CELL KILLING BY FROG VIRUS 3 : EVIDENCE FOR CELL KILLING BY SINGLE VIRAL PARTICLES OR SINGLE VIRAL SUBUNITS

R. DRILLIEN, D. SPEHNER and A. KIRN

Groupe de Recherches de l'I.N.S.E.R.M. sur la Pathogénie des Infections Virales, Laboratoire de Virologie de la Faculté de Médecine, Université Louis Pasteur, 3, rue Koeberlé, 67000 STRASBOURG FRANCE.

Received September 5,1977

SUMMARY : Cell killing by Frog Virus 3 was assayed after infection of chinese hamster ovary cells under non permissive conditions for virus multiplication. The kinetics of the loss in the efficiency of colony formation as a function of the virus multiplicity indicated that infection of a cell with a single viral particle brought about cell death. About 15 percent of the cells exhibited transient resistance to killing by single viral particles. Treatment of cells with proteins solubilized from Frog Virus 3 also resulted in cell killing with one hit kinetics thus implying that the interaction with a single viral subunit sufficed to entail cell death.

INTRODUCTION

Infection of cell cultures with a large variety of animal viruses results in inhibition of cellular macromolecular synthesis (1). FV 3, an icosahedral cytoplasmic DNA virus, rapidly inhibits host DNA, RNA and protein syntheses (2, 3). At temperatures above 33°C FV 3 is unable to multiply (4) and may therefore be considered as uninfectious for mammalian cells maintained at 37°C an optimal temperature for their growth. Nevertheless, inhibition of macromolecular metabolism by FV 3 is still observed at 37°C

Abbreviations : FV 3, Frog Virus 3 ; PFU, plaque forming units BHK 21, Baby hamster kidney cells ; CHO, Chinese hamster ovary cells ; DEAE, diethy-aminoethyl ; CKP, cell killing particle.

ISSN 0006-291X

(2, 5, 6). This suggests that shut off of cell functions by FV 3 does not require expression of the viral genome but is brought about by the structural viral proteins of the input inoculum. Additional evidence comes from the fact that neither gamma ray nor heat inactivation of virus infectivity abolishes shut off by FV 3 (5). Direct proof for the involvment of the structural viral proteins in the inhibitory effects has been obtained by showing that the solubilized viral proteins from FV 3 display inhibitory activities similar to those of the intact virion (7, 8). Logically enough, cell death must be the ultimate consequence of irreversible inhibition of host macromolecular metabolism. The degree of cytotoxicity of virus particles or solubilized viral proteins can best be evaluated by measuring the amount of material that is required to kill a single cell since the extent of inhibition of macromolecular metabolism varies with the time of infection and may conceivably be reversible in some instances whereas cell death is definitive. The results presented in this paper illustrate the potency of the FV 3 toxic proteins by demonstrating that cell killing by virus particles or the solubilized virus proteins follows one hit kinetics thus suggesting that a single viral particle or a single subunit from FV 3 may bring about cell death.

MATERIAL AND METHODS

Virus and cells

The production and purification of FV 3 (9) as well as the preparation of a soluble viral extract (10) containing FV 3 structural proteins have been previously described. Both the virus suspensions and the soluble viral extract were sterilized by filtration through sterile 0.22 nm nitrocellulose filters. The virus titer was determined in PFU by the plaque assay at 29°C on monolayers of BHK 21 cells. BHK 21 cells were grown in BHK medium (Eurobio, Paris) supplemented with 10% calf serum. CHO cells (CHO proline auxotroph originally supplied by Dr. L. Siminovitch) were obtained from Dr. J.L. Mandel and

grown in alpha medium (Eurobio) supplemented with 10% calf serum. In this medium the efficiency of plating was 50% the cell count.

Cell killing assay.

The loss of the cell's ability to give rise to a colony was chosen as a criterion for cell death. In experiments, monolayers of CHO cells (about 5.10^6 cells) were infected with 2 ml of medium containing various concentrations of a purified virus suspension or the soluble viral extract. DEAE dextran (Pharmacia) 100 µg per ml was included with the soluble viral extract in order to facilitate uptake of the soluble proteins. DEAE dextran alone had no effect on the efficiency of plating. After one hour of adsorption at room temperature, virus or the soluble viral extract was removed, fresh medium without DEAE dextran was added and the cultures were incubated at 37°C for four hours. After this period the medium was discarded and cells were removed from the culture dishes by rapid treatment with a solution containing trypsin (2 mg/ml) and ethylenediaminetetraacetic acid (0,15 mg/ml) in calcium, magnesium free phosphate buffered saline. The same number of cells (determined by counting in a hemocytometer) were recovered from uninfected and infected plates. Cell aggregates were disrupted by extensive pippetings and serial five fold dilutions were made. After each dilution cells were distributed into two 60 x 15 mm plastic dishes and incubated at 37°C. Seven days afterwards the colonies were fixed in methanol, stained with giemsa and count d.

RESULTS

CHO cells were infected with FV 3, incubated at 37°C for four hours and then plated for colony formation at 37°C. Virus multiplication was prevented by maintaining the cells at 37°C, a temperature at which FV 3 is uninfectious. A typical experiment giving the number of cell colonies obtained after infection with various concentrations of virus is presented in figure one. Cell survival is plotted on a logarithmic scale against the viral multiplicity. At low multiplicities of infection (less than 0.3 PFU per cell) there was a linear decrease in the fraction of cells surviving infection with increasing amounts of virus. This kind of relationship is characteristic of one hit kinetics and suggests that a single viral particle is sufficient to kill a single cell. When cell killing is considered, viral particles have been referred to as cell killing particles (CKP) (11, 12).

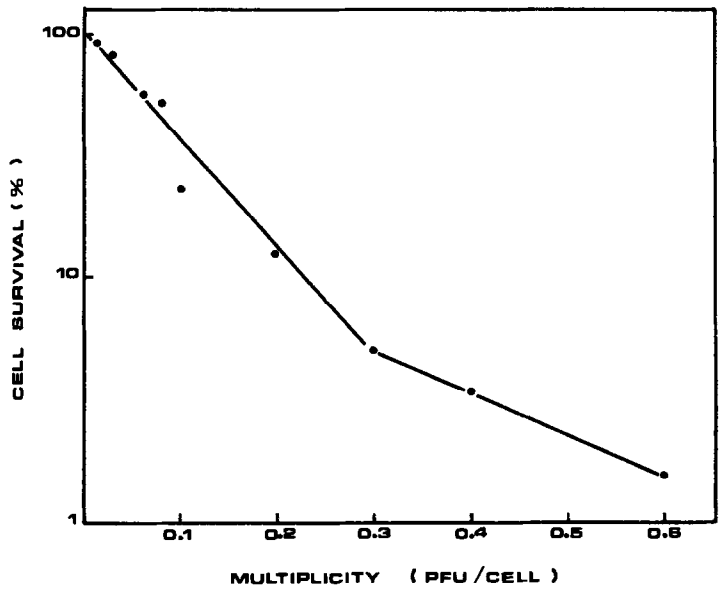

Figure 1 : Colony formation after infection with various multiplicities of FV 3.

CHO monolayers containing approximately 5.10^6 cells were infected with an ultrasonically treated virus suspension and processed as described in methods. The percentage of survival as measured by colony formation was calculated relative to the number of colonies obtained after a mock infection.

A CKP multiplicity of one is equivalent to the amount of virus necessary to decrease cell survival to 37%. According to figure 1, one CKP determined on CHO cells corresponds to 0.1 PFU. When the CKP multiplicity was assayed on BHK cells a similar ratio was found. These results suggest that the FV 3 suspension contains a large number of CKP that are defective in productive infection. However, one must take into account the fact that cell killing was assayed at 37°C whereas FV 3 plaques were made at 29°C therefore the difference between the CKP and PFU multiplicities may be partly due to a more efficient uptake of virus particles at 37°C.

At multiplicities of infection higher than 0.3 PFU per cell

the loss in survival no longer followed one hit kinetics (figure one). Thus, extrapolation of the second portion of the curve to the ordinate indicates that approximatively 15% of the cells are partially resistent to the cytotoxic effect of FV 3 and must be infected by more than one particle per cell in order to be killed. It seems unlikely that this was due to the presence of a sub-population of genetically resistent cells since CHO cells were clonally purified several weeks before the experiment. Appearance of mutations within the population could also explain the presence of a partially resistent cell fraction although the frequency would be extremely high. This has been ruled out also since several clones isolated among the survivors obtained after a high multiplicity of infection did not exhibit any phenotypic resistance. Therefore, 15% of the cells were transiently resistent to cell killing. Such transient resistance could be related to the physiological state of the cell although not governed by the cell cycle since experiments where cells were synchronized have failed to show any period of the cell cycle during which cells are not susceptible to the cytotoxic effects of FV 3 (unpublished results).

The extent of cell killing was also followed after treatment of the cells with various concentrations of the FV 3 soluble viral extract (figure 2). As obtained after virus infection, cell killing followed one hit kinetics thus suggesting that interaction of a single subunit of the FV 3 particle with a single cell can result in cell death. Work is currently being done to determine whether the cytotoxic viral subunit is a single polypeptide or an association of polypeptides. A small fraction of the cells (3%) were partially resistent to cell killing. Again, this was not genetic resistance since several clones surviving treatment with

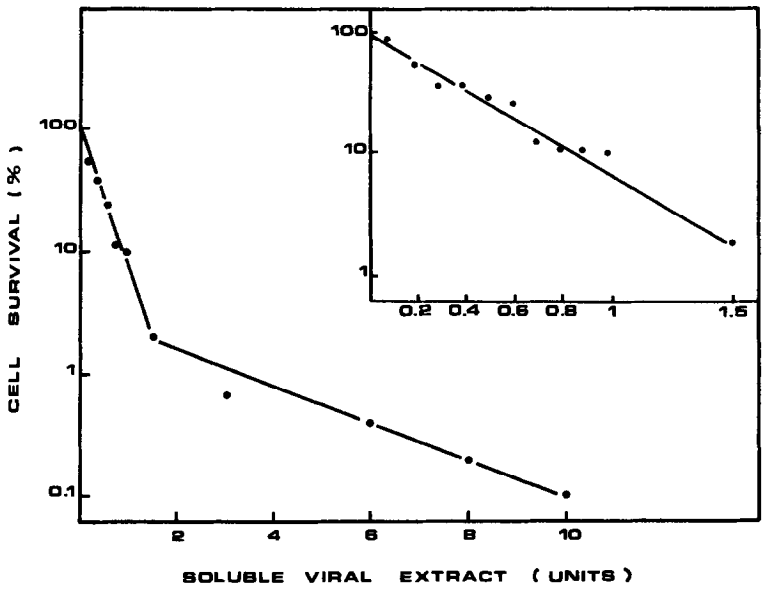

Figure 2 : Colony formation after treatment with the FV 3 soluble viral extract.

CHO cells were treated with various concentrations of the soluble viral extract and processed as described in methods. The concentration of the viral extract is expressed in arbitrary units (10 units correspond to approximately 1 mg protein per ml). The percentage of survival was calculated relative to the number of colonies obtained after mock treatment with DEAE dextran (100 µg per ml). The inset includes the results obtained with small doses of the soluble viral extract.

high doses have been isolated and found to be sensitive.

DISCUSSION

The results reported in this paper are to our knowledge unique in that evidence is presented for cell killing not only by single uninfectious viral particles but also by single toxic subunits obtained from a viral particle. Cell killing by single virus particles is not a surprising event when it is considered that toxic viral molecules accumulate in the cell during multiplication of the virus. This is the case for some if not most of the cytocidal viruses such as Newcastle disease virus (11,

12) or Vesicular stomatitis virus (13) where cell killing requires at least partial expression of the viral genome. In other cases as the one reported here and under suitable conditions as recently reported for Sendaï virus (14) the interaction of a cell with a single uninfectious virus particle may lead to cell death. The finding that single viral subunits of the FV 3 virion may also bring about cell death circumvents the problem of possible residual expression of inactive virus genomes and illustrates the potency of this toxic material. Equally sensitive cell killing effects are known to occur for other proteins as for example the effect of colicins on bacteria (15). Exactly how the toxic viral proteins bring about cell death remains to be solved however the fact that few molecules per cell need be involved suggests that a catalytic or amplifying mechanism of some sort occurs.

ACKNOWLEDGEMENTS :

We are grateful to Anne-Marie AUBERTIN for her advice and interest. This work was supported in part by a grant from the D.R.M.E.

REFERENCES :

1. Bablanian, R. (1975) Progr. med. Virol., 19, pp. 40-83, Karger, Basel.
2. Maes, R., and Granoff, A. (1967) Virology 33, 491-501.
3. Kucera, L.S., and Granoff, A. (1968) Virology 34, 240-249.
4. Granoff, A., Came, P.E., and Breeze, D.C. (1966) Virology 29, 133-148.
5. Gravell, M. (1969) Bacteriological Proceedings p. 182.
6. Guir, J., Braunwald, J., and Kirn, A. (1971) J. Gen. Virol. 12, 293-301.
7. Aubertin, A.M., Hirth, C., Travo, C., Nonnenmacher, H., and Kirn, A. (1973) J. Virol. 11, 694-701.
8. Aubertin, A.M., Travo, C., and Kirn, A., (1976) J. Virol. 18, 34-41.
9. Tripier, F., Markovic, Lj., Braunwald, J., and Kirn, A. (1975) Ann. Microbiol. (Inst. Pasteur) 126 B, 447-460.
10. Aubertin, A.M., Anton, M., Bingen, A., Elharrar, M., and Kirn, A. (1977) Nature (London) 265, 456-457.
11. Marcus, P.I., and Puck, T.T. (1958) Virology 6, 405-423.
12. Marcus, P.I. (1959) Virology 9, 546-563.
13. Marcus, P.I., and Sekellick, M.J. (1974) Virology 57, 321-338.
14. Toyama, S., Toyama, S., and Uetake, H. (1977) Virology 76, 503-515.
15. Jacob, F., Siminovitch, L., and Wollman, E. (1952) Ann. Inst. Pasteur 83, 295-315.