ENTEROVIRUS ISOLATION FROM FOETAL AND PLACENTAL TISSUES


1Department of Virology, Institute of Microbiology, Federal University, 05040 Rio de Janeiro, and 2Fernando Figueira Institute, Rio de Janeiro, Brazil

Received October 21, 1983

Summary. — Four spontaneous abortions and two stillbirths occurred during a prospective survey following the teratogenicity of echoviruses in 80 pregnant women selected at random from the Antenatal Care Service. Echoviruses types 10, 27, and 32, Coxsackie B3 and B4 were isolated from placental and foetal tissues (brain, liver, kidney, heart, and spleen). The mothers also excreted the virus by faeces at least twenty days before abortion and responded serologically, indicating active virus infection. Almost all aborted children were anomalous with signs of viral infection.

Key words: enteroviruses; congenital infections; viral placentitis; foetal and placental tissues

Introduction

Since 1941 when Norman Gregg established the teratogenic role of rubella virus for humans, several authors have recognized viruses causing foetal infections (Aycock, 1941; Bates, 1955; Holowach et al., 1957; Greenwald, 1958; Rhodes, 1960). Infections by cytomegalovirus, herpes, rubella, varicella-zoster, vaccinia, variola, polio, and Coxsackie B viruses during pregnancy may damage the human foetus (Hanshaw and Dudgeon, 1978).

The reports on viruses potentially causing congenital defects, stillbirth, or neonatal complications are still controversial. Of these, echoviruses have caught our attention, because their adverse effect on the foetus has not been accepted yet, although their capacity to cross the placenta had already been demonstrated (Rantsalo et al., 1960; Kleinman et al., 1962; Berkovich and Smithwick, 1968).

Hovatta et al. (1983) analysing the intrauterine deaths during a period of 6 years, considering the clinical and autopsy findings, registered a case of
enterovirus isolation from tissues of a stillborn. In Brazil, echovirus and Coxsackie B were isolated from placentas and foetal organs (Fonseca et al., 1982). There have been reports dealing with the occurrence of fatal infections "in utero", caused by echoviruses types 9 and 11 (Landsman et al., 1964; Brown and Karunas, 1977; Jones et al., 1980; Modlin, 1980). The present paper is the result of a prospective study made with a group of pregnant women, where the objective was the detection of maternal infection by echoviruses during the period of pregnancy and its possible damage for the foetus.

**Materials and Methods**

**Patients.** The studied population consisted of a group of 78 pregnant women that had been enrolled to the Antenatal Care Service from IPP in Rio de Janeiro, Brazil. The women were observed during the whole gestation period. Blood samples were collected monthly and two blood samples were taken: one at the beginning and one at the end of pregnancy for viral and serological studies, respectively. Placental tissue samples were collected, during delivery, for viral, histopathological, and electron microscopic studies. In stillbirth cases, tissue samples were collected from organs such as liver, brain, spleen, kidney, and lungs.

**Virologic studies.** Suspensions from placetas, foetal organs, and steel samples were inoculated into following cell cultures: LLC-MK2, VERO, and CRFK. When positive for cytopathic effects, cell suspensions were titrated and the viruses identified in LLC-MK2 cells with McNab's standard sera against enteroviruses.

**Serological studies.** Both blood samples collected from each mother were assayed against the virus isolated from placetas by neutralization test. Assays were performed to exclude other concomitant infections such as rubella, cytomegalovirus, toxoplasmosis, syphilis, listeriosis, and brucellosis. In addition, data were determined such as Rb factor, glycemia, and blood pressure that could be important during delivery.

**Immunofluorescence tests** were made in paraffin-embedded tissue samples. The sections were deparaffinized with xylene (Merck) for 20 min, washed several times with PBS pH 7.2, and overlaid with specific purified rabbit antiserum against the isolated viruses for one hour at 37°C. The sections were then washed with PBS pH 7.2, covered with calf antiserum against rabbit serum labelled with fluoresceine (Flushing Lab reagents). After one hour immersion at 37°C, the sections were washed with PBS, air dried, and mounted (Lactic et al. and Schmidt, 1976).

**Electron microscopy.** The fresh tissues immediately after labour were cut into small pieces and fixed in 2.5% glutaraldehyde (Merck) for 2 hr, washed with pH 7.2 cacodylate-citrate buffer, post-fixed by osmium tetroxide (Sigma) dehydrated in a series of alcohols and embedded in Polylyte resin (Resina). The resulting blocks were cut in an ultramicrotome. The sections were contrasted with uranyl acetate and lead citrate (Merck) and observed in electron microscope EM 301, Philips.

**Results**

Two stillbirth cases and three spontaneous abortions occurred in the selected group. The abortions took place between the third (one case) and fifth month of pregnancy (two cases). Five per cent (5%) of the studied pregnancies were lost by spontaneous abortion and 2.5% by stillbirth. The virus was often isolated from placenta (64.1%) rather than from foetal specimens (37.8%). About 47% of the foetal organs collected at autopsy harboured the viruses. Enterovirus had been isolated from 3 maternal foetal specimens, just before miscarriage; the same viruses were isolated from placenta and all respective foetal organs such as brain, liver, kidney, and heart. Echoviruses type 19, 27, and 33 had been isolated from 4 cases.
Abortion occurred in the third (one case) and fourth (one case) trimester of pregnancy. In the third trimester, 4 cases were isolated from 6 cases of abortion, and in the fourth trimester, 1 case was isolated from 4 cases of abortion. The virus was isolated from the fourth trimester rather than from the third trimester. The abortus was collected at 5 months of gestation. Virus shedding also occurred in electron micrographs of abortus tissue.

<table>
<thead>
<tr>
<th>Cases</th>
<th>Gestational period</th>
<th>Occurrence</th>
<th>Virus isolation from</th>
<th>Virus type</th>
<th>Serology</th>
<th>Toxoplasmosis</th>
<th>Syphilis</th>
<th>Brucellosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5th month</td>
<td>Spontaneous abortion</td>
<td>Placenta, feces (8 days before abortion)</td>
<td>Echovirus 33</td>
<td>NT: 0</td>
<td>640</td>
<td>64</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>5th month</td>
<td>Spontaneous abortion</td>
<td>Placenta, liver, kidney</td>
<td>Echovirus 33</td>
<td>CF: 80</td>
<td>640</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>3rd month</td>
<td>Spontaneous abortion</td>
<td>Placenta, liver, brain</td>
<td>Coxsackie B1</td>
<td>1st serum: 0</td>
<td>40</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>9th month</td>
<td>Stillbirth</td>
<td>Placenta, heart, kidney, spleen</td>
<td>Echovirus 21</td>
<td>2nd serum: ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>9th month</td>
<td>Stillbirth</td>
<td>Placenta, brain, liver, brain</td>
<td>Coxsackie B1</td>
<td>1st serum: ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

- = negative, ND = not done, CF = Complement fixation, NT = Neutralization test for isolated enterovirus, Toxo = toxoplasmosis, Syph = syphilis, Brucel = brucellosis
Fig. 1.
Specific immunofluorescence in placental tissue confirming infection with Echovirus type 27 (case 4). Scattered foci of immunofluorescence (arrows), magn. 400×.

Fig. 2.
Part of foetal hepatocyte infected with Echovirus type 27; dilatation of endoplasmic reticulum in the vicinity of virus-like particles (arrow). The cell cytoplasm is lysed showing aggregates of ribosomes. Bar = 160 nm.

of abortion and stillbirth (Table 1). Echovirus type 27 were isolated from cases of abortion.

Enterovirus specific markers were also observed in all cases of abortion. The children were born with syphilis, and listeriosis, as well as rubella and mumps, conversions.

The presence of viral antigens was confirmed by immunofluorescence staining, mainly in the villi and amniotic fluid of cases as well as the sera of mothers who had been investigated. In some instances, alterations were also seen. We found assessment of the foetal tissues from the rupture of the chorion, and the cytotoxophoblast, and formation of multinucleated giant cells (Figs. 3, and 4).

Part of a cytotoxophoblast
N = nucleus, Bar = 175 nm
of abortion and stillbirth and Coxsackie B types 2 and 6 from two cases (Table 1). Echoviruses types 18, 21, and 25, Coxsackie B types 2 and 6 were isolated from cases of placental tissue only. Enterovirus specific serological conversions in mothers were observed in three cases of abortion. In the group where only placentalitis occurred and the children were born without abnormalities, serological conversions were also observed in all mother’s sera. Rubella, cytomegalovirus, toxoplasmosis, syphilis, and listeriosis serology were performed without positive serological conversions.

The presence of viral antigens of the enteroviruses here involved, was confirmed by immunofluorescence test. It was positive in the placentas mainly in the villi and Langhan’s cells (Fig. 1). All placentas from abortion cases as well as the foetal organs, from where enteroviruses were isolated, had been investigated by electron microscopy. The presented morphological alterations were possibly related to virus replication, but no virions were seen. We found assembly of long polyribosomes attached to enlarged elements from the rough endoplasmic reticulum and electrondense aggregates in the cytotrophoblast. Further alterations were enlargement of mitochondria and formation of vacuoles with multiple concentric membranes (Figs. 2, 3, and 4).
Discussion

Despite of the relatively large number of known factors such as chromosomal aberrations, endocrine disturbances, drugs, radiation, physical injuries, malnutrition, and infections, which were well documented, more than 50% of anomalous births remain unexplained (Brown and Arbor, 1968), specially the abortions and stillbirth cases (Mueller et al., 1983). In fact, surveys have estimated that less than 20% of anomalous infants were known to have been exposed to proved teratogenic agents. In Brazil, unfortunately, no statistical approach has been adopted for anomalous births but it can be assumed high (Radis, 1968). The incidence of infectious diseases with contamination of the placenta, specially by haematogenic route is high and includes: bacterial, fungal, viral, and protozootic agents. Concerning virus infections, the following have been detected: rubella, cytomegalovirus, mumps, varicella-zoster, and herpes simplex (Garcia et al., 1985) and as noticed recently, Coxsackie B and echoviruses (Fonseca et al., 1982).

It is well known that the hypothesis on viral aetiology of congenital malformations is based mostly on the studies with rubella and cytomegalovirus. Both are also incriminated as agents of stillbirth and spontaneous abortion (Hurley, 1981). Effective researches have lead researchers (Hovata et al., 1983; Newborns) to observe the mothers and the children born after studied pregnancies were normal babies, it is important to viruses to affect the omental tissues. Some maternal (foetal extract) had been done so that they experienced the occurrence of the infection would enable the small virus to reach the foetal and to reach the foetus are probably the best choice.

The occurrence of abortion can be explained by foetal congenital infection in the term delivered normal children. On the other hand, the cases occurred probably by the maternal active virus infection.

One patient had investigated to rubella virus, without seroconversion, placenta and from the spontaneous abortion occurred presented a respiratory discharge followed soon by a spontaneous abortion and foetal tissues. Measles (Rubella virus 33) infection by viral antigens in the placenta.

Although the child had signs of illness during the first days, the child became a healthy child (Molin, 1986).

Due to the importance of the abortion or stillbirth cause and the enterovirus infection there are viruses to infect placenta and the foetus, causing serious problems.
factors such as chromo-
ad documented, more than
radiotherapy. In fact, 
infants were known
in Brazil, unfortunately,
nalous births but it can
infectious diseases with
ovarian route is high and
ptoms. Concerning virus
abellat, cytomegalovirus,
ies et al., 1985) and as
vaca et al., 1982).
ology of congenital mal-
lla and cytomegalovirus.
spontaneous abortion
(Hurley, 1981). Efforts to incriminate additional teratogens were per-
formed, employing the retrospective analysis or prospective investigation
(Hovata et al., 1983; Modlin, 1986). This prospective study permitted us to
observe the mothers through pregnancy; the data that accumulated so far,
provided us some information such as the high incidence of enterovirus par-
ticles present in the placenta from pregnancies considered normal. The
children were born apparently without any abnormalities. Since 5% of the
studied pregnancies were lost and 95% of the mothers delivered apparently
normal babies, it is supposed that the placental barrier does not permit
viruses to affect the child, although they are able to replicate in the pla-
cental tissues. Some mothers from whom viruses were isolated (placental and
foetal extracts) had serological conversions to the virus, thus confirming
that they experienced virus infection during pregnancy. The widespread
occurrence of the infections was subclinical in mothers but the viraemia
would enable the small particles (25 nm) to cross the placental barrier easily
and to reach the foetus. The age and the degree of placental differen-
tiation are probably the best explanation for the ability of virus to reach the foetus.
The occurrence of abortion was noticed in those patients who had experi-
enced infection in the first months of pregnancy while those who had de-
ivered normal children, had enterovirus infection at the end of gestation.
On the other hand, the isolation of echovirus from placenta could be ex-
plained by foetal contamination during labour. If the same viruses have
been found in the children tissues, this would suggests that the infection
occurred probably by haemogenic route. The same viruses were also isolated
from maternal foecal samples about 20 days before abortion, stressing an
active virus infection, followed by viraemia.

One patient had contact with rubella-like exanthem but serological in-
vestigation to rubella virus demonstrated her previous immunity against that
virus, without seroconversion and Coxsackie B type 2 was isolated from her
placenta and from the child autopsy organs (brain and heart). The sponta-
aneous abortion occurred at the third month of pregnancy. Other patient
presented a respiratory-like illness with high fever and chills; vagina blood
discharge followed soon thereafter with uterine contraction. The mother
had a spontaneous abortion and Echovirus 33 was isolated from placenta
and foetal tissues. Mother serology confirmed an acute enterovirus (Echov-
irus 33) infection. Immunofluorescence tests confirmed the presence of
viral antigens in the placenta and foetal organs.

Although the children were born apparently normal and did not show
signs of illness during their first days of life at the hospital, we are not sure
whether they became carriers of the virus or if they were responsible for
a nursery outbreak of enteroviruses infections as discussed elsewhere (Mo-
dlin, 1986).

Due to the importance of knowledge about the agents that could trigger
abortion or stillbirth cases Brown and Karunas, 1977), we call attention to
enterovirus infection during pregnancy and to the capacity of such vi-
ruses to infect, to replicate in its tissues and eventually to reach the foetus, causing serious lesions to both, specially to the child that would
show, subsequently, several signs of virus infection. Our results also indicate that primacy infection occurring in the beginning of pregnancy eventually could be harmful to the foetus probably resulting in spontaneous abortion. In the fact, as stressed by Modlin (1980), "whereas acute illness in the mother before birth often precedes neonatal Echovirus infection and the infection transmitted vertically from mother to infant may be severe, postnatal transmission of the same serotype results in milder disease".

When completing this manuscript we noticed that also Parvoviruses have been recently isolated from cases of abortion and stillbirth (Brown et al., 1982; Knott et al., 1984; Hall, 1985); thus, the number of viruses related with pregnancy hazard increases without causing apparent clinical illness in the mother, or, as described in some cases, causing a rubella-like illness. The findings for parvovirus association with clinical and congenital diseases are similar to our findings.

Acknowledgements. We are in debt with CNPq and FINEP (Brazil) that awarded us with a grant for this research. We would like to thank to Elizabeth Brito Ferrarezi and Paulo Roberto de Andrade Rios, for their technical help.

References


Our results also indicate of pregnancy eventually in spontaneous abortion.CASE ACUTE ILLNESS IN THE virus infection and the fact may be severe, post-milder disease*.

At also Parvoviruses have a stillbirth (Brown et al., number of viruses related apparent clinical illness in a rubella-like illness, and congenital diseases

(Brazil) that awarded us with Drito Ferrarezi and Paulo Ro-


ation due to Echovirus type 22, and heart disease. Am. Health

genital anomalies and maternal


at 1933–1934.


Lee, M. E. F., and Wigg, M. D.

embryo illustrated by the effect 94, 77–108.

C. German measles in the mother.


The fevers and newborn; W. B.

mental defects in infants following 5.


