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A comparison of RK13, vervet monkey kidney and patas monkey kidney cell cultures for the isolation of rubella virus

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INTRODUCTION

Clinicians have become increasingly concerned with the public health hazard of rubella because of the relationship between maternal rubella in early pregnancy and subsequent congenital malformation (Gregg, 1941). Laboratory techniques are now available to assist clinicians in their diagnosis. Studies on the immunological status of patients are of limited use, but direct demonstration of excretion of virus is particularly valuable in the investigation of maternal rubella and newborn infants with the rubella syndrome (Plotkin, 1964).

Different workers favour different methods for virus isolation. The interference technique employing primary vervet monkey kidney cultures (Parkman, Buescher & Artenstein, 1962) is generally used for routine purposes in the U.S.A., where such cultures are readily available commercially. In Britain, where these cultures are more difficult to obtain, most workers employ a continuous rabbit kidney cell culture line, RK 13 (Beale, Christofinis & Furminger, 1963), the presence of rubella virus (RV) being detected by the appearance of a characteristic cytopathic effect (McCarthy, Taylor-Robinson & Pillinger, 1963). Patas monkey kidney cell cultures have been shown to support the growth of RV (M. Butler, personal communication), but studies on their use for virus isolation from clinical specimens have not been reported.

This paper presents the results of a comparison of the sensitivity and reliability of different cell cultures for the isolation of RV from clinical specimens. These were tested simultaneously under identical conditions.

MATERIALS AND METHODS

Tissue culture

We compared RK 13, primary vervet monkey kidney (VMK) and patas monkey kidney (PMK) cell cultures in parallel. A limited number of specimens were also tested on a continuous line of rabbit kidney, LLC-RK 1 (Hull & Butorac, 1966). Although this line has recently been shown to be sensitive to RV and its cytopathic effect (CPE) is easy to detect, its use for RV isolations from clinical specimens has not been reported.

Primary VMK and PMK cell suspensions ready for dispensing into tubes were

supplied by the M.R.C. Hampstead, and Wellcome Research Laboratories respectively. These cell cultures were generally confluent in 5–6 days. The RK 13 cell cultures were subcultured weekly, using a trypsin (0·125 %)/versene (0·05 %) mixture. Confluent cell sheets were obtained within 2–3 days. The trypsin/versene mixture was also used for subculturing the LLC–RK 1 cell cultures. These cell cultures required at least 7 days and 1–2 changes of medium before becoming confluent.

Table 1

Tissue culture system	Approximate no. cells inoculated per tube	Growth medium	Maintenance medium
VMK	15,000–20,000 clumps in 1 ml. amounts	Hanks's LaH Calf serum 5 % Glucose 1 % Sodium bicarbonate 0·088 % SV 5 antiserum 0·2 %	Earle's LaH Foetal calf serum 2 % Sodium bicarbonate 0·2 % SV 5 antiserum 0·2 %
PMK	125,000 cells in 1 ml. amounts	Earle's LaH Calf serum 2·5 % Sodium bicarbonate 0·1 % SV 5 antiserum 0·2 %	As for VMK
RK 13	100,000 cells in 0·5 ml. amounts	Medium 199 Calf serum (Flow Laboratories) 5 % Sodium bicarbonate 0·1 %	Medium 199 Inactivated rabbit serum 4 % Sodium bicarbonate 0·132 %
LLC-RK_1	100,000 cells in 1 ml. amounts	Medium 199 Calf serum 5 % Foetal calf serum 5 % Sodium bicarbonate 0·1 %	Medium 199 Foetal calf serum 2 % Sodium bicarbonate 0·154 %

Antibiotics, penicillin 200 units/ml. streptomycin 200 μg/ml. were included in all media.

The media required for growth and maintenance of the four types of cell culture are shown in Table 1. All cultures were incubated in stationary racks at 36° C. until ready for use.

The addition of amphotericin B (1·6 μg/ml.) to RK 13 cultures occasionally produced vacuolation, and its use was therefore unsatisfactory. Mycostatin (30 units/ml.) provided a satisfactory alternative antifungal agent. M & B 938 has also been found to be a satisfactory alternative (M. Butler, personal communication).

Initially, 3 lines of continuous vervet monkey kidney cell cultures which were reported suitable for the recovery of RV were also included in the investigation; GLV 3 (Christofinis, personal communication), BSC-1 (Parkman, Buescher *et al.* 1964), and GMK-AH 1 (Günalp, 1965). Although these lines supported the growth of RV, we found them insensitive to small amounts of virus such as might be obtained in clinical specimens. These continuous vervet cultures were therefore excluded from the investigation.

Specimens tested

Pharyngeal swabs were obtained from thirteen patients with rubella at St Thomas's Hospital. Two swabs were simultaneously obtained from each patient and immediately immersed in a bottle containing 4–5 ml. Hanks's BSS containing bovine plasma albumen 0·5 %, penicillin 500 units/ml., streptomycin 500 μg/ml., amphotericin B 8 μg/ml. Two swabs were obtained from each patient in order to inoculate as much virus as possible on to the cell sheet. In addition, specimens consisting of early passage tissue culture fluid from either RK 13 or VMK cell cultures were obtained from other laboratories. All material was 'snap' frozen and stored at $-70°$ C. until tested.

Inoculation of specimens

Whenever possible, all three tissue culture systems were inoculated in parallel with 0·1 ml. of the specimen undiluted and at 10^{-1} and 10^{-2} dilutions. Three tubes per dilution were used for RK 13 and four tubes per dilution for VMK and PMK; all specimens were adsorbed for 1 hr. at room temperature. Better adsorption of virus was found to occur if specimens were inoculated directly onto the cell sheet rather than onto cell cultures on maintenance medium. The process appeared to be more effective at room temperature than at 37° C., at which temperature some inactivation of virus may have taken place. All tubes were thereafter rolled at 36° C. Medium was changed on the RK 13 cultures after 18–24 hr. incubation in order to eliminate some of the swab or cellular material found to cause non-specific effects.

Control titrations

A control titration of a pool of RV (Judith) was included with each test, virus being inoculated in parallel in each cell culture system at virus dilution 10^{-1} to 10^{-7}. Virus titres were estimated according to the method described by Reed & Muench (1938).

Identification of virus

VMK and PMK cell cultures were challenged on the 10th day, and checked for the presence of interference 3 days later. Echovirus type 11 (100–1000 TCD 50/ml.) was used to challenge VMK cell cultures. Since echovirus 11 produces a poor CPE in PMK cell cultures, a bovine enterovirus, M 6 (100–1000 TCD 50/ml.), was used to challenge these cell cultures (M. Butler, personal communication).

RK 13 and LLC-RK 1 cell cultures were examined daily for 10 days. If CPE occurred following primary inoculation, cells and fluid were passed into fresh cultures to confirm the presence of CPE, since non-specific effects were invariably encountered when original specimens were inoculated. These effects may be confused with CPE. In all cell culture systems, two further passages following primary inoculation were carried out before specimens were declared negative.

Neutralization tests to confirm the identity of viruses were performed, employing sera from rabbits that had received six intravenous injections at weekly intervals of RV (Judith).

Serum neutralization tests

Neutralization tests on sera from seven patients were carried out simultaneously in RK 13 and PMK cell cultures. RV was used at 100 TCD 50 in RK 13, and 50 InD 50 (50% interference dose) in PMK. PMK cell cultures were challenged on the tenth day after inoculation. Sera were not inactivated. The serum-virus mixtures were incubated at 37° C. before inoculation (Dudgeon, Butler & Plotkin, 1964; Parkman, Mundon, McCown & Buescher, 1964). Serum titres were estimated by the method of Reed & Muench (1938).

RESULTS

Sensitivity

The number of isolations from throat swab fluid, together with the number of passes and time taken to give an unequivocal result are shown in Table 2. Although CPE could be detected in RK 13 cell cultures as early as 3–4 days after inoculation of specimens, a passage in fresh cultures was always necessary in order to eliminate non-specific effects. Despite this, RV was detected both more quickly and more frequently in RK 13 than in VMK or PMK cell cultures. Furthermore, titration of original throat swab fluid revealed a titre of 10 or greater, in five of thirteen specimens tested in RK 13, but in only one of the twelve tested in both VMK and PMK cell cultures. Although VMK cultures appeared more sensitive overall than PMK cell cultures, these two cell culture systems were almost equally sensitive on primary inoculation.

Table 2. *Efficiency of isolation*

	cell system		
	RK 13	VMK	PMK
Number of isolations on primary inoculation	10	5	6
Number of isolations on first pass	2	5	1
Number of isolations on second pass	—	1	2
Total number of isolations/number tested	12/13	11/12	9/12
Mean time in days required to give an unequivocal result	10*	21	23

* This includes time to confirm CPE.

In order to determine which culture system was most effective in detecting small amounts of virus, a number of specimens known to contain low titres, including harvests from organ culture experiments in progress, were titrated in parallel. Table 3 shows that the highest titres and highest isolation rates were obtained in RK 13. Although RK 13 cell cultures were the most sensitive in the above tests, virus that had been originally isolated in VMK in other laboratories, when titrated in parallel gave higher titres in VMK and PMK than in RK 13 (Table 4). This difference was, however, not observed in the high passage strain, R 12 and the attenuated vaccine strain, HPV 77 (Parkman, Meyer, Kirschstein & Hopps, 1966).

A series of specimens, including some clinical specimens previously found to be negative, were passed in parallel in LLC-RK 1 and RK 13. Higher titres were

consistently obtained in LLC-RK 1. In addition three specimens which previously yielded no virus in RK 13 were found to be positive in LLC-RK 1 (Table 5).

Reliability

Fig. 1 shows that the titre of our standard pool of virus varied less in RK 13 than in either VMK or PMK cell cultures. It was found that poor titres of standard

Table 3. *Ability to detect small amounts of virus*

Specimens	Cell system Titre of virus (log_{10} TCD 50 or InD 50/0·1 ml.) shown where applicable		
	RK 13	VMK	PMK
RK 13*a*	2 passes	—	—
RK 13*f*	1 pass	—	1 pass
RK 13*g*	1 pass	—	2 passes
RK 13*i*	2 passes	—	—
O.C. 1 30/4	1·25	1·25	N.T.
O.C. 1 2/5	< 1·00	—	N.T.
O.C. 1. 4/5	1·30	0·50	N.T.
O.C. 1. 6/5	< 1·00	0·50	N.T.
O.C. 1. 7/5	< 1·00	—	—
O.C. 3. 16/5	1·50	1·00	1·50
O.C. 3. 20/5	1·00	N.T.	1 pass
TL P1824	1·00	—	1 pass
Mean number of days required to give virus titre	6·50	13	13
Total no. positive/no. tested.	12/12	4/11	5/8

When no virus was detected on primary inoculation, the number of passages required to detect virus are shown.

N.T. = not tested

O.C. = organ culture material (human embryonic trachea and nose).

RK 13*a*—*i* = specimens containing small amounts of RK 13 grown virus.

Table 4. *Comparative titres of passage material*

Specimen	Details	Titre (log_{10}TCD 50 or InD50/0·1 ml.) Pass no.	RK 13	VMK	PMK
	A. *Initial passages in VMK or PMK*				
HPV77	Attenuated RV Vaccine strain	79	2·50	2·75	2·75
R 12	High Pass. Toronto	25	2·70	2·75	2·75
TL	T/S. Infants. Rubella syndrome	3	1·30	2·00	1·75
DM		1	< 1·00	1·25	2·20
Ca		2	1·00	2·25	> 2·50
Gi		2	1·75	> 2·50	> 2·50
Hi		2	1·50	2·20	2·20
Si		2	1·75	2·00	2·50
	B. *Initial passages in RK* 13				
AL T/S	Infants. Rubella syndrome	1	> 2·50	1·50	2·00
AL CSF		1	> 2·50	> 2·50	> 2·50
BH T/S		1	4·75	4·50	1·50
HM T/S		5	2·50	2·75	1·00

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Table 5. *Comparison of RV isolations in RK* 13 *and LLC-RK* 1

Specimen	Details	Cell culture system Titre of virus (log_{10}TCD 50/0·1 ml.) shown where applicable: LLC-RKI	RK 13
RV 5	Standard strain (Judith)	5·00	3·50
HPV 77	Attenuated RV vaccine strain	3·50	2·50
HS	T/S. Nurse. Rubella	1·50	1·00
DC*	T/S. Doctor. Rubella	+	+
SP	T/S. Nurse. Rubella	> 1·50	< 1·00
OC 13 15/5	Organ culture (H. embryonic trachea)	1·00	1 pass
OC 3 16/5	Organ culture (H. embryonic trachea)	> 1·50	0·75
BW*	T/S. Infant. Rubella syndrome	+	–
ST*	T/S. Child. Rubella	+	1 pass
YC*	T/S. Child. Rubella	+	–
AH*	T/S. Child. Rubella	+	–
ST	T/S. Child. Rubella	–	–
DM	T/S. Infant. Rubella syndrome	–	–
J.G. (1)	T/S. Infant. Rubella syndrome	–	–
J.G. (2)	T/S. Infant. Rubella syndrome	–	–
No. Positive		11/15	8/15

When no virus was detected on primary inoculation the number of passages required to detect virus are shown.

* Specimens which were not titrated.

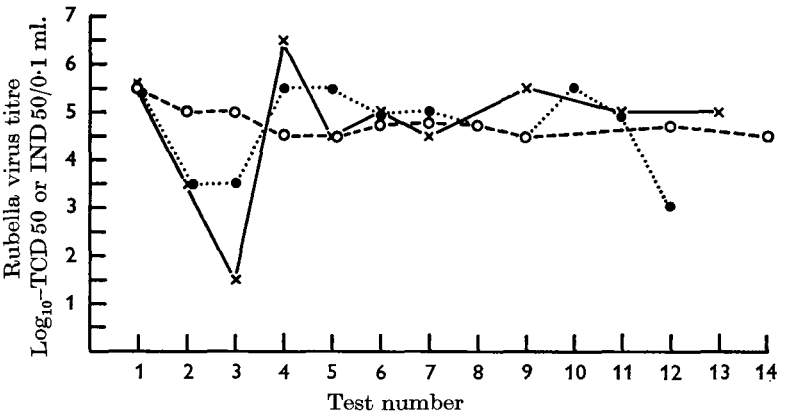

Fig. 1. Serial titrations of RV (Judith) in RK 13 (○—○) VMK (×—×) and PMK (●...●) cell cultures.

virus often coincided with poor cell sheets. However, the presence of simian syncytial and vacuolating agents did not usually cause widespread degeneration of the cell sheets or affect virus titre. The number of successful tests, with reasons for the failure of the others, are shown in Table 6. RK 13 cultures were usually maintained for two weeks without difficulty.

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Serum neutralization tests

Results of serum neutralization tests with clinical details of patients studied are shown in Table 7. It can be seen that similar antibody titres were obtained in each system. Results were obtained by the 4th day in RK 13, but not until the 12–13th day in PMK cell cultures.

Table 6. *Condition of cultures*

	RK 13	V MK	PMK
No. satisfactory tests/no. tests performed	8/12	7/11	5/12
Difficulties encountered:			
Vacuolation	4	—	—
Failure to maintain	—	4	5
Primary MK cells not available, secondaries used	—	—	2

Table 7

Serum specimen	Details	Cell culture systems RK 13	PMK
S.C.	Convalescent serum. Rubella	48*	48
J.G.	6-month-old infant. Rubella syndrome	48	48
M.G.	Mother of J.G.	48	48
B.Gr.	3-year-old child. Bilateral perceptive deafness	64	48
M.Gr.	Mother of B.Gr.	8	12
R.M.	Convalescent serum. Rubella	24	24
M.B.	Convalescent serum. Rubella	48	48

* Reciprocal antibody titre.

DISCUSSION

RK 13 cell cultures were seen to be the most sensitive for RV isolation, as shown by the higher isolation rates and greater capacity for detecting small amounts of virus. They were also the most reliable, as was shown by the comparative lack of variation in titres of the standard pool of RV. Being a continuous cell line, RK 13 cultures can be prepared whenever required; they also provided a result in a shorter time, a mean time of 9 days in this series, compared with 22 days by the interference technique.

However, the presence of RV in VMK and PMK cell cultures can be detected by the absence of CPE after challenge with echo 11 or M 6 more easily than by the CPE caused by RV in RK 13 cell cultures. This is of considerable advantage to inexperienced workers especially if large numbers of tubes are being used. PMK cells are more readily available commercially in this country than VMK, as patas monkeys are frequently used for vaccine production. Although secondary cells can be more readily transported than primary cells, no results have been reported for the comparative sensitivity of primary and secondary PMK and VMK cell cultures for RV isolation.

More specimens were found positive and even higher titres of virus were obtained in LLC-RK1 than in RK13. If sufficient virus was present it brought about complete destruction of the cell sheet in 5–7 days, this being more easily detected than the diffuse CPE obtained in RK13. However, an insufficient number of specimens were compared in parallel in LLC-RK1 and RK13 to draw firm conclusions.

Neutralization tests on patients' sera showed comparable antibody titres. The RK13 cell system (Dudgeon *et al.* 1964) is used as the method of choice in this laboratory, as cell cultures can be prepared whenever required and a result is usually obtained 8 days earlier than by the interference technique.

This paper gives the results of a necessarily limited series of experiments. The shortage of suitable clinical material and the difficulties encountered in obtaining a regular supply of PMK and VMK cell cultures made a larger series impracticable. Nevertheless, even this small series demonstrated that RV isolation in RK13 and LLC-RK1 cell cultures was superior to monkey kidney cultures in both sensitivity and speed. However, good cell sheets are essential and experience in the evaluation of changes in these cultures is necessary. Because variations in the quality of cell cultures is frequently encountered and since clinicians, particularly in the assessment of rubella during pregnancy, require an accurate diagnosis as quickly as possible, it is advisable to employ two cell culture systems in parallel, e.g. RK13 and either PMK or VMK. It is useful to have a reserve of RK13 cells kept frozen by means of liquid nitrogen (Nagington & Greaves, 1962). This can be reconstituted whenever difficulties are encountered, as it may take some weeks to adapt a line imported from another laboratory.

SUMMARY

RK13 and primary PMK and VMK cell cultures were compared for the isolation of RV by means of the simultaneous inoculation of original specimens and early passage material. RK13 was found to be the most sensitive and reliable and provided a result for both isolation and neutralization in the shortest time. As the interpretation of CPE and the propagation of these cultures is sometimes difficult, the simultaneous use of a second system in which RV is easy to identify, e.g. VMK or PMK cell cultures, is recommended. Both PMK cell cultures and LLC-RK1 were suitable for isolating RV from clinical specimens. Preliminary studies with LLC-RK1 indicate that it may provide an even more sensitive alternative to RK13, but further studies employing clinical material require to be carried out before firm conclusions can be reached.

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