausible underlying explanation, we cannot rule out that this was a chance observation.

Our study did not concern itself with diagnosis of first trimester influenza infection, and therefore we cannot draw conclusions concerning an effect of influenza infection at this stage of pregnancy. Indeed, such a study would present considerable practical difficulties, as ideally it would necessitate the collection of serum samples from women prior to confirmation of their pregnancy. However, it should be emphasised that it was second trimester influenza infection that was reported to increase the risk of the development of schizophrenia in the offspring[7,8]. Similarly, we cannot comment on a recent report suggesting influenza infection may increase the risk of spontaneous miscarriage[17].

This study was undertaken to provide data relating to two potential pathogenic mechanisms, whereby maternal influenza virus infection during pregnancy could give rise to deleterious effects on the fetus, namely transplacental transmission of infection and induction of auto-reactive antibodies with subsequent transplacental passage.

Viraemic spread of influenza virus beyond the respiratory tract during acute infection is unusual. Occasional case reports indicate that influenza virus may infect the placenta and fetus[18–20], including the fetal brain[21]. Diagnosis of viral infection of the fetus during pregnancy is not straightforward. The presence of virus specific IgM in cord serum is a valuable marker of intrauterine infection in some infections (e.g. rubella and cytomegalovirus), but not others (e.g. parvovirus B19). Currently available assays for the detection of influenza IgM involve sucrose density gradient fractionation, IgM capture haemadsorption assay[22] and an indirect IgM enzyme linked immunosorbent assay[23]. Influenza infections in adults are frequently re-infections, for which IgM is not a reliable marker. However, in the specific case of transplacental infection of a fetus, which would clearly be a primary infection, it is possible that virus specific IgM might be a useful marker of fetal infection. We were unable to detect IgM in any of 138 cord sera derived from infants born to mothers who had undergone influenza A infection in pregnancy or their matched controls. IgM antibodies following acute viral infections are short-lived and may have disappeared from the fetal circulation before birth. However, this is unlikely to provide an explanation for lack of IgM in any of the cord sera, as 64 of the cord sera from cases were taken between November 1993 and January 1994, when influenza A H3 N2 infection is highly likely to have occurred in the months close to delivery. Persistence of IgG is an accepted alternative criterion for the diagnosis of congenital infection (e.g. HIV), but no haemagglutination inhibition assay antibodies were detected in 12 postnatal blood samples taken at a time when maternal antibody would be expected to have waned.

The titres of haemagglutination inhibition assay antibody in cord sera were compared with those in the respective postnatal maternal sera for 138 cases and their matched controls, as a significant elevation in cord serum titre might be adduced as evidence of fetal infection. While a fourfold increase in cord compared with postnatal serum was found in 27% of cases, compared with only 7% of controls, these results should be interpreted with caution, as the haemagglutination inhibition assay titres in the postnatal maternal sera were significantly different in the case and control *cohorts*. An active system for the transport of IgG across the placenta exists, resulting in higher antibody concentration on the fetal compared with the maternal side of the circulation[24], and thus the increased frequency of higher cord titres in the influenza cases may simply reflect the fact that in the control cases there was very little or no maternal haemagglutination inhibition assay antibody to become concentrated in the cord serum. Overall, our data suggest that transplacental transfer of influenza would be a rare outcome of maternal infection in the second or third trimester of pregnancy.

Transplacental transmission of maternal auto-antibodies is recognised as a cause of disease in the fetus/neonate (e.g. neonatal thyrotoxicosis, myasthenia gravis and congenital heart block). We have shown previously that in rabbits, live or inactivated influenza viruses elicit auto-antibodies against a 37 kDa brain-specific protein[25], which is expressed at high levels in the fetal brain, while influenza virus infection of humans has been shown to elicit lupus-related auto-antibodies[26]. The active transport of IgG antibodies across the placenta may enhance the pathogenic potential of any influenza-induced auto-antibodies in the fetus, as would the immaturity of the fetal blood–brain barrier, which allows maternal antibodies to reach the fetal brain in man[27]. In a large number of postnatal samples (n = 172) from women with intercurrent influenza virus infection, we were unable to demonstrate any increase in the frequency of detection of a range of organ-specific and nonorgan-specific auto-antibodies. However, as argued above for influenza IgM antibodies, we cannot rule out the transient production of auto-antibodies in response to acute infection, with subsequent loss of reactivity over time.

In summary, this study of a relatively large cohort of women, in whom 11% were infected with influenza viruses at some stage in their pregnancy, failed to find evidence for either transplacental transmission of influenza virus infection or for induction of potentially pathogenic auto-antibodies. This, given the frequency of intercurrent infection during pregnancy, provides reassuring data concerning the likelihood of influenza-induced abnormalities in fetal development.

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