J. Hyg., Camb. (1968), **66**, 407
Printed in Great Britain

Growth of rubella virus in human embryonic organ cultures

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(*Received* 3 *February* 1968)

INTRODUCTION

Organ cultures of foetal trachea and nasal epithelium have been used for the growth of viruses which cause acute respiratory infection (Hoorn & Tyrrell, 1965; Tyrrell & Bynoe, 1965, 1966; MacIntosh *et al.* 1967). This technique has been applied to the growth of rubella virus, since this virus can be isolated from the nasopharynx of both patients with rubella and infants with the rubella syndrome.

MATERIALS AND METHODS

Virus

Two strains of rubella virus were used, Judith (McCarthy & Taylor-Robinson, 1965) and Thomas, the latter having been isolated at St Thomas's Hospital in 1966 from an infant with congenital rubella. Judith was propagated in both RK 13 and BHK-21 cell cultures. Since its original isolation, Thomas had been passed eight times in RK 13 and BHK-21 cell cultures. Titres of virus used for inoculation varied from < 10 TCD 50/ml. to $10^{6.5}$ TCD 50/ml.

Organ cultures

Embryonic trachea, nasal mucosa and, on some occasions, pharynx, larynx, skin and brain were obtained from foetuses aged 15–28 weeks. These were dissected within 4–22 hr. of hysterotomy according to the method of Hoorn & Tyrrell (1965). Two or three pieces of trachea or one piece of nasal epithelium were placed in each Petri dish (Nunc brand) with 1·25 ml. medium for trachea and skin cultures and 2–3 ml. for nasal epithelium, pharynx, larynx and brain. The pieces of pharynx, larynx, skin and brain were cut into pieces 5–10 mm square.

Initially medium consisted of Medium 199 (Burroughs Wellcome and Co.), containing sodium bicarbonate 0·088 %, bovine plasma albumin 0·2 %, penicillin 200 units/ml., and streptomycin 200 μg/ml. Cultures were incubated at 36° C. in an atmosphere of 4 % CO_2 in air. In later experiments Leibovitz L 15 medium (Flow Laboratories Ltd.) containing foetal calf serum 2 %, glutamine 0·029 %, penicillin 200 units/ml. and streptomycin 200 μg./ml. was found to be satisfactory, since cultures could be incubated in a humidified box without added CO_2.

Organ cultures were inoculated 1–3 days after preparation. After removing medium, virus was dropped on to the pieces of organ culture. The maximum volume of virus inoculated was equal to the volume of medium initially in the Petri dish. Cultures were then replaced at 36° C. for approximately 6 hr. to allow adsorption of virus, after which the medium was changed. Control cultures were

either left uninoculated or were inoculated with an equal volume of tissue culture fluid which contained no virus. In some experiments a virus-control Petri dish was included containing virus, but no organ cultures. The inoculum in this dish was left unchanged when we wished to test the survival time of the inoculated virus.

In growth curve experiments the medium from each of the Petri dishes was harvested daily, 'snap' frozen and stored at −70° C., after which fresh medium was added. During other experiments the medium was changed at intervals of 2 or 3 days. Cultures were examined daily for ciliary activity.

Virus titrations

The harvested fluids from each experiment including those from all control cultures were titrated in parallel in RK 13 cell cultures (Best & Banatvala, 1967). In order to detect the presence of intracellular virus, cultures of trachea and nasal epithelium were inoculated with rubella virus, uninoculated controls being included in the test. Cultures of trachea were tested on days 4 and 12, and cultures of nasal epithelium on days 3 and 11; the trachea and nasal epithelium were obtained from different foetuses in these experiments. The pieces of tissue were weighed, washed twice with PBS (Dulbecco Solution A), ground with a pestle and mortar and a 1% (w/v) suspension prepared. This was then sonicated for 2 min. using an MSE ultrasonic unit. These suspensions were stored at −70° C. until they could be titrated.

Histological studies

Pieces of tissue were fixed in Bouin's fluid and embedded in paraffin wax 13 days after inoculation. Sections were stained with haematoxylin and eosin and were examined for structural changes.

RESULTS

Twenty-seven foetuses were used for the preparation of trachea and nasal epithelium organ cultures. Pharynx was used in three experiments, larynx in two, and both skin and brain in one experiment only. Material from two foetuses was discarded because of contamination and no virus was recovered from a further seven, but it was shown subsequently that insufficient virus had been inoculated. Organ cultures from the remaining eighteen foetuses supported the growth of rubella virus.

Titration of tracheal organ culture fluids from two typical experiments produced the growth curves shown in Fig. 1. No virus was detected in the organ culture fluids for 48 hr. after inoculation, after which virus titres rose from $10^{1.5}$ to $10^{2.2}$/ml., being maintained at approximately this level until the experiments were discontinued on day 11 and day 12. However, further experiments revealed that titres could be maintained at this level for at least 34 days following inoculation. Virus was not isolated from uninoculated control cultures, but some virus was recovered from the virus control dishes during the first 48 hr. after inoculation when the medium was not changed at 6 hr.

Table 1 shows that intracellular and organ-culture fluid virus titres were similar in both trachea and nasal epithelium when tested on two occasions.

Table 2 shows details of organ-culture experiments in which high titres were obtained in the organ culture fluid. Virus titres obtained in organ culture fluids tended to vary with the titre of virus in the original inoculum. The highest titre was obtained in nasal epithelium on day 9 in Expt. 22 ($10^{3\cdot7}$TCD 50/ml.), when 2·5 ml. of virus at a titre of $10^{6\cdot5}$/ml. was inoculated. Although virus of a similar titre was inoculated in Expt. 12, it failed to yield as high a titre; however, only

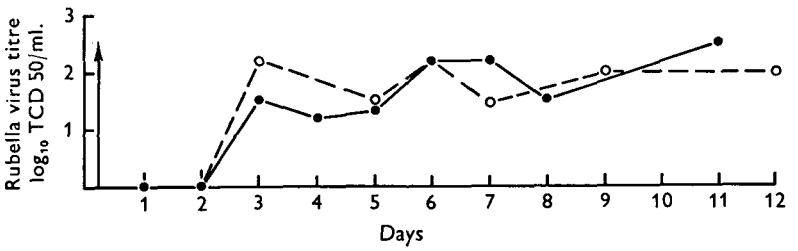

Fig. 1. Two typical growth-curve experiments, showing the multiplication of rubella virus in organ cultures of human embryonic trachea. The arrow indicates the titre of virus inoculated.

Table 1. *Comparison of intracellular and organ-culture fluid virus titres*

	Days after inoculation	Titre of rubella virus ($\log_{10}$TCD 50/ml.) Fluid	Tissue*
Trachea	4	1·2	1·2
	12	2·2	2·5
Nasal	3	1·7	2·2
	18	2·2	2·7

* 1 % (w/v) suspension prepared from organ culture tissue.

1·0 ml. of tissue culture fluid was inoculated and this was allowed to adsorb for only 3 hr. No virus was recovered in experiments in which < 10TCD 50/ml. of virus was inoculated. Rubella virus usually grew as well in pharynx, larynx and skin as in trachea and nasal epithelium. The only experiment in which all these organs were used from the same foetus produced the virus titres shown in Table 3. In the only experiment in which brain was included, rubella virus at a titre of $10^{1\cdot2}$TCD 50/ml. was recovered on day 8.

Rubella virus could be passed successfully in organ cultures. After two passages, Judith produced a titre of 10^3/ml. (Expt. 13), but this did not increase after two further passages (Expt. 18). Thomas was passed twice to produce a final titre of $10^{2\cdot4}$/ml.

Ciliary activity was maintained throughout in inoculated cultures of trachea, nasal epithelium, pharynx and larynx in all experiments. Histological studies

Table 2. *Details of organ-culture experiments in which high titres of rubella virus were obtained*

Expt. no.	Age of foetus (weeks)	Virus inoculum: Strain of virus	Virus inoculum: Titre (log_{10}TCD 50/ml.)	Virus inoculum: Volume inoculated: Trachea	Virus inoculum: Volume inoculated: NE*	Highest titre in organ culture fluid: Trachea: Titre (log_{10}TCD 50/ml.)	Highest titre in organ culture fluid: Trachea: Day obtained	Highest titre in organ culture fluid: Nasal epithelium: Titre (log_{10}TCD 50/ml.)	Highest titre in organ culture fluid: Nasal epithelium: Day obtained
				1st passage					
12	22	Judith (BHK)	6·0	1	1	1·5	4 and 23	1·4	4 and 23
19	16	Thomas (BHK)	5·1	1·25	2·5	2·4	11	3·3	11
22	23	Judith (BHK)	6·5	1·25	2·5	2·5	12	3·7	9
				2nd passage					
13	18	Judith (o.c. fluid)	2·0	0·6	0·75	3	21–27	1·4	14–27
20	17	Thomas (o.c. fluid)	2·0	1·25	2	< 1·0	3–13	2·4	13
				3rd passage					
16†	16	Judith (o.c. fluid)	3·0	0·4	0·4	3	9	2·4	9
				4th passage					
18	17	Judith (o.c. fluid)	1·5	1·25	2·5	3	18	2·4	34

* Nasal epithelium. † Larynx used instead of nasal epithelium in this experiment.

revealed no differences between control and inoculated sections of organ cultures. Cilia were clearly seen to be present and no degenerative changes or inclusion bodies were seen.

Table 3. *Results of organ culture fluid titrations using cultures derived from the same foetus*

Days after inoculation	Titre of rubella virus. $\log_{10}$TCD 50/ml.			
	Trachea	Nasal epithelium	Pharynx	Skin
5	1·5	3·4	1·5	2·5
7	0·5	3·4	0·5	2·2
9	0·5	3·7	0·5	2·2
12	2·5	3·4	—*	1·0

* Not tested.

DISCUSSION

These *in vitro* experiments show that rubella virus replicates consistently in human tissue derived from embryonic trachea and nasal mucosa and can persist in these cultures for up to 34 days without apparent damage to the ciliated epithelium. This contrasts with the effects of some viruses causing acute respiratory infection, which destroy the cilia and damage the epithelial surface (Tyrrell & Hoorn, 1966). However, electron-microscopic studies conducted on sections stained with osmium tetroxide derived from some of our organ cultures showed inclusion bodies containing vesicular structures 400–1200 Å in diameter in the mucous secreting cells and virus-like particles at the cell surface (Kistler, Best, Banatvala & Töndury, 1967). These particles were 400–700 Å in diameter and were morphologically similar to those described in other recent reports on the structure of rubella virus (Best *et al.* 1967; Holmes & Warburton, 1967).

Although congenital malformation is associated with maternal infection by rubella virus during the first trimester of pregnancy (Gregg, 1941; Manson, Logan & Loy, 1960; Lundström, 1952, 1962), these experiments show that organs from foetuses aged 15–28 weeks are capable of supporting the growth of rubella virus *in vitro*. It was not possible to study the features of infection in organ cultures derived from foetuses aged less than 15 weeks, since these are not generally delivered by hysterotomy. Whether the placenta forms an effective barrier to virus entering the foetal circulation after the first trimester or whether foetal infection occurs without causing any persisting damage has not been clearly determined. That virus may cross the placenta in late pregnancy is supported by a report of a case in which rubella occurred during the last week of pregnancy. An apparently healthy infant was born at term but virus was isolated from his blood on the day of delivery, and from the pharynx 3 days later (Banatvala *et al.* 1965).

Although a limited number of experiments were conducted with embryonic organs other than trachea and nasal epithelium, the replication of virus in pharynx, larynx, skin and brain suggests that many foetal organs will support the growth of

rubella virus *in vitro*. In addition growth of rubella virus in organ cultures of rabbit and hamster lung has been reported by Oxford & Schild (1967). These findings are consistent with the recovery of virus from a variety of different tissues derived from infants with the rubella syndrome (American Pediatric Society and Society for Pediatric Research, 1965). It was not possible to conduct histological studies on organ cultures other than trachea and nasal epithelium in this series, but it is hoped to conduct further studies on these and additional infected organs.

The only other report of the multiplication of rubella virus in human organ culture is by Albanese & Romano (1967), who infected human embryonic and rabbit trachea with the Judith strain of rubella virus and reported loss of ciliary activity and degenerative changes in the epithelium, loss of ciliary activity beginning 24 hr. or less after inoculation. However, they recovered virus from organ culture fluids on one occasion only, and failed to recover virus from both organ culture tissue and the remaining daily harvests of fluid. Using a similar or greater virus inoculum we have consistently been able to recover virus from the organ culture fluid and tissues, without observing any loss of ciliary activity. Oxford & Schild (1967) showed that ferret trachea would support the growth of rubella virus without damage to the cilia or epithelial structure.

Since rubella virus has been shown to replicate successfully in organ cultures of human embryonic tissues, this may provide a convenient experimental system for studying its pathogenesis, as the cells in these cultures unlike cells found in monolayer cell cultures are similar in structure and physiology to those of the intact human host.

SUMMARY

Two strains of rubella virus multiplied in organ cultures of human embryonic trachea, nasal epithelium, pharynx, larynx, skin and brain derived from foetuses aged 15–28 weeks. Growth curve experiments conducted on cultures of nasal epithelium and trachea showed that virus appeared in the culture fluid 72 hr. after inoculation and thereafter rose to titres varying from 10 to $10^{3 \cdot 7}$TCD 50/ml. These titres persisted for periods up to 34 days after inoculation. Intracellular and organ culture fluid virus titres were shown to be similar in specimens tested in both the early and late stages of the growth curve. No degenerative changes or loss of ciliary activity was observed in these cultures.

We are grateful to Dr H. E. M. Kay and his staff (Royal Marsden Hospital), for the supply of human embryos; Dr D. A. J. Tyrrell (M.R.C. Common Cold Research Unit, Salisbury) for his encouragement, and Miss G. E. Fairbairn (St Thomas's Hospital) for the histological preparations.

This research was supported by a grant from the Medical Research Council.

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