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COXSACKIEVIRUS B3 INFECTION IN PREGNANCY AND ITS INFLUENCE ON FOETAL HEART DEVELOPMENT

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Summary.—Infection of mice on the 12th or 14th day of pregnancy with Coxsackievirus B3 resulted in the birth of growth-retarded young which died soon after birth and exhibited an abnormal heart development. The ratio of heart weight to body weight in these offspring was higher than normal. The auricles were prominent and the ventricles developed such that the heart apex was bifid in appearance. This anomalous cardiac development may have been due to a direct viral pathogenicity in the developing tissue or, as seems more likely, resulted from a generalized disturbance in foetal growth attributable to a virus-induced pancreatic insufficiency in the mother. Retarded development in the pulmonary system also resulting from aberrations in foetal growth may have been contributory to impaired postnatal cardiac growth.

INFECTIONS with Coxsackieviruses of Group B have been associated with heart disease in newborn and young children for many years but, according to Grist and Bell (1974), good evidence for their causative role is hard to find. Although maternal infections with Coxsackieviruses in pregnancy may be a cause of congenital heart disease or malformations, this, too, has not been unequivocally substantiated. In contrast, the association between rubella infection and foetal heart disease was clearly recognized (Gregg, 1941: Swan *et al.*, 1943), where the diagnosis of infection was largely by the overtly recognizable maternal illness and the exanthematous rash. Coxsackievirus B infections however, are not usually accompanied by specific symptoms and may be inapparent in at least 50% of cases. Diagnosis of infections has relied on a patient's own observations or on retrospective serum antibody determinations (Brown and Evans, 1967). Thus a four-fold or greater increase in neutralizing antibodies would be regarded as positive evidence of a recent infection (Droughet and Rouquette, 1970). Current views on the possibility that Coxsackievirus B infections in human pregnancy may be a cause of congenital heart disease are based on this type of evidence.

In one such study, Brown and Karunas (1972) collected sera from 22,935 women in early pregnancy and again at delivery. They observed that cardiovascular abnormalities occurred more frequently in the children born to mothers having given evidence of infection with Coxsackievirus B3 or B4. The risk was increased in patients who showed signs of having been infected with more than one Group B virus. In this work, the heart malformations included patent ductus arteriosus, septal defect, coarctation of the aorta and transposition of the great vessels. Droughet and Rouquette (1970) believed that Coxsackieviruses B3 and B4 had an undeniable role in the aetiology of malformations and they assessed that the risk with B3 for congenital heart deformity would be approximately 1·3%.

Although the results of the epidemiological surveys point to a causal relationship between Coxsackievirus B infections

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and congenital heart disease, these infections are very common and most people possess antibodies to them (Grist and Bell, 1974). It may be that the higher the antibody titre, the greater is the probability of a recent infection having occurred. However, such is the variation between different people that a rising antibody titre may not be a reliable guide either to the time of infection or to its severity. A further factor in these surveys is the lack of an identification of viral antigen either in the maternal blood or in the cord blood. Maternal viraemia and foetal infection might well have been prevented by the presence of circulating antibodies arising from a previous infection (Droughet and Rouquette, 1970).

The results of the epidemiological studies relating to congenital heart disease where serological evidence of infection has been used contrast strongly with observations made in earlier work where virus was isolated from the hearts of children dying early in life with severe heart disease (Kilbrick and Benirischke, 1956, 1958), and who were presumed to have been infected prenatally. These cardiac lesions were a focal necrosis with inflammatory-cell infiltration and they were similar to those occurring when infection was acquired postnatally (Verlinde, van Tongeren and Kret, 1956). They also resembled those produced in experimental animals where Coxsackievirus was identified in areas of tissue damage by means of immunofluorescent antibody tests (De Pasquale *et al.*, 1966). Thus it seems more probable that in these children the cardiac changes were attributable to a direct viral pathogenicity resulting from a viral infection *in utero* or by an infection which was acquired perinatally. At present, Coxsackieviruses are believed to cross the human placenta but little information is available to indicate if the heart is susceptible to damage prenatally, and to what extent.

If group B Coxsackieviruses do not infect the foetus to affect cardiac development directly, then it would seem that the cardiac changes referred to in Brown's study (1967, 1972) are the result of an indirect action related to such factors as maternal stress, metabolic disturbance or fever in the pregnant mother, all of which have been shown to be responsible for human and other mammalian foetal malformations (Carter, 1967; Edwards, 1969; Thomas, 1975).

In view of the suggestive but as yet equivocal views on the role of Coxsackieviruses B in the aetiology of congenital heart disease in man, it is possible that more substantive information may be forthcoming from experimental studies in mice. This species was originally used to isolate and characterize Coxsackieviruses and has been used subsequently to study their pathogenicity in the pancreas (Coleman, Gamble and Taylor, 1973; Lansdown, 1976) and in the heart in young and adult animals (Grodums and Dempster, 1959; Woodruff and Woodruff, 1974; Miranda, Kirk and Beswick, 1973). On the basis of their experience in using this species, Gear and Measroch (1973) considered that the lesions caused by Coxsackievirus infections were not greatly dissimilar to those seen in human infections.

The present investigation was designed therefore to examine the possibility that Coxsackievirus B3 infection in mice at a susceptible stage in pregnancy will produce heart disease in the offspring similar to that reported in human babies presumed to have been infected prenatally.

MATERIAL AND METHODS

Virus stock.—The Coxsackievirus B3 used in these studies was derived from the original Nancy strain from the stock maintained at the Public Health Laboratory Service at Colindale (London). It had been passed through suckling mice, isolated in primary monkey kidney cells and through vervet monkey kidney cells (VERO) to give a final suspension with a tissue culture infective dose of $10^{6.85}$/ml ($TCID_{50}$).

Animals.—The mice used were of the "TO" outbred strain derived from the original Swiss stock (Theiler). Virgin females weighing between 18 and 25 g were used in all experiments. They were placed overnight with proven males and the morning when vaginal plugs were noted was designated the first day of pregnancy. At all times, the mice had free access to a standard laboratory animal diet and water *ad libitum*.

Experimental.—Groups of 15 pregnant mice were injected i.m. or i.p. with 0·3, 0·5 or 0·75 ml of undiluted Coxsackievirus B3 suspension in tissue culture fluid on the 12th or 14th day of pregnancy. Control animals received an equivalent dose of virus suspension inactivated by heating at 56° for 30 min.

The mice were allowed to litter normally and in preliminary experiments the neonates were observed for their ability to suckle and for their general viability. When the young born to severely affected mothers were seen to die soon after birth and become subject to maternal destruction, all subsequent litters were killed within 12 h of birth. Each neonate was freshly weighed and then dissected with the hearts and lungs being weighed and then fixed in phosphate-buffered formalin or in formol alcohol for histological examination. Histological sections were routinely stained by haematoxylin and eosin, oil red-O for neutral fat, and by the PAS technique for glycogen.

Virus isolation and immunofluorescence studies.—To relate histological changes in the hearts with the presence of viral antigen, some foetuses were obtained 2 to 3 days after infection and the heart removed aseptically for virus isolation. These hearts were homogenized in phosphate-buffered saline to give a final concentration of 10% w/v. This foetal heart cell homogenate was titrated in VERO cell tube cultures and incubated for 6 days. The tissue culture infective dose ($TCID_{50}$) was calculated after Karber's standard procedure.

Alternatively, the aseptically dissected foetal hearts were examined for presence of Coxsackieviral antigen by specific immunofluorescence. The hearts were snap-frozen in a mixture of isopentane and solid carbon dioxide and then sectioned at −30° using a refrigerated cryostat (Slee, London). Sections were exposed at room temperature to a 10^{-1} dilution of fluorescein isothiocyanate labelled Coxsackievirus B3 antiserum (Wellcome) and anti-rabbit immunoglobulin (Wellcome).

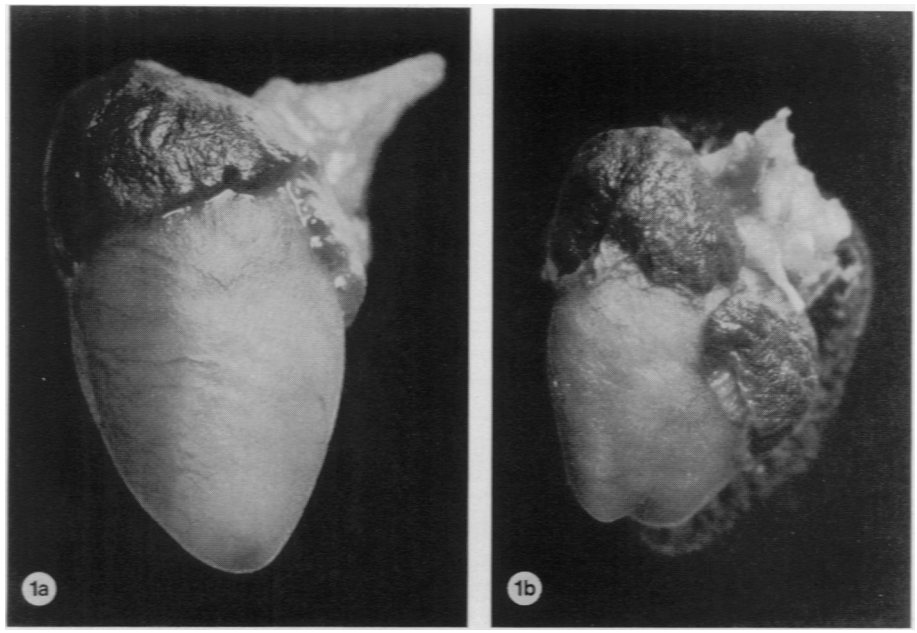
RESULTS

Mice injected with a low dose of Coxsackievirus B3 (0·3 ml) on the 14th day of pregnancy remained in overt good health throughout but delivered young that were significantly smaller than the corresponding group of controls ($P > 0.001$) (Table). In these neonates, the hearts weighed as much or more than those of the control group but the ratio of heart weight to body weight was higher than normal. In contrast, whereas the young born to mothers injected with virus on the 14th day of pregnancy usually survived for more than 3 days postnatally, those exposed to virus on the 12th day of pregnancy rarely survived for more than a few hours after birth and

TABLE.—*Influence of Prenatal Coxsackievirus B3 Infection on the Body Weight and Heart Weight in Neonatal Mice*

Treatment	No. foetuses	Mean body wt. of neonatal mice (g)	Mean heart wt. (g)	$\frac{\text{Heart wt.}}{\text{Foetal body wt.}} \times 100$
0·3 ml Cox. B3* injected i.m. on Day 14 of pregnancy	156	1·06±0·014	0·0122±0·0004	1·151
Control inj. 0·3 ml inac. Cox. B3 on Day 14 of pregnancy	143	1·510±0·025	0·0096±0·0003	0·637
0·5 ml Cox. B3 injected i.p. on Day 12 of pregnancy	46	0·878±0·034	0·0104±0·007	1·185
0·5 ml Cox. B3 injected i.m. on Day 12 of pregnancy	85	0·944±0·028	0·0098±0·0003	1·041
0·75 ml Cox. B3 injected i.p. on Day 12 of pregnancy	57	0·923±0·021	0·0105±0·0004	1·138
Control inj. 0·5 ml inac. Cox. B3 i.m. on Day 12 of pregnancy	80	1·539±0·016	0·0126±0·0003	0·819

* Suspension of Coxsackievirus B3 contains $10^{6.85}$/ml ($TCID_{50}$).



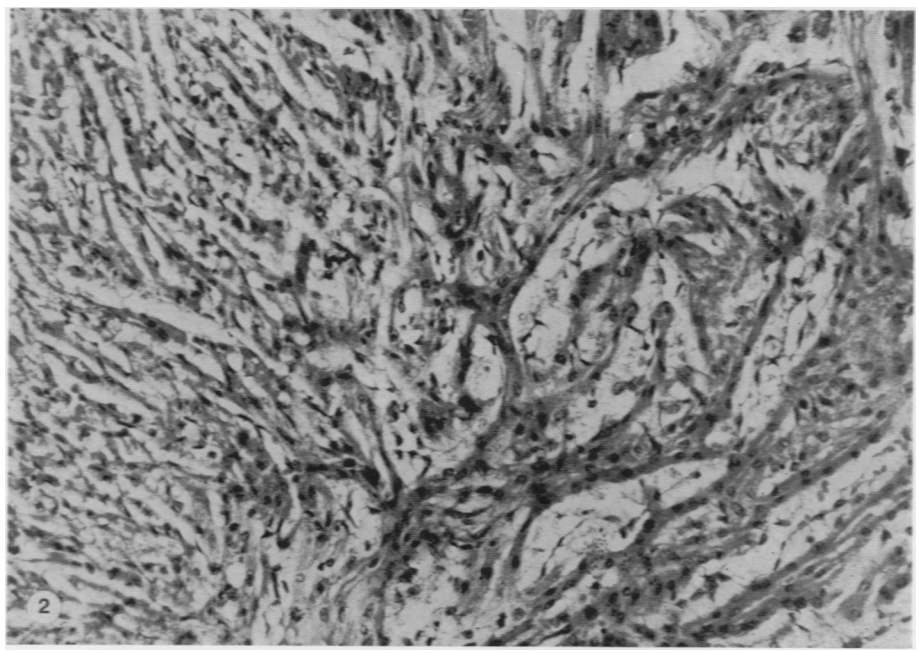


FIG. 1.—(a) Heart from a control 1-day-old mouse. × 20. (b) Heart from a 1-day-old mouse born to a mother injected on Day 12 of pregnancy with 0·5 ml of Coxsackievirus B3 showing hypertrophy of the right ventricle and the bifid apical condition. × 20.

FIG. 2.—Interfibrous vacuolation in the myocardium of the right ventricle of a control 1-day-old mouse. H. and E. × 90.

were frequently subject to maternal destruction.

Macroscopically, the hearts of the young from infected mothers differed from the controls of the same age in having prominent auricles and an abnormal ventricular form (Fig. 1). The ventricular development was such that the heart apex was bifid in appearance reflecting an increased size of the right ventricle relative to the left. Transverse sectioning of the affected hearts in the transauricular and transventricular planes did not reveal septal or valve defects, an open foramen ovale was not seen in any animal examined and the intraventricular septum was continuous. The wall of the right ventricle was appreciably thinner than that of the left ventricle in most cases. In the controls there was little clear difference in the dimensions of the ventricular walls. The major blood vessels in the proximo-cardiac region did not reveal a clear patent ductus arteriosus and the state of development did not differ from that in the controls.

Histologically, the hearts of the neonates from infected mothers did not differ from those of control animals. The myocardial fibres in the abnormally shaped hearts were morphologically normal and the valves and septae were not affected by the treatment given. A variable amount of interfibrous vacuolation was present in infected and control tissues in the subendocardial regions of the auricles and ventricles (Fig. 2, 3). This condition, which was more frequently present in the right ventricle, was not histochemically associated with a deposition of neutral lipid or glycogen. In both infected and control animals, the elastic laminae in the aorta and pulmonary arteries were well developed and did not differ according to the shape of the organ.

Virus isolation and immunofluorescence

Virus isolation studies revealed no antigen in the cardiac tissue from neonates from mothers infected with Coxsackievirus B3 on Day 14 of pregnancy and examined on the 16th or 17th day of gestation. Immunofluorescence studies revealed only a mildly positive staining reaction indicative of the presence of virus in the hearts of young mice from mothers injected with virus on Day 12 or 14 of pregnancy and killed 2 to 3 days later. In these tissues, the immunofluorescence was evenly distributed through the auricular and ventricular myocardia and did not exhibit any specific localization.

Lung pathology

The lungs taken from normal 1-day-old mice were usually well expanded with clear alveoli, thin interalveolar septae and clear airways (Fig. 4). On occasions, some small alveoli were seen associated with deposits of proteinaceous material and red blood corpuscles. This condition was not marked in any animal examined. In contrast, in the lungs from the neonates of infected mothers, the alveoli were appreciably smaller, less expanded and the tissues generally more solid than normal. The interalveolar septae were thicker and many alveoli contained red blood corpuscles and protein (Fig. 5). The protein in some infected neonatal lungs was apposed to the septae in the form of the hyaline membrane condition. The bronchioles in the infected neonates were frequently distended but the mucosae were histologically normal. There was a clear correlation between birth weight and the developmental state of the lungs.

DISCUSSION

Infection with Coxsackievirus B3 in mice during late pregnancy resulted in severe intrauterine growth retardation leading to the birth of small-for-dates offspring which were less viable than normal and which exhibited anomalous heart development. Although viruses of the Coxsackie B group are known to infect the murine foetus and placenta (Soike, 1967; Selzer, 1969), little evidence

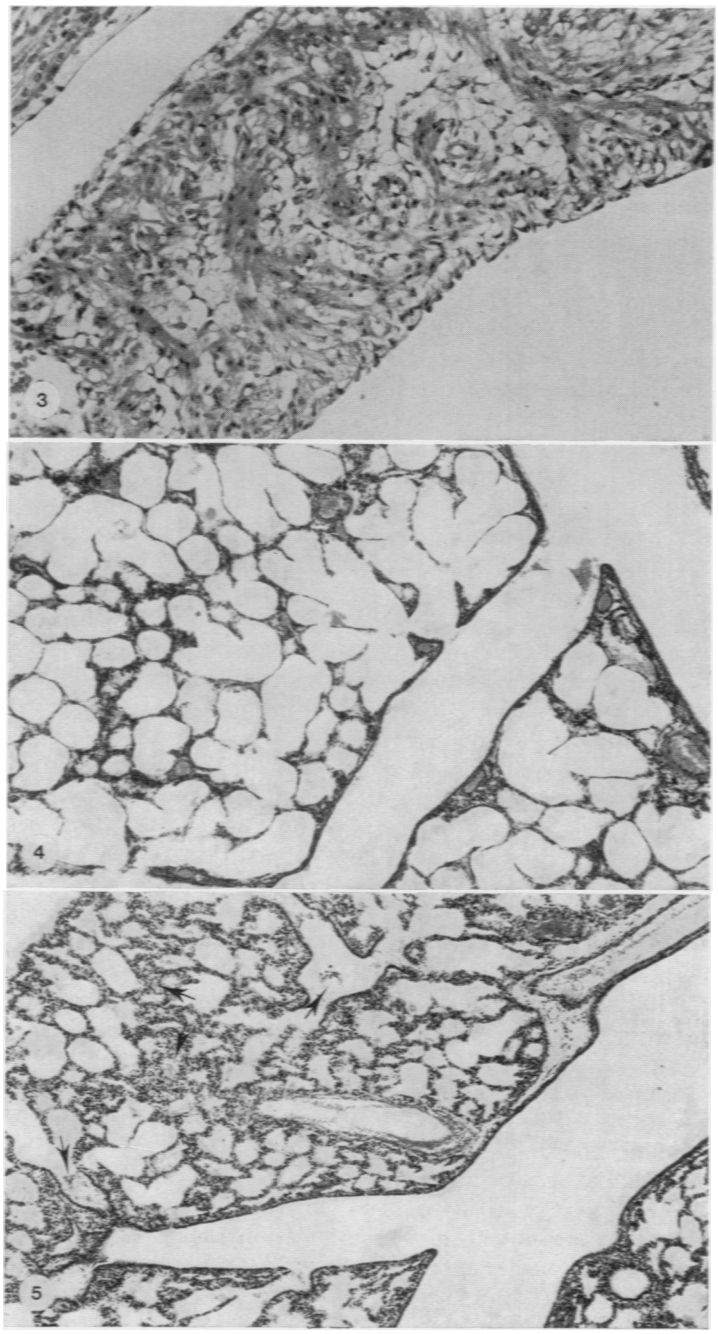


FIG. 3.—Subendocardial vacuolation in the auricle of a control 1-day-old mouse. H. and E. × 90.
FIG. 4.—Lung section from a control 1-day-old mouse showing clear airways and well expanded alveoli with thin intra-alveolar septae. H. and E. × 225.
FIG. 5.—Lung section from a 1-day-old mouse following Coxsackievirus B3 infection of the mother in pregnancy (0·5 ml virus on Day 12) showing well expanded airways but with the alveoli generally small and undilated and containing protein and red blood corpuscles (arrowed). H. and E. × 225.

was presented in the present study to implicate direct viral pathogenicity in the aetiology of the heart deformity. There was little indication for viral replication at this site 2 to 3 days after maternal infection when foetal viral titres are high (Droughet and Levantis, 1968), and no inflammatory or necrotic changes of the type associated with viral antigen following postnatal infection were identified (Grodums and Dempster, 1959; Woodruff and Kilbourne, 1970).

In previous studies, anomalous heart development in experimental teratology has been reported following maternal exposure to trypan blue dye (Waddington and Carter, 1953), increased levels of oestrogens and progestogens (Heinonen *et al.*, 1977) and hypoxia (Haring, 1965). The types of anomaly reported varied from those observed in the present work. They included pericardial enlargement oedema (trypan blue dye), failure in the closure of the intraventricular septum, pulmonary artery stenosis, ventricular hypertrophy, and transposition of the major arteries (hypoxia, oestrogens). The difference between this range of abnormalities and those seen in mice exposed to Coxsackievirus B infection may be in part explained by differences in the times of exposure to the agencies in pregnancy. In the teratological experiment resulting in abnormal foetal heart development, the environmental agent was administered at an earlier stage in development than that used in the present work, after the major part of the differentiation process had taken place. Even so, Haring (1965) produced defects in the closure of the foetal rat intraventricular septum following exposure to hypoxia at 15–16 days gestation (rat gestation time 22–23 days), and in the mouse the ductus arteriosus is patent and the intraventricular septum and foramen ovale are still open until near term (19 days' gestation). Theoretically, therefore, if Coxsackievirus B3 is cardiotropic in this strain of mice, more evidence of malformation would be expected, possibly associated with a focal or diffuse inflammatory response of the type seen following postnatal infections.

Two mechanisms are suggested for the abnormal development that was seen following Coxsackievirus B3 infection in pregnancy. They are, that due to protein malnutrition resulting from virus-induced pancreatic insufficiency in the mother (Lansdown, 1975), the differentiation pattern in the foetus is retarded or distorted. Retarded development has been demonstrated in the lungs in this study, and in the development of serum proteins, the skin and the thymus in previous work (Coid and Ramsden, 1973; Lansdown, 1975*a*; 1977). It may be that the retarded development in the lungs is contributory in the formation of the prominent auricles and the disproportionate ventricular growth seen in the heart, which is particularly obvious in those young dying 2 to 3 days after birth. The alternative explanation suggested is that although the immunofluorescent antibody test indicated only a low level of virus in the heart, it is conceivable that even this level may be sufficient to cause some subtle changes in the rapidly dividing and differentiating myocardial cells to produce the gross changes seen.

The conclusion reached here is that abnormal cardiac development is probably part of a generalized disturbance in foetal growth related to virus-induced ill-health in the mothers. A similar explanation may account for the possible teratogenic effects attributed to Coxsackievirus B infection in human babies.

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