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G. I. Fokina, et al

Army Medical Research Institute of Infectious Diseases
Frederick, Maryland

1972

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MULTIPLICATION OF COXSACKIE GROUP A VIRUSES IN HUMAN EMBRYO LUNG DIPLOID CELLS

G.I. Fokina, Z.F. Zubova, R.I. Rapoport
Moscow Viral Preparations Research Institute - Published 9/17/1969

Vop. Virusol. 15:3:346-350 (1970)

Human diploid cells have attracted the attention of virologists as a convenient cellular system for studying the interaction of viruses and cells since they are homogeneous in terms of morphological characteristics, free from latent viruses and transforming agents, and possess a wide range of sensitivity. In a preceeding report[2] we demonstrated that a whole series of Coxsackie group a viruses causes degeneration of diploid cells and accumulates up to titers of 10^6-10^7 CPD_{50}/ml.

We have studied the multiplication pattern of these viruses in diploid cells, the dynamics of viral accumulation in the cells and in culture fluid, and we have explained the relation of the point at which degeneration begins and the maximum accumulation of the virus to the value of the inoculating dose.

Materials and Methods

All experiments were carried out on diploid lung cells of human embryos (strains L-45 and Wl-38). The cell culture was raised in an Igla IX medium, 30% of which was made up of a 0.5% solution of hydrolyzate of milk albumin and 10% calf serum. Coxsackie group a type 13, 18, 20, and 21 (Kuykendall strain) viruses were used. Each of these viruses had a titer in diploid cells of $10^{5.5}$-10^7 CPD_{50}/ml. In order to study the dynamics of accumulation, the cell culture was inoculated with these viruses in doses ranging from 0.0001 to 0.5 CPD_{50}/cell. The multiplication cycle followed the model of A21 virus, Kuykendall strain. The inoculating viral doses were 16 and 1.6 CPD_{50}/cell. Viruses were in contact with the culture tissue for two hours at 37°, after which the cultures were carefully washed and medium No. 199 was added. Separating flasks with the inoculated cells were incubated at 37°. Every two hours during inoculation with large doses (1.6 and 16 CPD_{50}/cell) and daily during inoculation with small doses (0.0001-0.5 CPD_{50}/cell) flasks were selected for processing: the culture fluid was poured off, the cells washed five times in a phosphate buffer, No. 199 medium was added up to the original volume, and the culture was frozen. Degenerated cells were removed from the decanted culture fluid by centrifugation at 1500 rpm for ten minutes.

Subsequently, the cells and culture fluid were frozen and thawed three times,

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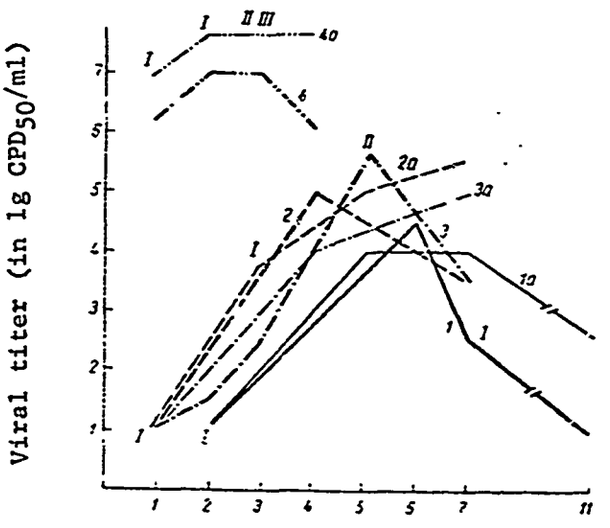
after which the viral titer was determined (according to cytopathological effect). The titer of the total virus was simultaneously made beginning after nine hours. Samples were titrated according to cytopathological effect. The degree of cytopathological change was judged during microscope observation of the inoculated culture under low magnification. Study of the multiplication cycle was accompanied by morphological investigations 2, 6, 8, 11, 14, and 20 hours after inoculation (fixation in Buena liquid, dyeing with hematoxiline-eosin).

Results

The experimental results indicated that the dynamics of accumulation of all varieties of investigated Coxsackie group a viruses follow the same pattern. The accumulation of intracellular virus parallels that in culture fluid up to the moment of degeneration of over 50% of the cells. Up to this time the intracellular quantity of virus is lower than that in the culture fluid (Fig. 1). This is apparently related to the destruction of the majority of cells and the transition of intracellular virus to the culture fluid. The period of maximum accumulation of the virus in the cells, as well as the point at which degeneration begins, depends on the value of the inoculation and not on viral type. Thus, with an inoculating dose of 0.01 CPD_{50} cell for virus A20 and A21 the period of maximum accumulation of virus in cells was four to five days, while when 0.5 CPD_{50}/cell dose was used for viruses A13 and A18, the period was two days. The point at which degeneration begins also depends on the quantity of the inoculation; viral inoculation with a 0.5 CPD_{50}/cell dose causes degeneration beginning on the third day, with a dose of 0.01 CPD_{50}/cell on the fourth to fifth day, and with a 0.0001 CPD_{50}/cell dose only on the eleventh day.

Fig. 1. Dynamics of accumulation of Coxsackie group a virus in diploid lung cells of human embryos.
1 - A21 virus in cells and medium (1a) during inoculation with a 0.0001 CPD_{50}/cell dose; 2 - A22 virus and medium (2a) during inoculation with a 0.01 CPD_{50}/cell dose; 3 - A20 virus in cells and medium (3a) during inoculation with a 0.01 CPD_{50}/cell dose; 4 - A13 virus in cells and medium (4a) during inoculation with a 0.5 CPD_{50}/cell dose; I - absence of cytopathological effect; II - degeneration of 20-30% of the cells; III - degeneration of over 50% of the cells.



We noticed in these experiments that before the appearance of cytopathological effect the virus accumulates in the cells and attains high titers ($10^{6,5-7}$ CPD_{50}/ml for viruses A13 and A18; $10^{4-4.5}$ CPD_{50}/ml for viruses A20 and A21). The maximum accumulation of virus in cells was noticed either in the absence of cytopathological effect or at the beginning of degeneration when 20-30% of the cells were involved in the process. During the action of low inoculating doses 0.0001 CPD_{50}/cell the cellular viruses accumulated to a maximum titer of $10^{4.5}$ CPD_{50}/cell on the sixth day, and subsequently the titer dropped to 10^{2} CPD_{50}/ml in the absence of any cytopathological changes during the usual microscopic observation. Only on the eleventh day could the initial signs of degeneration be seen, and by this time the viral titer in the cells had lowered to 10^{1} CPD_{50}/ml. The viral titer in culture fluid was $10^{2.5}$ CPD_{50}/ml. The maximum titer in culture fluid on the sixth day was 10^{4} CFD50/ml, also without cytopathological changes (see Fig. 1). It may be assumed that in this case only a very small percentage of the cells were inoculated and produced virus. By multiplying, the remaining cells create the impression that the cell layer is intact. However, it is possible that a special interaction of the chronic infection type takes place here between virus and cell.

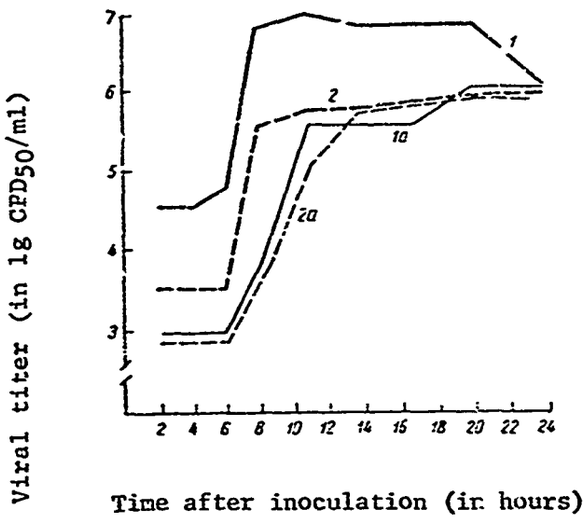
The multiplication pattern of Coxsackie group a virus in diploid cells was studied with A21 Kuykendall strain virus. Under conditions of high multiplicity with inoculations of 16 and 1.6 CPD_{50}/cell one multiplication cycle (Fig. 2) could be seen. During inoculation of 16 CPD_{50}/cell for four hours after terminating contact or six hours from the moment of inoculation, the number of virus in cells and nutrient medium did not increase. Eight hours after inoculation the number of virus in cells sharply increased, attaining a maximum titer of $10^{6.75}$ CPD_{50}/ml. At the same time a slight increase in the titer of virus in culture fluid began. Sharp increase in the viral titer to $10^{5.5}$ CPD_{50}/ml was identified three hours later. A gradual increase took place in subsequent viral titers in culture fluid. The quantity of intracellular virus reached the same maximum level after 20 hours and after 24 hours had experienced only a slight drop (to 10^{6} CPD_{50}/ml). However, the viral titer in cells was always higher than in culture fluid. As for the general viral number, only 11 hours after inoculation it reached maximum titer in both 16 and 1.6 CPD_{50}/cell doses and remained at the same level to the twenty-fourth hour (16 CPD_{50}/cell) and fourty-eighth hour (1.6 CPD_{50}/cell). The same principles were observed for the inoculating dose of 1.6 CPD_{50}/cell, although the viral titer was somewhat lower and was still rising after 24 hours.

In these experiments as in the preceeding ones, the maximum titers in cells were attained in the absence of cytopathological changes. The latter was observed only 11 hours after inoculation and was expressed by the 10-20% rate of appearance of round, strongly refracted light of the cells. Titers of intracellular virus reached their maximum level at this time. Thinning of the cell layer and significant degeneration of over 50% of the cells was visible only 24 hours after inoculation, when the titer inside cells had begun to decrease. During the morphological investigation of fixed and dyed preparations the number of rounded, specifically modified cells with crescent-shaped, pyknotic nuclei pushed to the edge of the eosinphil mass reached 16% after 11 hours, 26% after 14, and 33% after 20 hours.

In a parallel control inoculation of a culture of diploid poliomyelitis type I Brunden cells with a 1.6 CPD_{50}/cell dose, the same principles were observed in regard to multiplication and accumulation of viruses, but cytopathological changes became visible earlier (after five hours when the titers had still not reached their maximum).

In order to determine the viral site during various phases of its development the following experiment was devised.[1] Diploid cell cultures in flasks were inoculated with Coxsackie A21 virus (0.16 CPD_{50}/cell). Samples were then taken after incubation at 37° for 15 minutes and 2, 3, and 4 hours. In one-third of the flasks the cells were

Fig. 2. The multiplication cycle of Coxsackie A21 Kuykendall strain virus. 1 - virus in cells and medium (1a) during inoculating dose of 16 CPD_{50}/cell; 2 - virus in cells and medium (2a) during inoculating dose of 1.6 CPD_{50}/cell.



destroyed by freezing, one-third were treated with homologous serum in a 1:10 solution (40 neutralizing units) with subsequent freezing, and in one-third cell destruction was begun by freezing and the serum was then added in the same solution.

Samples were titrated in one experiment (see Table).

Penetration and accumulation of Coxsackie A21 virus into human diploid cells

Test material	Cell virus level (in 1g CPD_{50}/ml) After 15 min	After 2 hours	After 3 hours	After 4 hours
Inoculated cells treated with medium No 199 and destroyed	3.5	3.0	3.5	4.0
Inoculated cells treated with serum, then destroyed	1	0	2.5	2.75
Inoculated cells destroyed, then treated with serum	0	0	0	0

After two hours in the preparations treated with serum no intracellular virus was observed; after three to four hours synthesized virus appeared again. The serum dose was sufficient to neutralize destroyed virus completely.

Discussion

Knowledge of the principles of accumulation and the multiplication pattern of viruses in cells permits the required inoculating dose to be selected and the optimal time for collecting the viruses to be determined. In this regard we studied the multiplication cycle in diploid cells (inoculating dose 16 CPD_{50}/cell) in A21

Kuykendall strain virus. During the first six hours after inoculation the number of virus did not increase in either cells or medium, although at this time virus was located in both. This is apparently explained by the adsorption of virus on the surface of cells and on cell walls.

A sharp increase in the number of intracellular virus up to the maximum titer was observed eight hours after inoculation. In culture fluid virus was located at the same time in low titers, and subsequently, after three hours, the viral titer sharply increased and continued rising up to the twenty-fourth hour.

The amount of intracellular virus exceeded that in the culture fluid throughout the observation period (see Fig. 2).

The rate of maximum accumulation of virus and time of beginning of degeneration depend on the value of the inoculating dose. The data we obtained on Coxsackie A virus multiplication in human lung embryo diploid cells are analogous to the results of other authors who have studied the cycles of poliomyelitis and Coxsackie group a and b multiplication in cultures of other cells.[1, 3-5, 7]

During the study of Coxsackie group a multiplication we observed that the maximum accumulation of virus in cells was reached either in the absence of cytopathological changes or during degeneration of 10-30% of the cells. Later on the remaining cells degenerated, but the viral titer did not increase. These findings correlate with the results of Dunnebacke and Mattern's[4] investigations, which, like ours, showed the absence of a correlation between cytopathological changes and viral accumulation in cells during the action of Coxsackie group a (A9 and A10) virus in the absence of poliomyelitis virus.[6]

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Summary

The pattern of multiplication and the dynamics of accumulation in cells and culture fluid of Coxsackie Group A viruses (types 13, 18, 20, 21) were studied in human embryo lung diploid cells. The dynamics of accumulation of different types of Coxsackie A viruses was found to be no different. The increase of virus in cells and the culture fluid was parallel up to the moment of degeneration of over 50% of cells after which time the titer of the cellular virus dropped and that of the culture fluid persisted. The latter appeared to be due to transition of intracellular virus into the medium when the majority of the cells were destroyed. The time of maximum increase of virus in cells and the time of degeneration were inversely related to the size of the infecting dose and did not depend upon the virus type. Coxsackie A 21 virus, Kuykendall strain at a high multiplicity of infection was found to penetrate into the cell in 15 min but could not be detected in 2 hours. Newly synthesized virus appeared in the cells in 3—4 hours.

The virus reached maximum titer in the cells 8 hours after inoculation, and in the culture fluid 3 hours later. By 11 hours after inoculation the total amount of virus reached maximum titer. The amount of intracellular virus throughout the observation period exceeded that in the culture fluid.

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