A Study of Microfoci and Inclusion Bodies produced by Rubella Virus in the RK-13 Cell Line

By G. KOURI, A. AGUILERA, PILAR RODRIGUEZ AND M. KOROLEV
Laboratories of Virology and Electron Microscopy, National Center for Scientific Research, Havana, Cuba

(Accepted 21 August 1973)

SUMMARY

A study was made of the mechanism of microfocus formation and of the nature of the inclusion bodies that appear in the cytoplasm of RK-13 cells infected by rubella virus.

Direct passage of virus particles from one cell to another, at least in the early stages of the infection, is posed as a possibility, and the lysosomal nature of the cytoplasmic inclusion bodies is demonstrated by histochemical methods. A description is given of conditions required in the monolayer for the formation of a microfocus, which is the characteristic manifestation of the c.p.e. of the rubella virus in this cellular system.

INTRODUCTION

The c.p.e. of rubella virus in RK-13 cells was described by McCarthy Taylor-Robinson & Pillinger (1963). In 1964, Taylor-Robinson et al. drew attention to the presence in inoculated monolayers of minute conglomerations of from 10 to 50 cells which tended to degenerate into granular, lobulated, amorphous masses. The author called these alterations microfoci, and pointed out that their number on the third day post-inoculation is proportional to the virus doses employed. A simple and practical method of titrating the virus was proposed.

In the same year Dudgeon, Butler & Plotkin (1964), stated that the c.p.e. of rubella virus on RK-13 cells is characterized by focal changes, and reported the presence of inclusion bodies in infected cells.

In a later paper, McCarthy (1969) described microfocus development as observed by means of time-lapse cine film techniques, which showed that focal changes begin at roughly 40 h after inoculation, followed by infection of contiguous cells and their incorporation into the tiny heap of originally infected cells.

Our studies (Kouri et al. 1971) corroborated the findings of McCarthy, but detected the onset of the local changes by the twelfth hour after inoculation. The possibility of the lysosomal nature of inclusion bodies was also suggested (Kouri et al. 1971; Korolev et al. 1973) on the basis of both optical and electron microscopical evidence.

The present paper describes a study of the mechanism of microfocus formation and presents further evidence concerning the nature of inclusion bodies, employing histochemical techniques, phase contrast microscopy, and electron microscopy.
Methods

Tissue cultures. The rabbit kidney continuous culture cell line (RK-13) was obtained from the American Type Culture Collection and grown in our laboratory in '199' medium, supplemented with 10% calf serum. Penicillin and streptomycin were used at the usual concentrations.

Virus. The Judith strain of rubella virus, generously made available to us by Professor K. McCarthy, Medical School, Liverpool University, was used with a titre of $2.3 \times 10^5$ TCD$_{50}$/ml.

Inoculation. An input multiplicity of 0.1 TCD$_{50}$/ml was used on a confluent cell monolayer composed of healthy polygonal cells. The maintenance medium was the same but with 2% heat-inactivated calf serum, and the incubation temperature was 35°C.

Histochemical techniques

Acridine orange. A vital technique was employed with a 1:1000 concentration of acridine orange in a saline solution. The staining time was five min at room temperature. Microscopic observation was carried out with an HBO-200 lamp with appropriate filters.

Acid phosphatase. Cells were fixed with 2% glutaraldehyde in cacodylate, using the classical Gomori technique, with 50 min incubation.

Inhibition of the microfoci. Cultures in tubes were inoculated with 100 and 1000 TCD$_{50}$ for an adsorption period of 2 h at 35°C. The inoculum was then extracted and the cell sheet was washed several times with phosphate-buffered saline (PBS). The maintenance medium containing an excess of anti-rubella immune serum was added. Simultaneously and in the same experimental conditions, a neutralization test was performed utilizing 300 TCD$_{50}$ and 4 units of the same serum, as well as tubes inoculated with 100 and 1000 TCD$_{50}$ without specific serum as our positive controls.

Electron microscopy. The cell monolayers on acrylic coverslips two or three days after inoculation were washed with PBS and fixed with a solution of 2.5% glutaraldehyde. They were post-fixed in 1% osmic acid and dehydrated by repeated passing through ethanol. Araldite was used for infiltration and inclusion. The use of a flat embedding method made possible electron microscopy of specific areas of the microfoci, which had been selected beforehand by optical microscopy. The ultratome LKB 8800 and the Hitachi HU-11 A electron microscope were used.

Results

Phase contrast microscopy

Twelve hours after inoculation, conglomerations of cells with well-defined structure appeared on the monolayer. At 48 h the microfoci were clearly evident (Fig. 1A). They were composed of from 10 to 50 cells projecting into the medium. Surrounding cells were disposed in radiated form and contained very large inclusion bodies (Fig. 1D). At greater magnification (Fig. 1C) it was seen that the centre of each microfocus was composed of a granular mass from which cell structure had disappeared, surrounded by apparently normal cells.

Acridine orange

After vital staining with acridine orange, the microfoci showed an intense red-orange fluorescence (Fig. 2B), indicating concentration of the vital stain at this site. The large inclusion bodies were also seen at the periphery (Fig. 2C) with an intense red-orange fluorescence.
Rubella virus microfoci and inclusion bodies

Fig. 1. A, Microfocus (M) surrounded by radially-arranged cells (×480). B, Normal RK-13 cells. C, Centre of microfocus composed of granular mass, from which all cell structure has disappeared (×1600). D, Large cytoplasmic inclusion body (L) rejecting the nucleus (N).
Acridine orange vital technique. A, Normal cells. B, Microfoci (M) display intense red-orange colouration. C, Very large inclusion body takes on intense red-orange colour (L).

Acid phosphatase

In the centre of the microfocus a diffuse positive reaction was observed within the cell cytoplasm (Fig. 3B), while cells in the periphery contained large cytoplasmic inclusion bodies with an intense positive reaction (Fig. 3C).
Inhibition of the microfoci

Our results confirm that microfoci appear in cultures inoculated with 100 and 1000 TCD\textsubscript{50} with an excess of anti-rubella serum present in the medium, although the appearance of the lesion was delayed and less intense than in the positive controls. Our results confirmed the neutralizing effect of the serum used.
Fig. 4. Electronic microscope technique. A and B, Cells with phagosomes (P) in varying stages of formation, cellular fusion (→). C, Dilation of the golgi complex with typical virus particles (→).

Electron microscopy

The centre of the microfocus was composed of cellular detritus and this zone was surrounded by totally or partially destroyed cells. The cytoplasm of the majority of these cells contained detritus-filled vacuoles and in some of these the remains of cell organelles could be seen.
Cells with phagosomes in varying stages of formation were visible. We also observed images in the microfoci which suggested the possibility of fusion between adjacent cells (Fig. 4A and B). Outside (at the outer edge of the microfocus) the cells were practically normal but presented the interesting detail of dilatation of the Golgi complex with the occasional presence in their dilated ends of typical virus particles in varying stages of formation (Fig. 4C).

DISCUSSION

Microfoci constitute the primary and most characteristic lesion of the c.p.e. of rubella virus in RK-13 cells, as several authors (Taylor-Robinson et al. 1964; McCarthy, 1969; Kouri et al. 1971) have pointed out. We have demonstrated that the microfoci begin to form twelve h after inoculation, although we concur with McCarthy that they are not well differentiated until 40 h.

Direct cell-to-cell spreading of virus particles may be the mechanism for the formation of these lesions. This would explain the progressive involvement of cells immediately adjacent to the cells originally affected. The results of attempts to inhibit microfoci with anti-rubella immune serum support this possibility.

The phagocytosis of cells bordering the originally infected cells could be an additional factor in the spreading of the infection. It is possible that the retardation of the c.p.e. observed in our experiment on “inhibition of the microfocus” can be explained by the inactivating effect of anti-rubella serum on the virus particles, which then pass into the medium, unable to penetrate other cells. This would leave the direct spreading of particles from one cell to another as the only mechanism for the formation of microfoci.

Electron microscopic evidence presented in an earlier paper (Korolev et al. 1973), as well as the results obtained in the present study, seems to confirm this hypothesis. In brief, a negative gradient of cellular destruction is observed from the centre to the periphery of the microfocus, while cells on the periphery are seen to present images which suggest an active phagocytosis. At the same time, zones of genuine cellular fusion are observed, which might serve as bridges facilitating the exchange of cytoplasmatic material with consequent migration of virus particles, or, more likely, of their genetic material.

The golgi dilatation and the presence of the particles formed is observed in the peripheral cells. This direct passage of virus particles could explain, in the event that this occurs in natural infection, the persistence of virus over a long period in foetuses affected in utero, despite the existence of circulating neutralizing antibodies. It could, similarly, explain a certain state of latency of the virus in the mother’s body, thus providing experimental confirmation of the possibility, proposed by Plotkin & Mellman (1970), that the virus may affect foetuses in pregnancies after that in which the initial infection occurred.

The evidence would appear to strengthen the hypothesis of Taylor-Robinson et al. (1964) that the number of microfoci is proportional to the virus titre, since, at least during the first two or three days of the infection, the basic manner of spreading of the virus is by direct extension via cellular bridges, and that, therefore, during this stage, all the microfoci can be considered primary lesions.

Our histochemical findings in the microfoci are in accordance with their ultrastructure, since the diffusion of the reaction observed can be explained by the fact that the centres of the microfoci are made up of cellular material captured by the normal peripheral cells.

It is essential to point out that the formation of the microfoci requires standardization of culture techniques to provide ideal conditions in the monolayer. This must be uniform; the cells must be healthy and clearly polygonal with adequate concentration. If these condi-
tions are not assured, the development of the c.p.e. is altered. This fact is easily explained, since, as we have indicated, the formation of the microfocus is closely related, in the first stage, to the direct passing of the virus particles from cell to cell via cellular bridges and, possibly, as well, to peripheral phagocytosis. In the case of either mechanism, there must be adequate contact between the cells, and their activity must not be affected. Our histochemical and electron microscopic studies show that the cytoplasmic inclusion bodies previously observed in this cell line (Dudgeon et al. 1964; Kouri et al. 1972) are true 'giant lysosomes' or 'macrolysosomes', which are linked to cellular activity and not to the intimate mechanism of virus replication.

The results obtained in the reaction of acid phosphatase, showed that, especially in the microfoci, the cells displayed an intense enzymatic activity diffused throughout the cytoplasm. Considering that the microfocus is the primary and specific lesion produced by this virus, we are able to affirm that those changes are related to the virus infection, although we cannot state specially whether this diffusion is the cause or the effect of cell damage. Nevertheless, the role of the lysosomes in the rubella virus RK-13 cells interaction has been demonstrated for the first time, allowing comparison with what has been described previously in other virus-cell systems (Allison, 1967).

The competent assistance of Marjorie Moore is gratefully acknowledged.

REFERENCES


(Received 5 April 1973)