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Further studies on the growth of rubella virus in human embryonic organ cultures: preliminary observations on interferon production in these cultures

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SUMMARY

Organ cultures prepared from 15 different organs obtained from 43 fetuses were consistently found to support the growth of rubella virus, irrespective of the gestational age of the fetus or the strain of rubella virus inoculated. Although rubella virus replicated in fetal lenses, adult lenses did not support the growth of rubella virus. Organs obtained from four fetuses between 8 and 17 weeks gestational age produced similar titres of an inhibitor which had the characteristics of interferon. The use of Trowell T8 medium and incubation in a mixture of 5% CO_2 in oxygen provided the most suitable conditions for the maintenance of most organ cultures. Under these circumstances it was possible to obtain adequate histological preparations from these organs, but light microscopy studies revealed no significant differences in sections of rubella inoculated and control organ cultures.

INTRODUCTION

Many viruses which cause acute repiratory infections can be cultivated in human embryonic organ cultures, particularly trachea and nasal epithelium (Hoorn & Tyrrell, 1965). This system provides a convenient method for determining the properties of some viruses which can only be cultivated with difficulty in monolayer cell cultures (Tyrrell & Bynoe, 1965; Almeida & Tyrrell, 1967). We previously established that rubella virus could be propagated in embryonic organ cultures derived from the upper respiratory tract (Best, Banatvala & Moore, 1968) and showed that virus could be detected electron microscopically in ultra-thin sections prepared from these cultures (Kistler, Best, Banatvala & Töndury, 1967). This communication describes our investigations on the growth of rubella virus in numerous other organ cultures derived from fetuses of varying gestational age and includes preliminary studies on the production of interferon in organ cultures derived from four of these fetuses. In addition, we have investigated different conditions for maintaining various organ cultures in a satisfactory condition for histological studies.

MATERIALS AND METHODS

Organ cultures

Organs were obtained within 1–22 hr. of hysterotomy from 'healthy' human fetuses varying in age from 8 to 28 weeks gestation. Fifteen different organs were used in these studies: brain (cerebrum), heart, kidneys, lens, liver, lung, nasal epithelium, trachea, pharynx, larynx, retina, skin, spleen, adrenal and chorion. Considerable care was taken in the preparation of organ cultures since we were interested not only in producing the best possible conditions for histological studies by light microscopy but also in preserving the cellular ultrastructure in a satisfactory condition for electron microscopic studies, details of which will be published later. Organs were cut into pieces no larger than 2 $mm.^3$ using new razor blades which were discarded after three cuts. Fragments were handled with strips of filter paper in order to reduce mechanical damage to a minimum. The virus inoculum was allowed to adsorb for approximately 2 hr. at room temperature or 4° C. and was then replaced with sufficient medium to ensure that all the fragments in the dish were completely covered. The medium was changed every 1–2 days. Control cultures were inoculated with cell culture fluid from non-infected cell cultures.

Growth of rubella virus in fetal lenses was compared with that in adult lenses which were obtained from eyes removed as soon as possible after death.

The following media were used for the maintenance of organ cultures:

(1) Medium 199 (Wellcome Reagents) containing 0·088 % sodium bicarbonate and either 0·2 % bovine plasma albumin or fetal calf serum ranging between 2 and 50 %.

(2) Medium Trowell T8 (Difco Laboratories) containing 10 % fetal calf serum (Trowell, 1959).

(3) Leibovitz L15 medium (Flow Laboratories Ltd.) containing 2 % fetal calf serum and 0·029 % glutamine. This medium enabled cultures to be incubated in air without added CO_2.

(4) Since the aqueous humour of the adult lens contains a high concentration of ascorbic acid, medium NCTC 135 (Flow Laboratories Ltd.), a medium which contains a high concentration (49·9 mg./l.) of ascorbic acid, was used for the maintenance of lenses with the addition of 50 % fetal calf serum.

All media contained 200 units/ml. penicillin and 200 μg./ml. streptomycin.

Cultures were incubated at 36° C. in an atmosphere of 5 % CO_2 in air or 5 % CO_2 in oxygen.

Inoculation of rubella virus

Four strains of rubella virus were used for inoculation of organ cultures. Strain Judith, a well-adapted laboratory strain (McCarthy & Taylor-Robinson, 1965) was propagated in RK-13 and BHK-21 cell cultures. Strains Giguere and Thomas were isolated from infants with congenital rubella in 1964 and 1966 respectively, while Portsmouth was isolated in 1967 from a child with postnatally acquired

rubella. These three strains had been passed less than 10 times in RK-13 and BHK-21 cell cultures.

Organ cultures were inoculated either on the day of preparation or 1–2 days later. Virus samples used for inoculation varied in titre from 10 to $10^{7.5}$ TCD50/ml. Although organ cultures could be infected by inoculating as little as 0·2 ml. rubella virus, in most experiments the virus inoculum was sufficient to cover the organ fragments. The inoculum was allowed to adsorb at room temperature or 4° C. for periods varying from 2 to 8 hr., after which it was replaced with fresh medium.

Control cultures were included in each experiment and were either left uninoculated or were inoculated with an equal volume of fluid from non-infected cell cultures. In order to test the survival time of the inoculated virus, a virus control dish containing virus but no organ culture was included in some experiments.

Virus titrations

Organ culture fluids were harvested at intervals, 'snap' frozen and stored at −70° C. until they could be titrated in parallel in RK-13 cell cultures (Best & Banatvala, 1967).

Interferon assay

In order to determine whether infection by rubella virus resulted in interferon production, fluids from rubella inoculated cultures prepared from four different fetuses were harvested daily for up to 5 days. A sample was taken from each harvest for virus titration and the remainder was adjusted to pH 2 by dialysis against glycine buffer pH 2 for 48 hr., followed by dialysis against phosphate buffered saline (PBS), pH 7·2 for 24 hr. to restore the pH to neutrality. Interferon assays were carried out in cultures of a continuous line of vervet monkey kidney, V3A (K. H. Fantes, personal communication). Cultures were challenged with 100 TCD50/ml. Sindbis virus 18 hr. after the inoculation of twofold dilutions of organ culture fluid and results were recorded when the Sindbis controls showed 75–100% cytopathic effect. The titre of interferon was taken as that dilution giving 50% inhibition of cytopathic effect. A standard human interferon sample of known titre was included in each test.

Histological studies

Organ fragments were fixed in Bouin's fluid and embedded in paraffin wax for sectioning. Preparations were stained with haematoxylin and eosin.

RESULTS

Maintenance of organ cultures

Although trachea and nasal epithelium could be maintained in a satisfactory condition for at least 34 days in medium 199 containing 0·2% bovine plasma albumin or 2% fetal calf serum in an atmosphere of 5% CO_2 in air, most other organs became rather friable after 2–3 days incubation in this medium, although degeneration did not occur so quickly when higher concentrations (10–50%) of fetal calf serum were used.

Table 1. *The effect of fetal age on rubella virus titres*

Gestational age of fetus (weeks)	Highest rubella virus titre detected in the organ culture fluid*												
	Cerebrum	Heart	Kidney	Lens	Liver	Lung	Nasal epithelium	Pharynx	Retina	Skin	Spleen	Suprarenal	Trachea
8	1·5†	—	1·5	—	—	< 1·5	—	—	—	—	—	—	—
11	—	—	2·8	—	—	2·0	—	—	—	—	—	—	2·5
11½	—	—	3·5	2·3	—	3·5	—	—	—	—	2·5	—	2·2
12	1·8	< 1·5	3·2	—	1·5	2·0	—	—	—	—	—	—	2·5
12	—	—	2·7	2·5	3·0	2·5	—	—	—	—	2·2	—	—
14	—	2·5	—	1·8	—	2·5	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—	—	—	—	—	3·5
15	2·2	2·5	—	2·2	2·5	2·6	—	—	2·0	2·5	2·0	—	2·8
15	—	—	1·5	—	—	—	—	—	—	—	1·4	—	—
15	—	—	—	2·3	—	—	—	—	—	—	—	—	—
15½	—	1·5	1·6	1·5	—	1·3	—	—	—	1·5	2·5	—	< 1·0
16	—	—	—	—	—	—	3·3	—	—	—	—	—	2·4
16	—	—	—	—	—	—	1·5	—	—	—	—	—	1·0
16	—	1·8	1·3	—	—	2·5	—	—	—	< 1·5	—	1·5	—
16½	2·0	—	> 2·5	2·0	2·0	2·1	2·5	—	—	2·0	1·5	—	—
17	—	—	—	—	—	—	—	—	—	—	3·8	—	2·2
17	—	3·0	—	2·2	—	2·5	—	—	1·6	—	2·6	—	—
18	2·0	—	—	—	—	—	4·0	—	—	—	—	—	2·8
19	2·5	—	—	—	—	2·5	—	—	—	—	—	—	—
19	—	—	2·3	—	—	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	3·7	1·5	—	2·5	—	—	2·5

* Organ cultures were inoculated with 10^5–$10^{7\cdot5}$ TCD 50/ml. rubella virus. † $\log_{10}$ TCD 50/ml.

Use of Leibovitz L15 medium had the advantage that a CO_2 incubator was not necessary for incubation of cultures. Although cultures maintained in this medium continued to produce virus for at least 12 days they did not maintain in satisfactory condition for detailed histological studies.

Medium Trowell T8 containing at least 10% fetal calf serum and incubation in an atmosphere of 5% CO_2 in oxygen provided the most suitable conditions for the maintenance of organ cultures, since most organs remained in good histological condition for at least 7 days in this medium. However, both fetal and adult lenses became opaque in this medium but when they were incubated in medium NCTC 135 containing 50% fetal calf serum in an atmosphere of 5% CO_2 in air they could be maintained for at least 13 days without becoming opaque. Cultures of retina were also incubated in 5% CO_2 in air since it has been reported that high concentrations of oxygen result in oxygen poisoning of the retina (Lucas & Trowell, 1958).

Growth of rubella virus in different organs

Organ cultures prepared from 43 different fetuses obtained between 8 and 28 weeks gestation supported the growth of rubella virus when inoculated with $\geqslant$ 10 TCD50/ml. No rubella virus was recovered in one experiment in which tracheal and spleen organ cultures prepared from a $14\frac{1}{2}$-week fetus were inoculated with 10^5 TCD50/ml. strain Thomas.

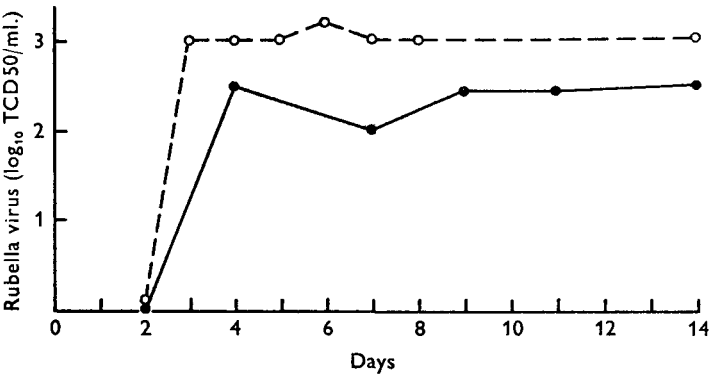

Fig. 1. Two typical growth curves showing the multiplication of rubella virus in organ cultures of spleen from two fetuses aged 16 and 17 weeks.

All 15 organs so far tested supported the growth of rubella virus. Table 1 shows that titres of between $10^{1.5}$ and 10^3 TCD50/ml. were usually obtained irrespective of the gestational age of the fetus. Experiments in which a virus inoculum of $< 10^5$ TCD50/ml. was used are not included in this table. The well-adapted laboratory strain Judith was employed in the majority of experiments but when Portsmouth, Giguere and Thomas, the low passage strains, were used similar titres were obtained. The highest titre obtained was 10^4 TCD50/ml. from cultures of nasal epithelium from an 18-week fetus. The variations in the titre of rubella virus recovered may have been influenced by variation in size of organ fragments

placed in each dish. Some low titres may also be explained by the fact that although some cultures appeared macroscopically to be in good condition, when examined microscopically they were found to have undergone considerable degeneration.

Typical growth curves obtained from organ cultures of spleen are illustrated in Fig. 1. The titre of virus in the surrounding fluid was $10^{2\cdot5} - 10^{3}$ TCD50/ml. on day 3 and was maintained at approximately this level until experiments were discontinued on day 14. Most organs continued to produce similar titres of virus as long as the organ fragments remained in a healthy condition. Since it was found

Table 2. *Rubella infected organ cultures from fetus aged* 15 *weeks.* 10^{6} *TCD*50*/ml. virus* (*Judith*) *inoculated*

Organ	Infected cultures Titre ($\log_{10}$ TCD 50/ml.) Day 4	Day 7	Condition of inoculated and control cultures
Trachea	2·8	2·2	Good
Lung	2·6	2·6	Good
Liver	2·5	2·0	Good
Cerebrum	2·2	1·5	Slightly friable
Spleen	2·0	1·6	Good
Heart	2·5	2·5	Good
Skin	2·6	2·5	Good
Lens	2·2	1·8	Clear
Retina	1·0	2·0	Good

No virus was recovered from control cultures.

that low titres of rubella virus could be recovered from virus control dishes inoculated with approximately 10^{6} TCD50/ml. for 2 days after inoculation, organ culture fluids from days 1 and 2 were not titrated. Results of a typical experiment using fragments of 9 different organs obtained from a 15-week fetus and maintained in medium Trowell T8 are shown in Table 2.

Growth of rubella virus in the lens

Although rubella virus was found to multiply consistently in 13 lenses obtained from fetuses of $11\frac{1}{2}$ to 19 weeks gestation, no rubella virus was recovered from five adult lenses inoculated and maintained under identical conditions.

Interferon production

All organs tested from four fetuses obtained between 8 and 17 weeks gestation produced an interferon-like inhibitor in the organ culture fluid which was detected within 24 hr. of inoculating rubella virus. However, by day 3, when rubella virus titres began to increase, the amount of inhibitor decreased, being generally undetectable 4 days after virus inoculation (Table 3). No inhibitor was detected in control cultures. Increasing gestational age did not appear to be associated with an increased capacity to produce interferon; the amount of interferon present in organ culture fluid in response to infection by rubella virus was low in all four fetuses studied ($\leqslant$ 8 units/ml.).

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This inhibitor had the following characteristics which strongly suggested that it was interferon:

(1) It was non-dialysable.

(2) It was stable at pH 2.

(3) Its activity was destroyed by treatment with 0·1 % trypsin.

(4) It was inactivated by heating for 1 hr. at 56° C.

(5) It was not sedimented by centrifugation at 100,000 *g* for 1 hr.

(6) The inhibitor was not neutralized when a sample was incubated with rubella antiserum, thus excluding the possibility that the inhibitory effect was due to interference by residual rubella virus.

(7) It appeared to be species specific since it did not inhibit the growth of Sindbis virus in chick embryo fibroblasts.

Table 3. *Production of interferon by organ cultures*

Organ culture	Age of foetus in weeks	Inoculum	Interferon titre, units/ml. Day 1	2	3	4	5
Cerebrum	8	Judith	4	2	2	—	—
Kidney	—	10^7 TCD 50/ml.	4	3	< 2	—	—
Lung	—		4	4	3	—	—
		Control cultures	< 2	—	—	—	—
Spleen	16	Judith	8*	—	—	—	—
		$10^{4·3}$ TCD 50/ml.	—	—	—	—	—
		Control cultures	< 2*	—	—	—	—
Cerebrum	16½	Judith	4	8	8	< 2	—
Kidney		10^5 TCD 50/ml.	8	8	4	< 2	< 2
Lens			4	< 2	< 2	< 2	—
Liver			< 2	8	< 2	< 2	—
Lung			< 2	4	< 2	< 2	—
Nasal epithelium			< 2	8	4	< 2	—
Skin			4	4	2	< 2	< 2
Spleen			8	4	< 2	< 2	< 2
		Control cultures	< 2	< 2	—	—	—
Spleen	17	Judith	4(8)†	—	—	—	—
		10^6 TCD 50/ml.	< 4(8)	—	—	—	—
		Control cultures	< 4	—	—	—	—

* Harvests from days 1, 2 and 3 were pooled.

† Figures in parentheses show the intracellular interferon titre. Organ fragments were ground up and an approximate 4 % suspension prepared. After centrifugation at 2000 rev./min. for 10 min. the supernatant was tested for interferon.

Histological studies

Using medium 199 and Leibovitz L15 medium the condition of all organs except trachea and nasal epithelium was generally too poor for detailed histological examination, but considerable improvement was seen when smaller (< 2 mm.^{3}) pieces of each organ were maintained in T8 medium containing 10 % fetal calf serum in an atmosphere of 5 % CO_2 in oxygen, provided the medium was changed frequently (Table 2). When organ culture fragments maintained in this medium

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were examined 7 days after inoculation no gross histological changes or inclusion bodies were observed, although rubella virus was present at titres up to 10^4 TCD50/ml. in the surrounding fluid. Extensive vacuolation and capsular hyperplasia were seen in some rubella virus-infected fetal lenses, but these changes may have been artefacts since similar but less extensive effects were noted in some controls. Satisfactory sections could not be obtained from adult lenses, which were hard and difficult to cut.

DISCUSSION

In previous studies employing organ cultures of trachea and nasal epithelium, we found that both control and rubella virus-infected cultures could be maintained for at least 34 days without loss of ciliary activity. Histological examination showed that the epithelial surface was intact and cilia were present on both control and rubella virus-infected cultures. No degenerative changes, ulceration or inclusion bodies were present (Best, Banatvala & Moore, 1968).

Studies reported in this paper show that many other human embryonic organ cultures will support virus multiplication and may be maintained in a suitable condition for histological examination provided care is taken to ensure that small fragments are used and that these are maintained in a specialized medium, such as T8. Trowell (1959) originally recommended this synthetic medium for maintaining cultures derived from different organs of adult rats. Although it contains fewer total constituents than medium 199 this medium incorporates thymine and extra cysteine. Because the concentration of oxygen is likely to be at its lowest in the centre of organ fragments it is important to ensure that fragments are no larger than 2 mm.^{3} in size and since the solubility of oxygen in biological media is low, Trowell recommended the use of 5% CO_2 in oxygen rather than in air in order to achieve higher oxygen concentrations.

Although no histological changes were evident, rubella virus replicated consistently in fetal but not in adult lenses. That adult lenses were refractory to infection is consistent with the finding that lens lesions have not been reported following postnatally acquired infection. This may be because rubella virus cannot penetrate or infect the adult lens capsule or perhaps because adult lens cells will not themselves support the growth of rubella virus. We previously demonstrated rubella virus particles and osmiophilic inclusion bodies containing vesicular structures and 'myelin whorls' in ultra-thin sections of human embryonic trachea and nasal epithelium (Kistler *et al.* 1967). Preparations of all other organs except lens were satifactory for electron microscopy when they had been maintained in medium Trowell T8 containing at least 10% fetal calf serum. Lenses could be maintained in a satisfactory condition in medium NCTC 135 containing 50% fetal calf serum (Kistler, Best & Banatvala, unpublished observations).

Our experiments have shown that rubella virus replicates consistently *in vitro* in human embryonic organs of widely differing gestational age. This is consistent with recent reports that, after laboratory confirmed maternal rubella during the first trimester, if sensitive techniques for virus detection are used, rubella virus can be recovered from 92 to 95% of fetuses (Rawls, Desmyter & Melnick, 1968;

Thompson & Tobin, 1970) and also with reports that even if acquired after this time fetal infection may occur. However, in such cases developmental anomalies are usually more subtle than if infection is acquired earlier and may not be detected unless infants are followed-up for a prolonged period (Hardy, McCracken, Gilkeson & Sever, 1969). Even though maternal infection during the first trimester almost invariably results in fetal infection, it is unlikely that, if allowed to proceed to term, all infected fetuses would be affected adversely. Thus, the fetus may be capable of limiting or terminating infection and it has been suggested that interferon may be involved in this process (Mims, 1968; Rawls, 1968). However, there have been few studies on human fetal interferon production, although it has been shown that young chick and mouse embryo cells produce less interferon than older ones (Isaacs & Baron, 1960; Sawicki, 1961; Heineberg, Gold & Robbins, 1964). Our studies suggest that the fetus is capable of producing from an early gestational age a substance which has the physical properties of interferon, but this response does not increase as the fetus matures. The time sequence of its development closely paralleled that of interferon induced in calf trachea organ cultures infected with para-influenza and rhinoviruses (Smorodintsev, 1968).

Cantell, Strander, Saxen & Meyer, (1968) studied interferon responses of human lymphocytes obtained from fetuses, newborns, children and adults infected with Sendai virus and also demonstrated that, although competent at an early gestational age, the amount of interferon produced was relatively constant during intrauterine as well as postnatal life. In our studies only low interferon titres were produced by rubella virus, but it is likely that rubella is a poor interferon inducer in fetal cells, this being supported by our finding that in comparison to such viruses as the para-influenza viruses and some rhinoviruses rubella produces only small amounts of interferon in human embryonic lymphocytes (Banatvala & Bowern, unpublished observations). A persistent fetal infection frequently follows maternal infection during the first trimester. Although this may perhaps be in part because rubella virus is a relatively poor interferon inducer, Siewers, John & Medearis (1970) showed that young human fetal fibroblastic cell lines between the 10th and 50th passages infected with Sindbis and vesicular stomatitis viruses were less sensitive to the action of interferon than cells derived from older fetuses. We hope to determine the interferon sensitivity of primary fetal cell cultures of varying gestational age infected with rubella virus.

Our investigations have shown that human embryonic organ cultures provide a useful system for studying some aspects of the pathogenesis of fetal infection by rubella. They may provide a useful experimental system for further studies on the distribution and spread of virus in different organs and also its susceptibility to inhibitory and chemotherapeutic agents. Furthermore, for studies on the pathogenesis of infection, cells maintained as organ cultures have the additional advantage that, unlike those in monolayer cell cultures, they are similar in structure and physiology to those in the intact human host.

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