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# THE GROWTH OF REOVIRUS 3 IN CULTURED RAT EMBRYOS AND IMPLICATIONS FOR HUMAN REPRODUCTIVE FAILURE

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**Summary.—Ten-day rat embryos were exposed to different concentrations of reovirus 3, heat-inactivated virus or diluent and then cultured for 48 h. An effect on embryonic growth and development was observed that required live virus. Virus concentrations of $10^{6.0}$ $TCID_{50}$ were embryolethal while $10^{5.0}$ $TCID_{50}$ killed about 20% of embryos and caused varying degrees of growth retardation in the remainder. Growth of the agent in the visceral yolk sac and embryonic tissues was confirmed by virus titration and immunofluorescent studies. Infectious virus was detected in the medium after 6–8 h and increased exponentially throughout the 48 h culture period, except for a check around 18 h. The significance of the findings in relation to previous animal studies and the potential importance of reovirus as a cause of human reproductive failure are discussed.**

THE USE of cultured post-implantation rodent embryos in developmental biology has recently been facilitated by improvements to the available culture techniques (New, 1978). One area where they may be of value, but as yet have not been used, is in the study of infectious embryopathy.

In order to evaluate the potential of whole-embryo culture in infectivity studies, reovirus type 3 (reo 3) was chosen since this virus was shown to infect rat embryos *in vivo* (Margolis and Kilham, 1973). Reoviruses are members of the Reoviridae family having a double-stranded RNA genome made up of 10 separate segments (Andrewes, Pereira and Wildy, 1978). Three mammalian and 5 avian serotypes are recognized and there appears to be some species association of the different serotypes although this is not rigid (Rosen, 1968). Thus, reoviruses types 1 (reo 1) and 2 (reo 2) are most often associated with human and reo 3 with rodent infections. Although they are widely distributed throughout the animal kingdom they are seldom associated with any disease state.

Interest in congenital reovirus infections began with reports of experimental infections in mice (Hashimi *et al.*, 1966; Hassan and Cochran, 1966). One report suggested that a reo 2 of human origin could infect mouse foetuses *in utero* and cause a protracted postnatal disease in many of the offspring (Hashimi *et al.*, 1966). It was not certain, however, that the mice were actually infected *in utero* and the results were also readily explained as due to a post-partum infection from the mothers (Rosen, 1968; Kilham and Margolis, 1975). Hassan and Cochran demonstrated that reo 1 inoculation into mice caused an increase in the incidence of malformations, resorptions, late foetal deaths (Hassan and Cochran, 1966) and neonatal mortality (Hassan and Cochran, 1969). Congenital infection was confirmed by foetal virus isolations and foster nursing of the offspring after caesarian delivery. Experiments with reo 3 in

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pregnant hamsters and rats confirmed that the virus could cross the placenta in these species but no gross abnormalities were found (Kilham and Margolis, 1973, 1974; Margolis and Kilham, 1973). In the hamster model the virus caused an increase in the number of resorptions and persisted until after birth (Kilham and Margolis, 1974) but although some individuals had extensive histopathological lesions, newborns rarely appeared ill (Kilham and Margolis, 1975). Thus, a variety of consequences for the developing foetus have been reported to follow maternal infection with reovirus. A preliminary experiment with reo 1, Lang strain, showed no effect on embryonic development (unpublished observations) and so reo 3 was used in all further work. The rat was chosen for experiments described in this report because embryo culture techniques were more reliable.

## MATERIALS AND METHODS

*Animals.*—Specific pathogen-free Sprague–Dawley rats were supplied by the breeding unit of the Clinical Research Centre. The colony was serologically negative for the following viruses: reo 3, Minute Virus of Mice, Kilham Rat Virus, Toolan H-1 Virus, Sendai and Mouse Hepatitis Viruses. Timed-pregnancies were taken as Day 0 when a copulation plug was found following overnight mating.

*Embryo culture technique.*—Embryos were removed aseptically according to the procedure of New (1971). The deciduum from each implant was carefully removed with watchmakers forceps using a Wild dissecting microscope (E. Leitz Ltd., London). Then the Reichert's membrane with the trophoblast and parietal endoderm attached were torn back to the ectoplacental cone and trimmed. This left the embryo inside an intact visceral yolk sac ready for culture. Embryos at explanation were in Stage 15 (Witschi, 1962), *i.e.* having 5–12 somites, dorsally concave and open neural tube.

The culture medium consisted of rat serum prepared by the method of New, Coppola and Cockroft (1976) or diluted with an equal part of Dulbecco's modification of Eagles' Minimal Essential Medium (EDMEM, Flow Laboratories Ltd., Irvine) containing 100 I.U./ml penicillin and 100 $\mu$g/ml streptomycin.

The roller-bottle method was used to culture the embryos (New, Coppola and Terry, 1973) using a modified haematological roller (Priscott, 1979). All-glass, wide-necked reagent bottles (30 ml capacity) were filled with 4 ml of culture medium to which 3 or 4 embryos were added. The bottles were carefully gassed with a 20% $O_2$, 5% $CO_2$, 75% $N_2$ mixture for 2 min before sealing and placing on a roller apparatus at 37°. After 24 h the bottles were regassed with 35% $O_2$, 5% $CO_2$, 60% $N_2$, and cultured a further 24 h. The medium was not replaced during the 48 h culture period.

Embryos were assessed for their morphological development, the number of somites formed and the crown-rump length measured using an eye-piece micrometer fitted to the dissecting microscope.

*Virus assay.*—Reo 3, Dearing strain, was supplied by Dr D. A. J. Tyrrell, Common Cold Research Unit, Salisbury. The seed virus was passed in secondary monkey kidney cells and had a titre of $10^7$ $TCID_{50}$.

A modified microplate technique (Rosenbaum *et al.*, 1963) was used to assay the virus Virus dilutions (0·5 $\log_{10}$ intervals) were made and 0·1 ml added to 4 microplate wells per dilution. A further 0·1 ml of $10^5$ cells/ml suspension of L929 cells in EDMEM containing antibiotics and 10% foetal calf serum (FCS). The plates were then incubated in a humidified incubator gassed with 5% $CO_2$ for 7–8 days and the presence of virus determined by examination for cytopathic effect.

*Fluorescent-antibody technique.*—Tissues were snap-frozen in iso-pentane cooled with cardice after first being immersed in "O.C.T. Compound" (Ames Co., Elkhart, Indiana). Sections 6 $\mu$m thick were cut on a cryostat (Slee Ltd, London) and allowed to dry for 30 min at room temperature before fixation in acetone for 10 min. Reo 3-infected rat convalescent serum and pre-infection serum were used as positive and negative antisera after appropriate dilution with phosphate buffered saline (PBS). The indirect method of detecting virus-antiserum reaction was employed, using anti-rat immunoglobulin G (National Cancer Institute, Office of Programme resources, Bethesda,) together with a rhodamine counterstain. Antisera and conjugates were allowed to react with tissue for 15 min and after each reaction the slides were washed for 30 min in two changes of PBS with 0·1% FCS added. Finally, slides were drained and mounted in "Clearmount" (Gurr's Ltd, High Wycombe) before examining for fluorescence using an "Orthoplan" microscope (Leitz Instruments Ltd, London) with incident light and darkfield together with BG 38 exciting and K510 absorbing filters.

*Statistics.*—Differences between experimental treatments were tested for significance using Student's $t$ test.

## RESULTS

In preliminary experiments it was found that embryonic infection could be established by immersing the conceptus in a virus suspension for 30–90 min at 37° and thoroughly washing before culture. It was decided that 30 min was sufficient for virus adsorption and minimized the time before embryos could be returned to an appropriate culture medium and therefore this time was used for subsequent experiments. It was also established that there was no statistically significant difference ($P > 0{\cdot}10$) between embryonic crown-rump lengths after virus-free adsorptions using uninfected monkey kidney cells or diluent alone. Therefore, it was decided to use diluent alone for control adsorptions.

A dose-related effect on embryonic development was observed at virus inoculum concentrations between $10^7$ and $10^5$ $TCID_{50}$ (Table I). When heat-inactivated virus was used there was no significant effect on either crown-rump length ($P > 0{\cdot}5$) or somite development ($P > 0{\cdot}2$). At the higher virus concentration all embryos were lethally infected and were necrotic at the termination of cultures. Upon exposure to $10^5$ $TCID_{50}$ of virus, 5 of 25 (20%) were dead after culture. The survivors exhibited varying degrees of growth retardation (Fig. 1).

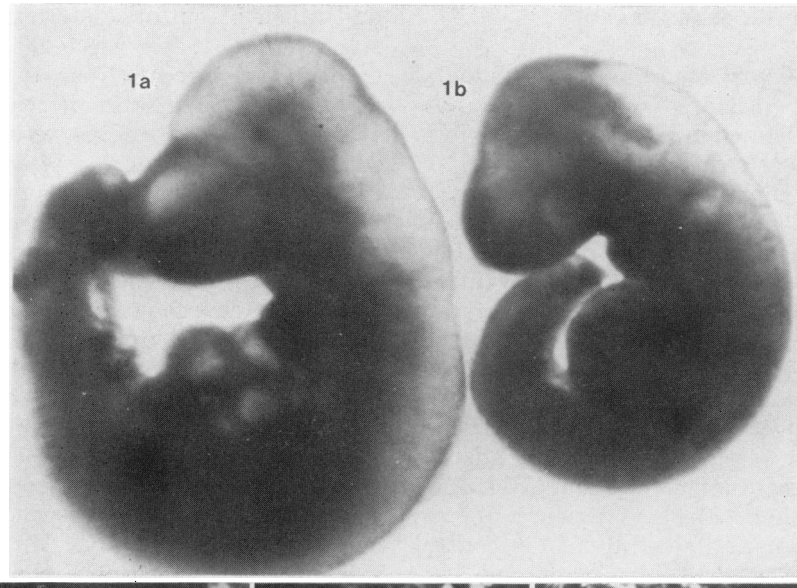
Having shown that there were live-virus dependent effects on embryonic development it remained to demonstrate virus replication in the conceptuses. Groups of 5 embryos were exposed to different virus concentrations, washed and virus concentrations at the start and finish of culture determined. It was shown that there was an increase in embryo-associated virus at all virus-infecting concentrations tested (Table II). The virus titration experiment was supported by immunofluorescent staining of frozen sections. Specific viral immunofluorescence was observed throughout the cytoplasm of cells in the yolk sac and outer aspects of the embryo (Fig. II). This was characteristic of cell-staining late in the infection cycle (Spendlove *et al.*, 1963). Occasionally foci of fluorescent cells were found within the embryo but the tissues involved were not identified. It is possible that they arose either through the ingestion of infected amniotic fluid or via the embryo-yolk sac blood circulation.

To further investigate the growth of virus in the system, an inoculum that caused a largely sub-lethal effect ($\sim 10^5$ $TCID_{50}$/ml) on the developing embryos was selected. After infection and thorough washing, embryos were cultured for 48 h and samples from the culture medium taken at different time intervals. The concentration of infectious virus present was then plotted against time in culture (Fig. 3); the data points being derived from 3 separate experiments. The culture medium showed a logarithmic increase in virus content from about 6 h until 18 h when the rate of increase slowed. Then another round of replication was observed to begin around 30 h and increased until cultures were stopped at 48 h. An overall increase in infectious virus of $10^5$ $TCID_{50}$/ml was observed in the culture medium during the 48 h of culture.

TABLE I.—*Effect of reovirus 3 on 10-day rat embryo growth and development*

| Treatment (n) | Inoculum ($log_{10}$ $TCID_{50}$) | Crown-rump length, mm ± s.e. mean | No. of somites ± s.e. mean |
|---|---|---|---|
| Live virus (11) | 6·0 | 1·65 ± 0·11* | NC |
| Live virus (25) | 5·0 | 3·32 ± 0·11* | 29·8 ± 0·8*[a] |
| Heat-inactivated virus[b] (11) | < 1·0 | 4·34 ± 0·07 | 31·7 ± 0·7 |
| Diluent (27) | — | 4·16 ± 0·12 | 32·8 ± 0·6 |

NC, not counted, somites indistinct,. [a] n = 23, somites indistinct in 2 embryos. [b] 60° for 1 h. * $P < 0{\cdot}001$. ** $P < 0{\cdot}005$.



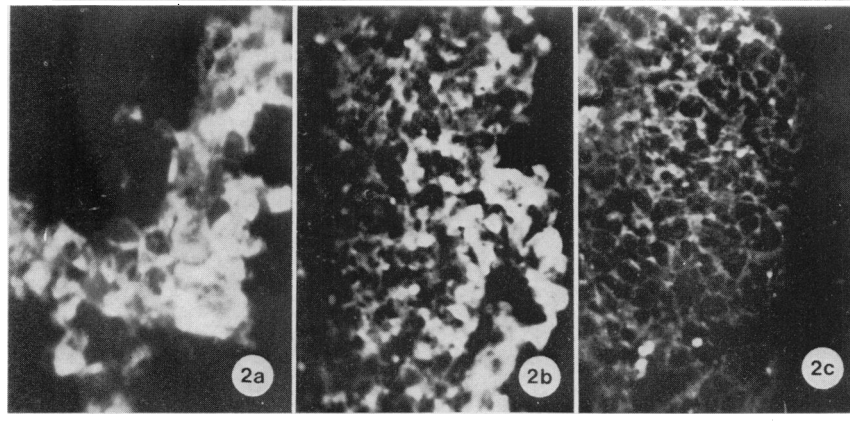




FIG. 1.—Gross appearance of uninfected (a) or reovirus 3 infected (b) embryos after 48 h culture from day 10 of gestation.

FIG. 2.—Immunofluorescent staining of cryostat sectioned conceptuses after infection with reovirus 3 at a concentration of $10^5$ $TCID_{50}$/ml; (a) infected yolk sac, (b) infected embryo, (d) uninfected embryo. Embryos were cultured for 48 h from day 10 of gestation.

TABLE II.—*Growth of reovirus 3 in 10-day rat embryos*

| Inoculum [a] | Pooled embryos (start) [a] | Pooled embryos (finish) [a] | $Log_{10}$ increase during culture |
|---|---|---|---|
| 5·8 | 6·4 | 7·7 | 1·3 |
| 5·3 | 5·2 | 7·3 | 2·1 |
| 5·1 | 4·5 | 6·9 | 2·4 |
| 4·3 | 4·3 | 6·5 | 2·2 |
| 3·5 | 3·0 | 5·4 | 2·4 |

[a] All virus concentrations expressed as $log_{10}$ $TCID_{50}$/ml.

## DISCUSSION

Many viruses have been implicated in the aetiology of reproductive failure but the mechanisms by which they damage the developing tissues are poorly understood. Animal experiments have been of value in studying the effects of maternal infection on embryonic development. However, the early embryo is relatively inaccessible, especially after implantation, and an imbalance in mater-

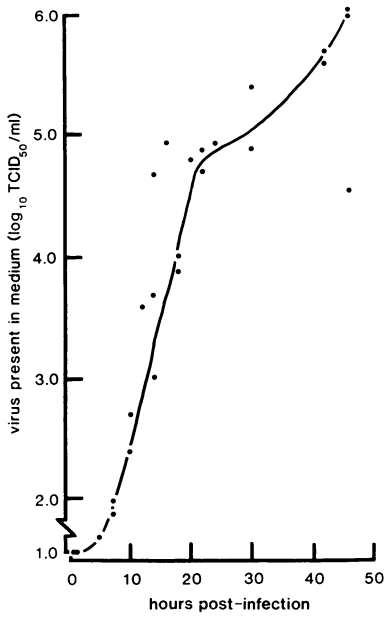



FIG. 3.—Graph of infectious virus released to the medium of cultures infected with $10^5$ $TCID_{50}$/ml at day 10 of gestation. Datum points are collected from 3 separate experiments.

nal physiology during infection has been shown to affect embryonic development (Coid and Wardman, 1972; Lansdown, Coid and Ramsden, 1975; Lansdown, 1977). In view of such limitations several *in vitro* approaches have been applied to study the direct effects of viruses upon embryonic tissues. These have included the culture of pre-implantation embryos and various tissues at different stages of development (reviewed by Heggie, 1979). The present study introduces the possibility of using cultured post-implantation embryos to study infectious embryopathy.

Reo 1 and 2 were the first to be associated with claims of teratogenicity in mice and although reo 1 was subsequently shown to infect mice and rats experimentally (Kilham and Margolis, 1969; Margolis and Kilham, 1969; Stanley, 1974), attempts to produce congenital disease have not been successful (Kilham and Margolis, 1974; Priscott, unpublished observation).

Reo 3 was selected for experiments with cultured embryos since it was known to naturally infect rodents (Kilham and Margolis, 1973). Infection of pregnant rats with reo 3 led to congenital infection but the embryos survived to birth and were grossly normal despite having histopathological lesions (Kilham and Margolis, 1973, 1974; Margolis and Kilham, 1973).

Infection of the cultured embryo with reo 3 resulted in a concentration-dependent effect that required live virus. At virus concentrations of $10^5$ $TCID_{50}$ a high proportion (0·75) of embryos survived to the end of the culture period and exhibited varying degrees of retardation. These results support the rat *in vivo* experiments. Higher virus concentrations were invariably lethal. Thus, it may be that the experimental protocol used in the mouse studies gave rise to a higher virus challenge with consequently greater effects on embryonic development (Hashimi *et al.*, 1966; Hassan and Cochran, 1966, 1969).

The virus titration and fluorescent-antibody studies confirmed that reo 3 replicated in the rat visceral yolk sac and embryonic tissue. Cell-associated virus increased by about $10^{2·3}$ $TCID_{50}$/ml, except at the highest virus inoculum concentration. This may have been the result of defective interfering particles in the inoculum (Nonoyama and Graham, 1970; Cole, 1975). Alternatively, the overwhelming infection of the embryo could have reduced the number of susceptible cells that were available for subsequent rounds of infection. The third possibility, that small amounts of interferon were present in the inocula from the original virus production, had interfered with the infection cycle seemed unlikely since interferon is species-specific in its effects (Baron, 1969) and stock virus was not grown in rat tissue.

Infectious virus was found to be released to the medium about 6–8 h after infection and increased exponentially throughout the culture period except for a check around 18 h. The reason for this

was not clear but may have resulted from a temporary lack of susceptible cells as all the yolk sac cells became infected, or a delay in the release of virus from infected embryonic cells since the embryo was enclosed by the intact yolk sac.

Reoviruses have not been implicated as causes of human intrauterine infection although there is no doubt that they can infect man (Rosen, 1968; Stanley, 1974). Serological surveys for reo 1 and 2 antibodies revealed a world-wide distribution (Taylor-Robinson, 1965; Brown and Taylor-Robinson, 1966). The incidence of adult individuals possessing antibodies was between 47 (England) and 100% (India and Uganda) for reo 1 and between 67 (Czechoslovakia, England, France, Italy and U.S.A.) and 100% (Australia, Chile, Jamaica, Lebanon and Malaya) for reo 2. Thus, the number of adults reaching child-bearing age that have not experienced reovirus infection is greater in the developed countries; for example 33–53% for reo 1 in the European population studied.

Another noteworthy feature of reovirus infection is that they are usually subclinical. Had it not been for the prominent exantham often seen during rubella virus infection, it is doubtful whether Gregg would have been able to make his classic association between rubella infection in pregnancy and certain types of birth defects (Gregg, 1941). More recently a third of rubella infections were shown to be sub-clinical (Fucillo and Sever, 1973) and in the case of the other principal human teratogen, cytomegalovirus, nearly all infections of the mother were clinically inapparent (Hanshaw and Dudgeon, 1978). Thus, reovirus has 3 of the characteristics of the 2 principal human teratogenic viruses, namely ability to invade embryonic tissues, a susceptible population at child-bearing age and largely sub-clinical infection.

The possibility that reoviruses might also infect the human foetus has been pointed out previously (Stanley, 1967; Kilham and Margolis, 1975) but, to date has not been included in any published prospective surveys into the possible infective cause of human developmental failure. Only 1 report has considered reovirus as a potential teratogen and this was a retrospective study conducted in Finland (Lapinleimu *et al.*, 1972). The experimental approach had several deficiencies which negated the conclusion that there was no association of reovirus infection with the malformations found. In particular, blood samples were obtained from mothers of malformed children approximately 2 months after delivery. This would be 6–9 months after the major period of organogenesis in the foetus had finished. If the virus was active in the community during that time the matched controls, who were mothers delivering normal infants immediately before the birth of the affected child at the same clinic, would be expected to have a similar incidence of antibodies.

Experimental animal models have shown that reoviruses infect the developing foetus where it may cause growth retardation and/or birth defects. The present study has demonstrated that the quantity of virus infecting the embryo is important in determining the outcome of the infection. It may therefore be postulated that individuals which develop a high circulating virus concentration during the viraemic phase or in which the placenta supports extensive viral replication would be at greater risk of reproductive failure in some form. However such information for the human infection is lacking and therefore the whole question as to whether reoviruses may be teratogenic or a cause of growth retardation in man remains open. It would thus seem prudent to include reoviruses in prospective surveys of human reproductive failure.

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