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ENTEROVIRUS ISOLATION FROM FOETAL AND PLACENTAL TISSUES

N. G. S. BASSO[1], M. E. F. FONSECA[1], A. G. P. GARCIA[2], J. A. T. ZUARDI[2], M. R. SILVA[1], H. OUTANI[2]

[1]Department of Virology, Institute of Microbiology, Federal University, 08040 Rio de Janeiro, and [2]Fernandes Figueira Institute, Rio de Janeiro, Brazil

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Summary. — Four spontaneous abortions and two stillbirth occurred during a prospective survey following the teratogenicity of echoviruses in 80 pregnant women selected at random from the Antenatal Care Service. Echovirus types 19, 27, and 33, Coxsackie B_2 and B_6 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 (Aycok, 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 controversal. 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).

Hovata *et al.* (1983) analysing the intrauterine deaths during a period of 6 years, considering the clinical and autopsy findings, registered a case of

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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, 1986). 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 IFF in Rio de Janeiro, Brazil. The women were observed during the whole gestation period. Stool 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 placentas, foetal organs, and stool samples were inoculated into following cell cultures: LLC-MK_2, VERO, and HEP_2. When positive for cytopathogenic effects, cell suspensions were titrated and the viruses identified in LLC-MK_2 cells with Melnick's standard sera against enteroviruses.

Serological studies. Both blood samples collected from each mother were assayed against the virus isolated from them by neutralization test. Assays were performed to exclude other concurrent infections such as rubella, cytomegalovirus, toxoplasmosis, syphilis, listeriosis, and brucellosis. In addition, data were determined such as Rh factor, glycaemia, 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, overlayed 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 (Boehring Laboratories). After one hour incubation at 37 °C, the sections were washed with PBS, air dried, and mounted (Lennette and Schmidt, 1979).

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-sucrose buffer, post-fixed by osmium tetroxide (Sigma) dehydrated in a series of alcohols and embedded in Polilyte resine (Resana). 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 faecal specimens (37.8%). About 47% of the foetal organs collected at autopsy harboured the viruses. Enterovirus had been isolated from 3 maternal faecal specimens, just before miscarriage; the same viruses were isolated from placentae and all respective foetal organs such as brain, liver, kidney, and heart. Echoviruses types 19, 27, and 33 had been isolated from 4 cases

Table 1. Abortions with enterovirus isolation

Cases	Gestational period	Occurrence	Virus isolation from	Virus type	Serology						Toxo	Syph	Brucel
					NT		CF / CMV		ELISA/Rubella				
					1st serum	2nd serum	1st serum	2nd serum	1st IgG	2nd IgG			
1	5th month	Spontaneous abortion	Placenta, faeces (10 days before abortion), brain, liver kidney	Echovirus 33	0	640	64	64	+	+	−	−	−
2	5th month	Spontaneous abortion	Placenta faeces (a week before abortion), liver, brain	Echovirus 33	80	640	8	8	+	+	−	−	−
3	3rd month	Spontaneous abortion	Placénta, liver, brain	Coxsackie B_2	0	40	8	8	+	+	−	−	−
4	9th month	Stillbirth	Placenta, heart, kidneys, spleen	Echovirus 27	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	9th month	Stillbirth	Placenta, brain, liver, brain, liver,	Coxsacke B_6	ND	ND	ND	ND	ND	ND	ND	ND	ND

− = negative, ND = not done, CF = Complement fixation, NT = Neutralization test for isolated enterovirus, Toxo = toxoplasmosis, Syph = syphilis, Brucel = brucellosis

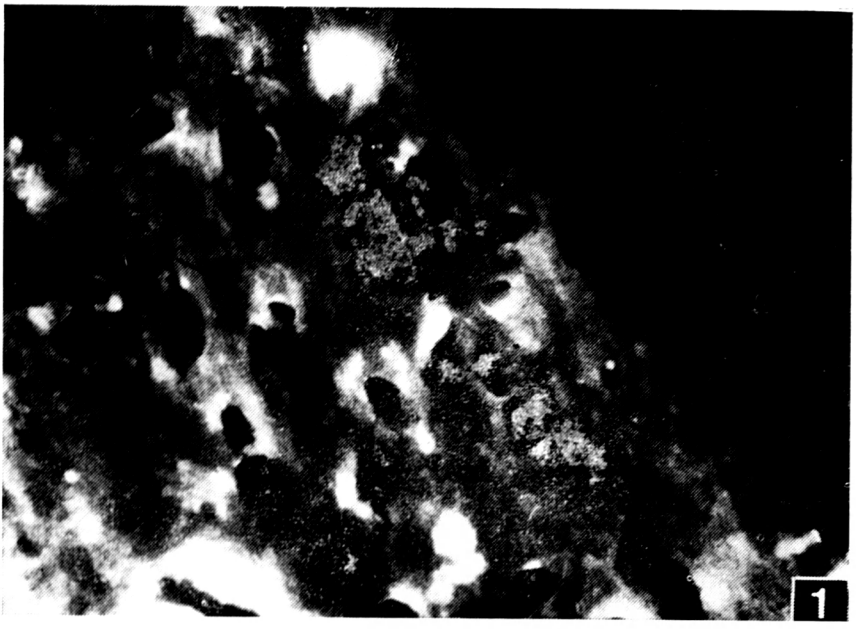

Fig. 1.
Specific immunofluorescence in placental tissue confirming infection with Echovirus type 27 (case 4). Scattered foci of immunofluorescence (arrows), magn. 400×.

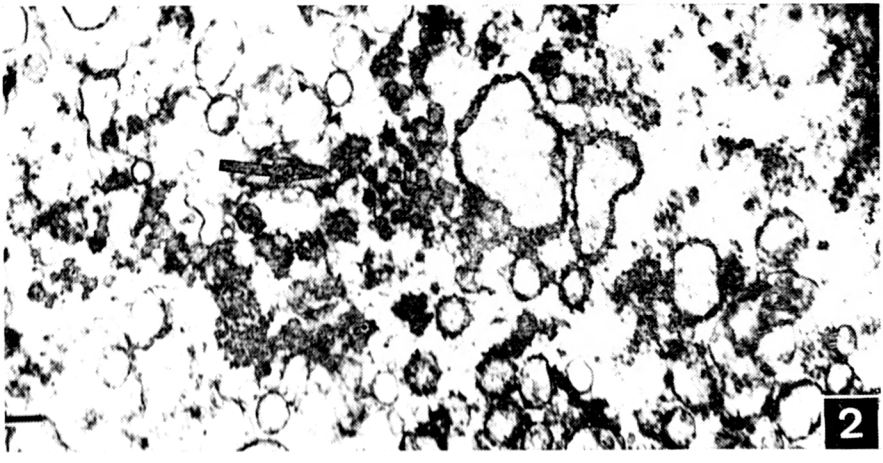

Fig. 2.
Part of foetal hepatocyte infected with Echovirus type 27; dilatation of endoplasmic reticulu in the vicinity of virus-like particles (arrow). The cell cytoplasm is lysed showing aggregates ribosomes. Bar = 160 nm.

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 placentitis only.

Enterovirus specific serological conversions in mothers were observed in three cases of abortion. In the group where only placentitis 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).

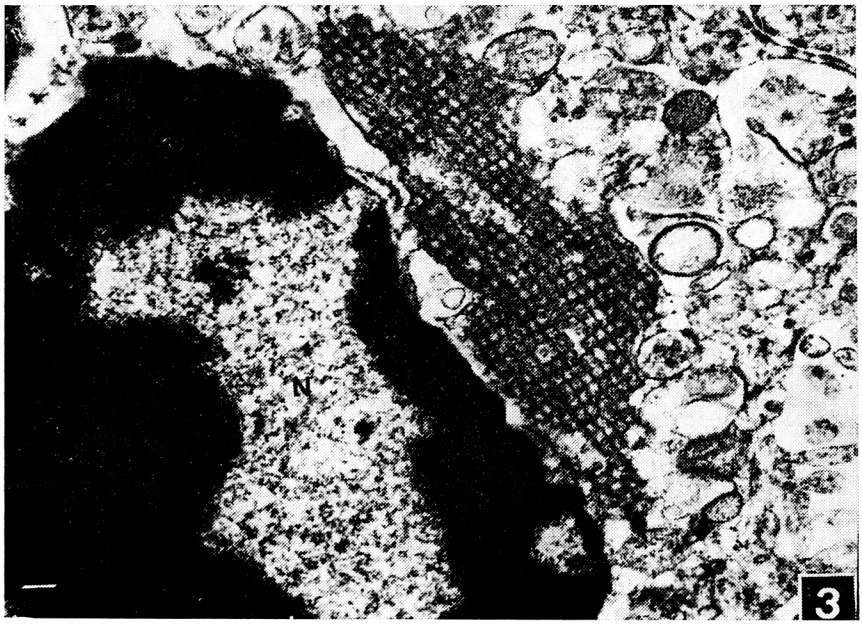

Fig. 3.

Part of a cytotrophoblast cell: arrangement of picorna virus-like crystals in the cytoplasm N = nucleus, Bar = 175 nm.

Discussion

Despite of the relatively large number of known factors such as chrom somal aberrations, endocrine disturbances, drugs, radiation, physical i juries, malnutrition, and infections, which were well documented, more th 50% of anomalous births remain unexplained (Brown and Arbor, 196 specially the abortions and stillbirth cases (Mueller *et al.*, 1983). In fa surveys have estimated that less than 20% of anomalous infants were kno to have been exposed to proved teratogenic agents. In Brazil, unfortunatel no statistical approach has been adopted for anomalous births but it c be assumed high (Radis, 1968). The incidence of infectious diseases wi contamination of the placenta, specially by haematogenic route is high a includes: bacterial, fungal, viral, and protozootic agents. Concerning vir infections, the following have been detected: rubella, cytomegaloviru mumps, varicella-zoster, and herpes simplex (Garcia *et al.*, 1985) and noticed recently, Coxsackie B and echoviruses (Fonseca *et al.*, 1982).

It is well known that the hypothesis on viral aetiology of congenital ma formations is based mostly on the studies with rubella and cytomegaloviru Both are also incriminated as agents of stillbirth and spontaneous aborti

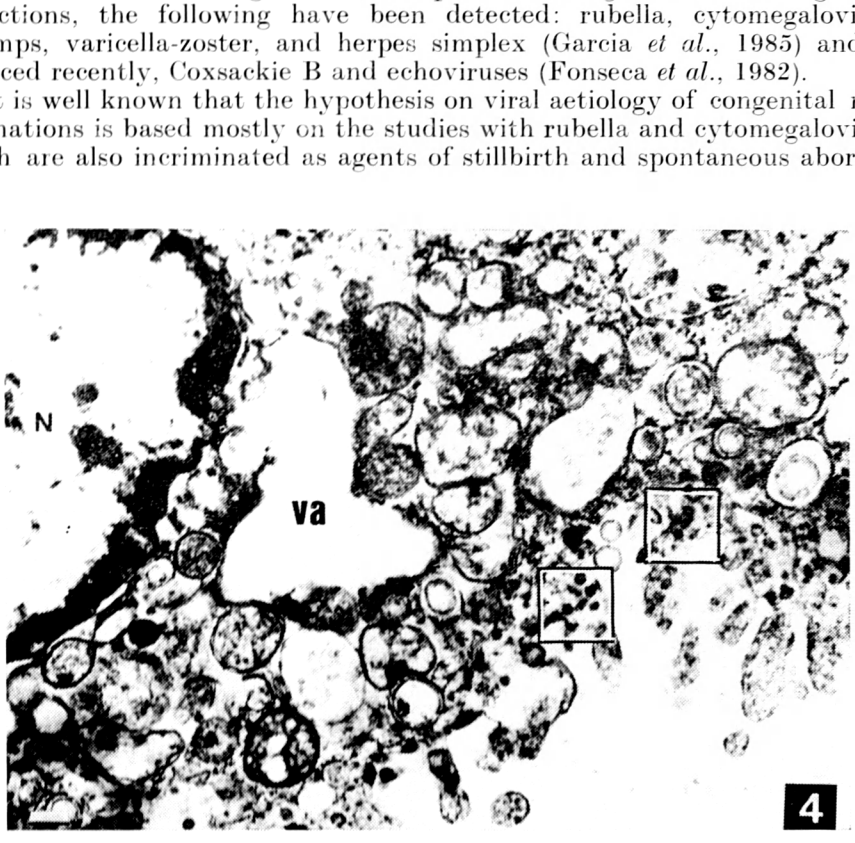

Fig. 4.

Part of a cytotrophoblast cell; picornavirus-like particles in the cytoplasm near to cell p trusions; the cell is lysed and vacuolated

N = nucleus, Va = vacuoles, Bar = 200 nm.

(Hurley, 1981). Efforts to incriminate additional teratogens were performed, 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 particles 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 placental 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 (28 nm) to cross the placental barrier easily and to reach the foetus. The age and the degree of placental differentiation 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 experienced infection in the first months of pregnancy while those who had delivered normal children, had enterovirus infection at the end of gestation. On the other hand, the isolation of echovirus from placenta could be explained by foecal 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 investigation 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 spontaneous 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 (Echovirus 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 (Modlin, 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 viruses to infect placenta, 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 indica that primaty infection occurring in the beginning of pregnancy eventuall could be harmful to the foetus probably resulting in spontaneous abortio In the fact, as stressed by Modlin (1986), "whereas acute illness in th mother before birth often precedes neonatal Echovirus infection and th infection transmitted vertically from mother to infant may be severe, pos natal transmission of the same serotype recults in milder disease".

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

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