Characterization of Growth of Rubella Virus in LLC-MK 2 Cells*

By

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With 7 Figures

Introduction

Since the first reports on tissue culture isolation of rubella virus (1, 2) a variety of tissue culture systems has been found to sustain growth of this agent. In our laboratory the continuous monkey kidney cell lines LCC-MK 2 (3) and BS-C-1(4) have been employed predominantly. Growth of rubella virus (RV) in both these cell lines has been previously reported (4, 5, 6). The purpose of this article is to characterize the cytopathogenic effect (CPE) which is seen in LLC-MK 2 cells infected with RV, present data on growth characteristics of RV in LLC-MK 2 cells, and report on the use of this cell line for neutralization tests. BS-C-1 cells, under very special experimental conditions, have yielded high titers of virus. Although this system has not lent itself to easy standardization, the types of experiments which have resulted in high virus titers, as well as the procedures followed in the purification and concentration of virus preparations, are presented. An evaluation of the usefulness and limitations, at present, of the CPE of RV in LLC-MK 2 cells is attempted.

Materials and Methods

1. Tissue cultures

a) LLC-MK 2 cells: stock cultures are grown and maintained in media 199, with 0.12 g% sodium bicarbonate and 1% horse serum. 2—3 hours adsorption at room temperature is used for virus inoculation,

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followed by change to maintenance media (Eagle's double strength, 1% HS). Bicarbonate is added as needed and the media changed once at day 5 or 6. In titrations, cultures are maintained for 12 days with 2—3 readings for rubella CPE and after 12—13 days 100 $TCID_{50}$ of ECHO-11 virus in 0.1 ml is added. Readings by interference of ECHO-11 growth are done when the controls show extensive or complete destruction. Details on the technique of hemagglutination of human O cells by ECHO-11 (challenging virus) are given in a previous paper (5).

b) BS-C-1 cells are grown in media 199, with 0.06 g % sodium bicarbonate, 0.1% yeast extract and 20% calf serum. For stock cultures yeast extract is not included. After inoculation, Eagle's basal medium with 2% horse serum is used as maintenance media and the feeding schedule is similar to that employed with LLC-MK 2 cells.

c) Roller cultures of BS-C-1 cells are prepared in round bottles (10× 6.5 cm), seeded with 40 ml of cell suspension ($5 \times 10^{4.5}$ cells/ml) in growth media and rotated at 1 revolution per 1—½ minute. After complete growth, which may require several changes of growth media at 2—3 day intervals, these cultures are washed with Hank's BSS, inoculated, and maintained with only 8 ml of maintenance media, rotating at the same speed.

2. Virus

The strains employed have been isolated in this laboratory from patients affected with typical rubella. Passage history is included in the text and figures. All titers referred to in this article were obtained in LLC-MK 2 cells by the interference technique as described by *Parkman* and *Buescher* (1), and are expressed as Interfering $Doses_{50}$ (InD_{50}) per ml. Titers obtained in this cell line are 0.5 to 1 log lower than simultaneous titrations of the same material in primary grivet monkey tissue culture.

3. Virus release curve

Three LLC-MK 2 culture tubes with an average of 231,000 cells per tube were inoculated with 1 ml of virus concentrated by centrifugation containing $10^{7.5}$ InD_{50}/ml. An equal number was inoculated with control fluid concentrated in the same manner. After an adsorption period of 1—1½ hour the inocula were removed, the monolayers washed three times and maintenance media added. At the intervals shown in Fig. 5 all the extracellular phase was removed and frozen and 1 ml of fresh media added. Cell counts were repeated at the end of the experiment in both virus and control inoculated tubes with an average of 113,000 and 525,000 cells per tube respectively. Titration of all aliquots and inoculum were carried out simultaneously.

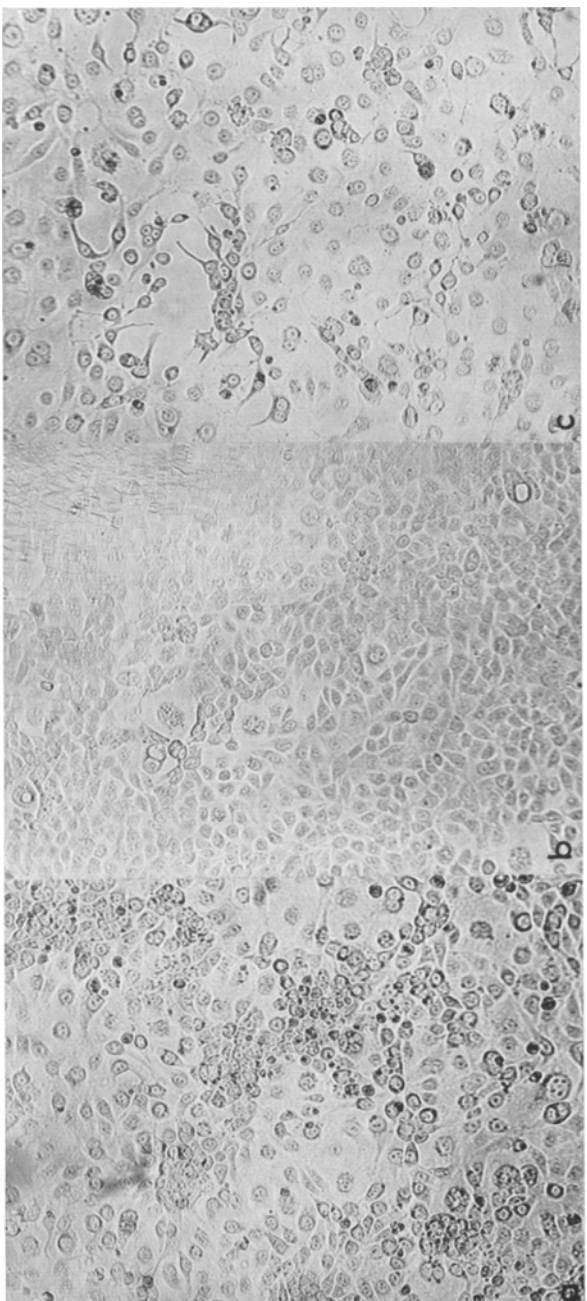


Fig. 1. a) Early RV-CPE. b) Control preparation. c) Advanced RV-CPE.

4. Thermal inactivation

Unconcentrated virus samples were held in a water bath at the temperatures indicated in Fig. 6. At the intervals indicated, 0.2 ml aliquots were removed, diluted and frozen. All aliquots were titrated simultaneously. Inactivation constants (slope of the curves) were calculated by the last square method and converted to natural logarithms. Half lives were calculated by the assumption of a first order reaction and energy of inactivation by the use of Arrhenius' formula, as used by *Price* et al. (7).

Observations and Results

1. Rubella virus CPE in LLC-MK2 cells

When 10^4 to 10^5 InD_{50} of rubella virus are inoculated into LLC-MK2 cells a characteristic CPE appears 4—6 days after inoculation; the first change noted is the appearance of dark, granular cells either in small irregular clusters or scattered among normal cells. Fig. 1a represents this type of picture which is referred to as "early CPE". Within a few days the number of these granular dark cells increases greatly and most of them float in the media. This leads to a fall in the cellularity of the monolayers; many of the remaining cells have granular cytoplasm and often intracytoplasmic inclusions can be observed even in unfixed preparations. This type of change is presented in Fig. 1c and is called "advanced CPE". Almost complete destruction is occasionally seen in tubes inoculated with large inocula and if earlier readings are not made it may lead to confusion at the time of interference readings. The time of appearance of RV-CPE is dependent on the inoculation dose, as shown in Fig. 2.

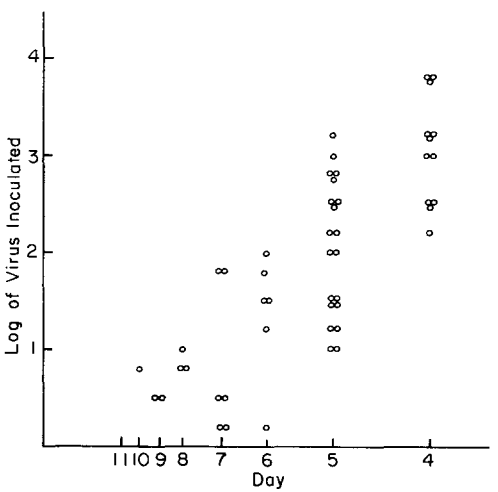


Fig. 2.

Although it seems that adaptation of RV to LLC-MK 2 cells results in enhanced CPE, in primary isolations in which CPE was especially looked for, it was recognized at times varying between 7 and 21 days following inoculation.

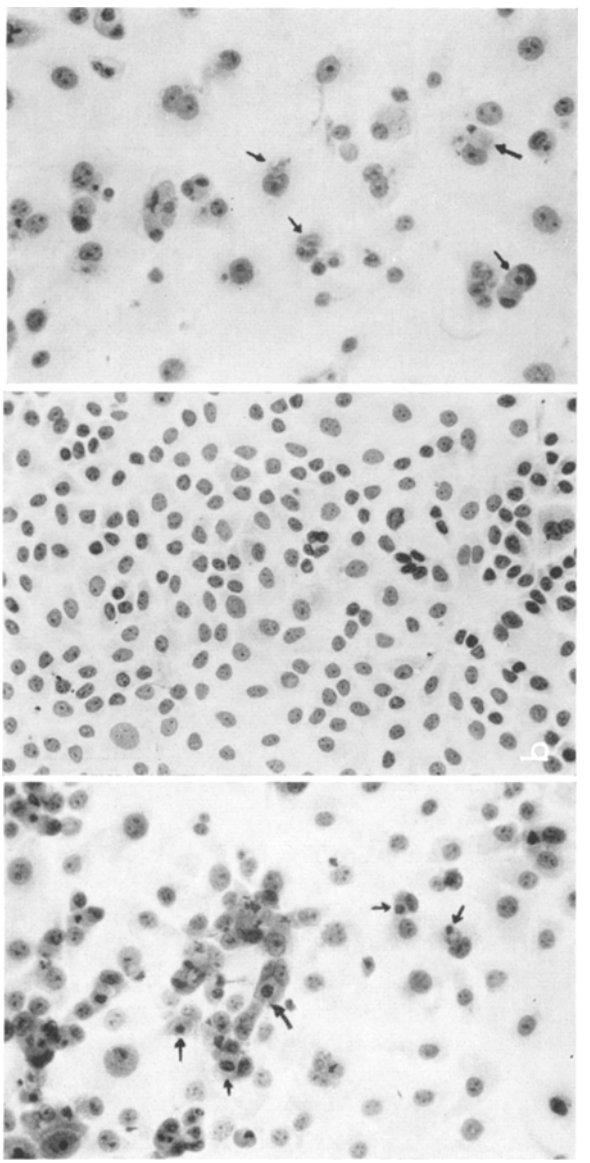


Fig. 3. Two fields showing advanced rubella virus-CPE (a and c) and control preparation (b). Hematoxylin-eosin stain. Arrows show inclusions.

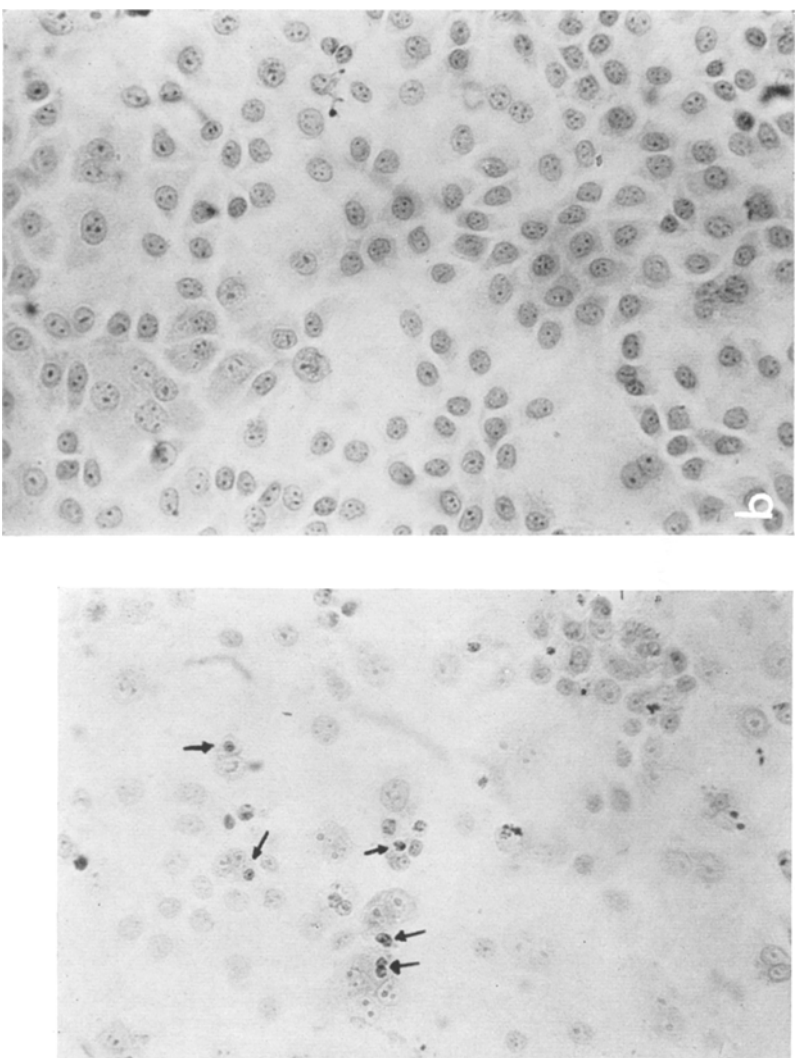


Fig. 4. Methyl-green pyronin stain. a) Advanced RV-CPE. b) Control preparation. Arrows point at blue granular inclusions surrounded by pyronophilic cytoplasm.

Preparations stained with hematoxylin-eosin give more detail of the cellular changes observed in the advanced stages of rubella CPE (Fig. 3). There is a decrease in cellularity and many cells present cytoplasmic inclusions which appear as eosinophilic bodies, often centered by basophilic material. Probably corresponding to this latter type of inclusion are those shown in Fig. 4 where, with a methyl green pyronin stain, red inclusions centered by a conglomerate of dark blue granules are seen. Since this agent seems to be an RNA virus (8), the probabilities are that these central

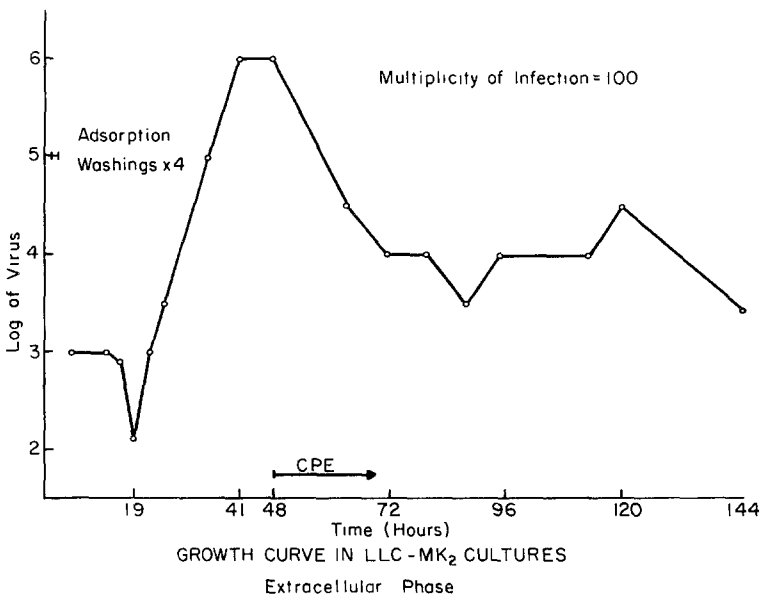


Fig. 5.

bodies are nuclear material. Basophilic inclusions were also observed by *Selzer* (8) in cells from a human embryo naturally infected with rubella virus, while eosinophilic inclusions have been described in infected primary human amnion cultures (2).

Of the nuclear changes in inclusion-bearing cells, the most evident is the replacement of the multiple fine nucleoli of normal cells by one or two large bodies.

2. Growth characteristics in LLC-MK 2 cells

With an inoculum of $10^{4.5}$ InD_{50}, *Maassab* (9) has found that maximum titers in the order of $10^{5.5}$ InD_{50}/ml are reached in 4 days. Intracellular virus appears earlier than virus in the extracellular fluid, but virus release proceeds rapidly enough to obtain similar titers in both phases by

the 4th—5th day. As long as the condition of the cultures is maintained titers persist for long periods with only small fluctuations.

Virus release curves following high multiplicity of infection have been obtained in the hope that under those conditions a single cycle of multiplication will be obtained; however, the simultaneous infection of all cells cannot be proved at the present time. As presented in Fig. 5, virus release becomes detectable at 19 hours post inoculation (end of the latent period), reaches a peak at 40—48 hours (10^6 InD_{50}/ml) and then declines. Concomitant with the maximum release of virus is the appearance of CPE which rapidly destroys the majority of cells. This lapse of 40—48 hours seems therefore to correspond to the duration of a single cycle of virus multiplication in the majority of cells, ending in their destruction. Neither the decline in virus titer nor the CPE are, however, complete and from 72 hours on, a steady submaximal titer in the order of 10^4 InD_{50}/ml is maintained by a very reduced cell population.

3. Neutralization tests using LLC-MK 2 cells

The use of LLC-MK 2 cells for neutralization tests is at present being standardized in our laboratory. Neutralizing antibody titers obtained in this system are similar to those obtained in others.

Two points which deserve mention at present are:

a) Results using RV-CPE or ECHO-11 interference as indices of virus growth give similar results, thus adding another proof of the specificity of the CPE described above.

b) The addition of fresh rabbit serum, proposed by *Weller* and *Neva* (2), for neutralization tests carried out in primary human amnion, permits in checkerboard tests, using LLC-MK 2 cells, greater neutralization titers at all levels of virus antibody mixtures.

4. High titers obtained in BS-C-1 cells

Roller BS-C-1 cultures which are maintained with only 8 ml of media (representing a 5-fold reduction in the fluid/cell ratio) have produced RV to titers as high as $10^{7.5}$ InD_{50}/ml. Due to the fluid reduction it has been necessary to change media every 2 days and to add sodium bicarbonate on alternate days.

Although many of these frequent harvestings have good titers, the overall yield of virus in this system is highly inconsistent.

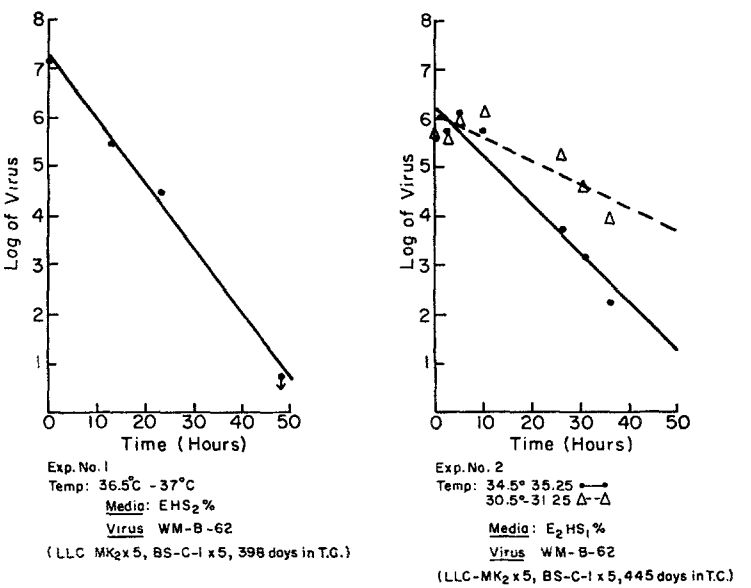
5. Thermal inactivation

An inactivation constant of -0.306 ln/hour was found for virus suspended in tissue culture fluid and held at 37° C. This corresponds to a half

life of 2—½ hours. Data for two other temperatures are presented in Fig. 6. Within the range explored (30—37° C) the estimated energy of inactivation is of 33,000 cal/mole.

6. Concentration and purification of virus

Our own experience is in agreement with that of other investigators in regard to the usefulness of carbowax concentration (10), and of high speed centrifugation (1, 2).



Temperature	Inactivation Constant K, ln / Hour	Half-life, Calculated From $V_1 = V_0 e^{-Kt}$
36.5-37° C	- 0.306	2 Hours and 15 min.
34.5-35.25°C	- 0.230	3 Hours
30.5-31.25°C	- 0.106	6 Hours 30 min.

Fig. 6.

Floatation experiments on media with 1,070 density resulted in recovery of 10% of the virus in the upper fraction, and potassium tartrate density gradient experiments exploring densities between 1.03 and 1.08 indicated a density between 1.05 and 1.06 for a similar proportion of the virus input. Experiments are in progress to explain the reasons for these low recoveries.

Filtration through Sephadex G-200 has proved to be of value in certain experiments. Fig. 7 shows a typical curve of virus recovery and the separa-

tion of the protein peak from the virus peak. Virus can be recovered in media with any desired salt composition, and in a typical experiment, the first two fractions containing virus represented 30% of the virus input, a 3-fold dilution of its volume and had only 7% of the protein present in the original preparation.

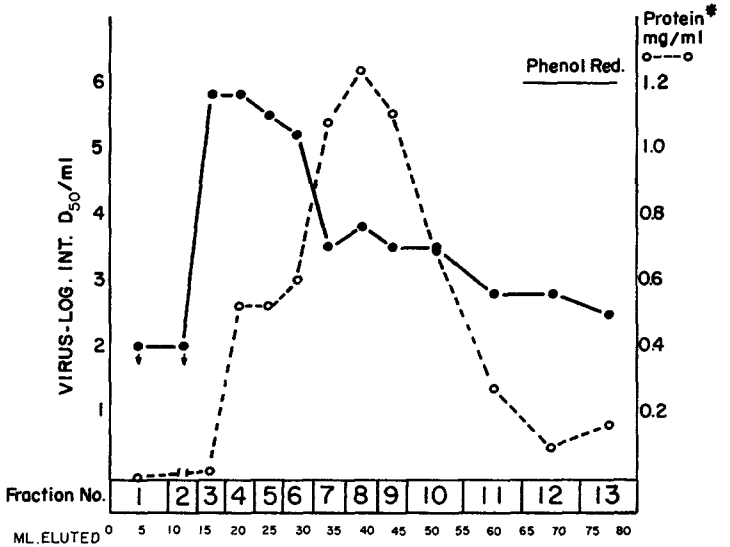


Fig. 7.

Discussion

The bases for the specificity of the characteristic CPE of RV in LLC-MK 2 cells are:

1) It is present in cultures infected with all strains used in this laboratory and absent in controls.

2) Its presence parallels the development of resistance to superinfection with ECHO-11 virus.

3) It is suppressed by rubella convalescent sera.

The presence of CPE in primary isolations indicates that it is not entirely dependent on virus adaptation although it is likely that repeated passage of virus in these cells may result in enhanced CPE. The long intervals sometimes required before CPE becomes obvious in primary isolations, discourages its use as the only criteria of virus growth, but when seen early it results in a more rapid diagnosis.

The inverse relation between time of appearance of CPE and dose inoculated is present in Fig. 2. With small doses (in the neighborhood of 1 InD_{50}) 12—14 days are sometimes required, which is similar to the time necessary to titrate RV in the same system by the interference technique. There is usually exact or close agreement between titrations read by CPE or by interference.

The time for obtaining the final endpoint in titrations read by CPE has been, therefore, no shorter than that required for titration by interference; since the former requires more experience for its recognition and is more variable, the interference test continues to be a very valuable index of growth of RV.

Nevertheless, the CPE measurement has several advantages. With well adapted strains this procedure can be substituted in all respects, and its recognition opens new and interesting lines for investigation, including possible dissociation of the direct or CPE effect from the indirect interference effect.

The submaximal steady titer found in virus release presented in Fig. 5 was an unexpected phenomenon. During this period, as well as during the plateau phase which obtains after 4—5 days following infection with moderate inocula, the rate of virus production can be assumed to equal that of thermal inactivation. The latter has been found to be 0.1 log/hour (Fig. 6) for virus maintained at 36—37° C in tissue culture media.

The yield per cell during the rapid release phase of the experiment presented in Fig. 5 is of 6—12 InD_{50}; the latter number includes the estimated proportion lost by thermal inactivation. Although this is a low yield as compared with other viruses it can be correlated with the virus production during the post-maximal phase of the same release curve. Supposing that a similar yield per cell obtains during that period and that the main loss occurs by thermal inactivation, only 1/10th of the remaining cells need to be actively producing virus at any given time to maintain the level observed. That this may be the case is also borne out in the observation of many histologically normal cells in infected cultures and of preliminary immunofluorescent studies.

The usual titers obtained in our laboratory in LLC-MK2 cells vary between $10^{4.5}$ and 10^6 InD_{50}/ml. Attempts to increase the number of cells per unit of media in roller cultures were not possible using LLC-MK2 cells because of cell clumping. BS-C-1 cells, on the other hand, yielded high titers of virus under the same conditions. Unfortunately, the yield of virus in this system is very irregular with titers ranging from 10^5 to $10^{5.7}$ InD_{50}/ml, but is generally far superior to virus yields obtained otherwise. Experiments are in progress to obtain a similar condition in the fluid to cells ratio in suspended cultures.

Summary

1. A characteristic CPE of rubella virus in LLC-MK 2 cells is described and its usefulness and limitations in experimental work are discussed.

2. Growth characteristics of this agent in LLC-MK 2 cells have been studied following moderate and high multiplicities of infection, and they are analyzed as preliminary information on the kinetics of virus production.

Incidental data on thermal inactivation, virus purification, and virus neutralization are reported.

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References

1. *Parkman, P. D., E. L. Buescher,* and *M. S. Artenstein:* Recovery of rubella virus from army recruits. Proc. Soc. Exp. Biol. and Med. **111,** 225 (1962).
2. *Weller, T. N.,* and *F. A. Neva:* Propagation in tissue culture of cytopathic agents from patients with rubella-like illness. Proc. Soc. Exp. Biol. and Med. **111,** 215 (1962).
3. *Hull, R. H., W. R. Cherry,* and *O. J. Tritch:* Growth characteristics of monkey kidney cell strains, LLC-MK 1, LLC-MK 2 and LLC-MK 3 (NCTC-3196) and their utility in virus research. J. Exp. Med. **115,** 903 (1962).
4. *Hopps, H. E., B. C. Bernheim, A. Nisalak, J. H. Tijio,* and *J. E. Smadel:* Biological characteristics of a continuous cell line derived from African green monkey. J. Immunol. **91,** 416 (1963).
5. *Veronelli, J. A., H. F. Maassab,* and *A. V. Hennessy:* Isolation in tissue culture of an interfering agent from patients with rubella. Proc. Soc. Exp. Biol. and Med. **111,** 472 (1962).
6. *Sigurdardottir, B., K. F. Givan, K. R. Rozee,* and *A. J. Rhodes:* Canad. Med. Ass. J. **88,** 128 (1963).
7. *Price, W. C.:* Thermal inactivation rates of four plant viruses. Arch. ges. Virusforsch. **1,** 373 (1940).
8. *Selzer, G.:* Virus isolation, inclusion bodies and chromosomes in rubella infected human embryo. Lancet **2,** 336 (1963).
9. *Maassab, H. F.,* and *K. W. Cochran:* The influence of 5-FUDR on growth characteristics of rubella virus. In press, Proc. Soc. Exp. Biol. Med. (1964).
10. *Norrby, E., P. Magnusson, B. Friding,* and *S. Gard:* A note on the morphology of rubella virus. Arch. ges. Virusforsch. **13,** 421 (1963).