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Nucleolar Fragmentation in Cells Infected with Alphaviruses[1] (39886)

G. JOHN STANTON, LLOYD C. OSBORNE, AND T. B. ALBRECHT

Department of Microbiology, The University of Texas Medical Branch, Galveston, Texas 77550

Alphaviruses have similar chemical, physical, and replicative properties (1-3). The earliest time of appearance of obvious viral cytopathic effects with these viruses varies from 16 to 48 hr or more depending on the cell type used. This is the first demonstration that nucleoli of cells became fragmented 2 hr after infection with several members of this group. No other RNA viruses have been shown to have a similar effect on nucleoli.

Materials and methods. Viruses and cell culture. Laboratory stocks of Semliki Forest virus (SFV), Western equine encephalitis (WEE), Eastern equine encephalitis (EEE), Venezuelan equine encephalitis strain BTC1 (VEE), Sindbis strain AR-339 (SIN), and Chikungunya virus, African strain, were prepared in primary chick embryo cells (CEC). Also, the Fleming and Highlands J strains of WEE were obtained from the NIAID, research reagents section, and passaged twice in CEC prior to use in the experiments. The titer of all stocks was determined by plaque assay by the methods previously described (4), and, in all experiments, cultures were infected with 10-50 plaque-forming units of virus (PFU) per cell. Studies were performed either in monolayer cultures of CEC prepared in Leighton tubes containing coverslips or in monolayer cultures of CEC, BHK-21, WISH, U, L-929, Vero, Muntjac, or primary embryonic mouse cells prepared in tissue culture dishes (60 by 15 mm, Falcon Plastics). Cultures were maintained and virus dilutions were prepared in Eagle's minimal essential medium (MEM) containing Earle's salts supplemented with 2% fetal bovine serum (FBS) and antibiotics (100 μg/ml of streptomycin and 100 units/ml of penicillin). All incubations were at 37° in a 5% CO_2 atmosphere.

Electron microscopy. Forty-eight-hour primary CEC were infected with WEE or SIN virus at a multiplicity of infection (MOI) of 10 PFU/cell. After 10 hr, infected and uninfected cells were fixed with 3% glutaraldehyde in Millonig's phosphate buffer for 1 hr followed by 1% osmium tetroxide for 1 hr. The cells were washed and poststained with a saturated solution of uranyl acetate and then rapidly dehydrated in a series of ethanol solutions of increasing concentration. The samples were then embedded in Epon 812 according to the method of Luft (5) and sectioned with a diamond knife on a MT2-B ultramicrotome. The sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined and photographed with a Phillips 300 electron microscope.

Light microscopy. Cells were fixed in Bouin's fluid according to the method of Lee (6) and stained with hematoxylin and eosin (H&E). The staining procedure included 20-min staining in Harris' hematoxylin (7), 15-sec destain in 0.4% HCl, and 10-min staining in an eosin-phloxine solution. For Giemsa staining, cells were fixed for 5 min in methanol and stained with a 1:2 dilution of Giemsa stain obtained from Gradwohl Laboratories. Specimens were examined and photographed with an Axiomat (Carl Zeiss) microscope using planapochromat objectives (50/1.00, 100/1.4).

Results. Fragmentation of cell nucleoli by SIN virus. Electron micrographs of CEC infected with SIN virus for 10 hr clearly showed that cell nucleoli were fragmented (Fig. 1a). Although the nucleoli were fragmented, the rest of the cell nucleus appeared normal, and fragmentation was not observed in either noninfected cells (Fig. 1b) or cells infected with WEE virus (Fig. 1c) at the same MOI (10 PFU/cell). However, the nucleoli of cells infected with WEE virus for 10 hr did appear more dense than nucleoli of uninfected cells, with fewer light areas, which suggests that the infection

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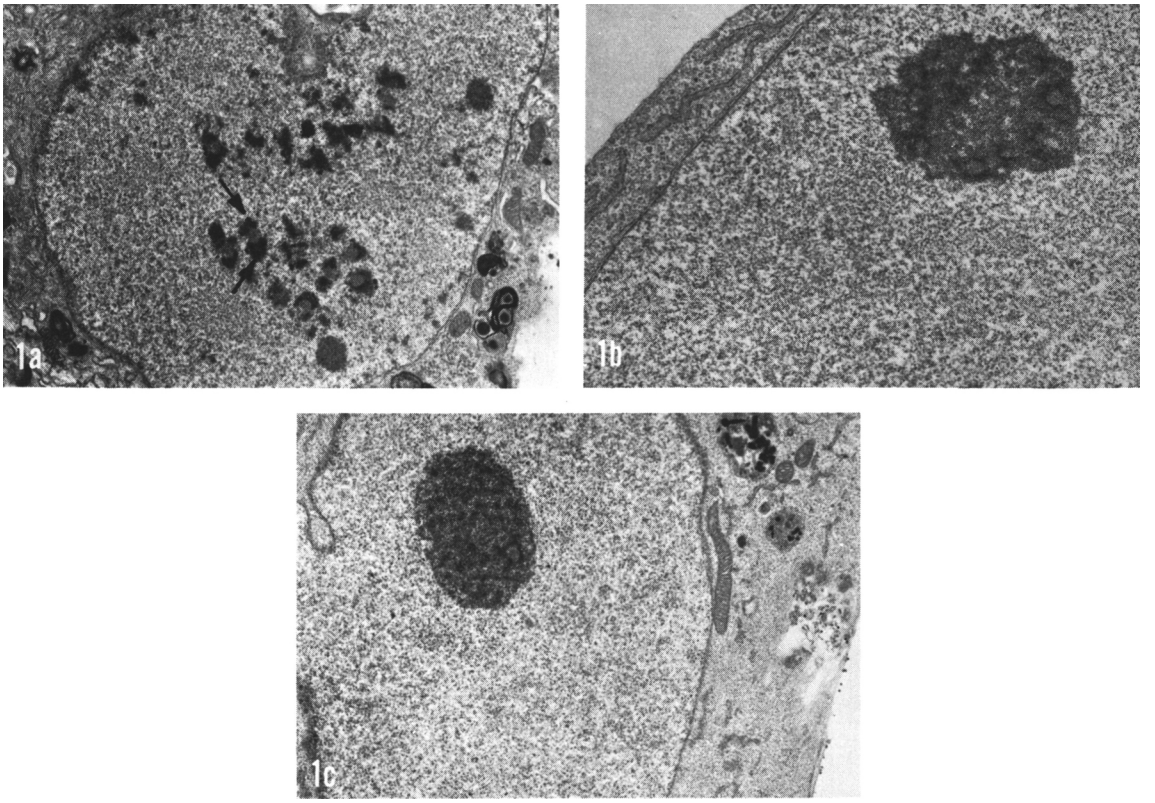

FIG. 1. (a) Nucleus of chick embryo cell infected with SIN virus, 10 hr postinoculation. Nucleolus is fragmented, and some fragments contain light and dark nucleolar "caps" (arrows) attached to a central body. (b) Nucleus of noninfected chick embryo cell. Nucleolus appears normal; fibrillar and granular components are typical of a normal cell. (c) Cell infected with WEE virus for 10 hr. Nucleolus remains intact, but appears more dense than nucleoli of noninfected cells.

may have affected these nucleoli, though in a different manner than SIN. Although electron micrographs of nuclei of cells infected with either WEE or SIN viruses were carefully examined for evidence of virions or nucleocapsids, none could be found. Studies using light microscopy gave similar results.

Nucleoli of cells infected with SIN virus and stained with H&E 10 hr postinoculation (PI) were fragmented. However, nucleoli of cells infected with any of three strains of WEE virus appeared indistinguishable from those of cell controls (Fig. 2a), suggesting that, at least in the case of WEE virus, the ability of viruses to fragment nucleoli appears to be independent of the virus strain.

Fragmented nucleoli were also observed following Giemsa or H&E staining of L-929 BHK-21, WISH, U, Vero, Muntjac, or primary embryonic mouse cells infected with SIN virus at 10–50 PFU/cell. Thus, fragmentation appears to be independent of the cell type used. At MOIs less than 10, the proportion of cells with fragmented nucleoli was a function of the MOI.

Fragmentation of nucleoli by other alphaviruses. Because WEE and SIN virus differed in their ability to cause nucleoli to fragment, other members of the alphavirus group were investigated for their abilities to cause fragmentation. Tube cultures of CEC infected with VEE, EEE, SFV, or Chikungunya virus were fixed with Bouin's solution 8 hr after virus infection, stained with H&E, and examined for nucleolar fragmentation. Cells infected with VEE or WEE virus had nucleoli indistinguishable from those of uninfected cells. Fragmented nucleoli similar to those seen in SIN virus-infected cells were observed in cells infected with SFV, Chikungunya, and EEE virus.

Kinetics of nucleolar fragmentation. Since

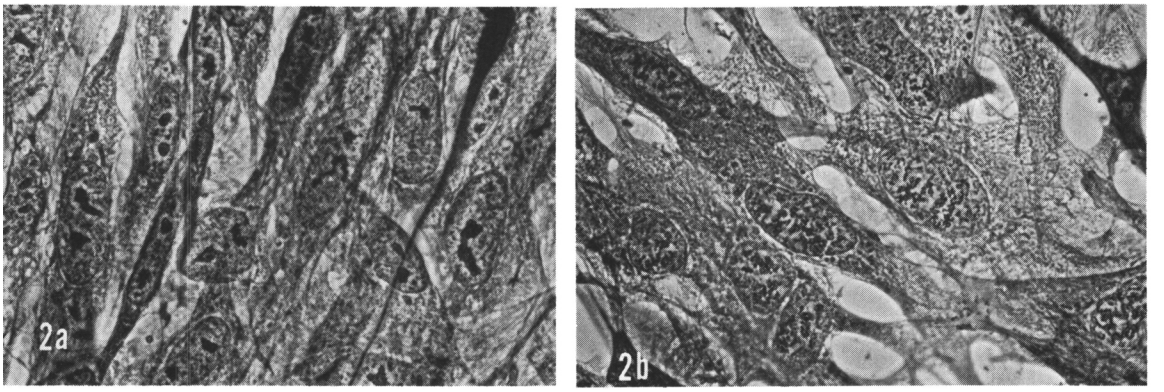

FIG. 2. (a) Noninfected chick embryo cells (CEC) with normal nucleoli. (b) CEC 3 hr after inoculation with SIN virus. Numerous small nucleolar fragments are dispersed throughout the nucleus.

several alphaviruses caused cell nucleoli to fragment, it was of interest to determine the kinetics of nucleolar fragmentation. Cultures infected with SFV, SIN, EEE, Chikungunya, WEE, and VEE virus and noninfected control cultures were fixed at hourly intervals up to 10 hr PI, stained with H&E, and the nucleoli were examined for fragmentation by light microscopy. Nucleolar fragmentation in cells infected with SFV or SIN progressed from a few large fragments at 2 hr PI to numerous small fragments at 3 hr PI (Fig. 2b). In cells infected with EEE or Chickungunya virus a similar course of fragmentation began at 4 hr PI and continued up to 6 hr PI. Nucleolar morphology in WEE or VEE virus-infected cultures 10 hr PI still resembled that of cell controls.

Discussion. Little is known about the mechanisms of early viral effects on cells and the dynamic molecular events required to maintain nucleolar organization and structure because of the lack of suitable model systems to study.

This is the first demonstration that certain alphaviruses (SFV, SIN, EEE, and Chikungunya viruses, but not WEE or VEE viruses) caused early and rapid disruption of nucleoli in infected cells, forming fragments which appeared to be scattered throughout the nucleus. No other nuclear abnormalities were detected, including the presence of distinguishable virions or nucleocapsids. Since fragmentation occurred in CEC, BHK-21, WISH, U, L-929, Vero, Muntjac, and primary embryonic mouse cells, it does not appear to be cell specific. At a MOI of less than 10 PFU/cell, the proportion of cells with fragmented nucleoli was a function of the MOI, but fragmentation was not enhanced by MOIs greater than 10 PFU/cell. Thus, some alphaviruses appear to have an early unique effect on a process responsible for maintaining nucleolar integrity which is common to most, if not all, cell types. This property could be used to subdivide the alphavirus group.

Although several other viral and chemical agents are known to have specific effects on nucleolar morphology, to our knowledge, none of these is able to fragment nucleoli in the manner observed in cells following alphavirus infection. For example, actinomycin D caused separation of fibrillar and granular components of nucleoli, resulting in light and dark nucleolar "caps" attached to a central body (8). Other chemical agents and some RNA- and DNA-containing viruses had different effects on nucleoli (9), but, in all cases, each nucleolus appeared to remain intact. It was interesting that, although actinomycin D did not fragment nucleoli, the morphology of individual nucleolar fragments in SIN virus-infected cells resembled whole nucleoli of cells treated with actinomycin D (Fig. 1). The significance of this is not known.

The early (2-hr) appearance of nucleolar fragmentation, possibly an hour or two prior to the times that alphaviruses have shut down the synthesis of cell protein and nucleic acid and before release of progeny virus, raises the possibility that this system could be used as a model for studying mechanisms of early viral effects on cells and the dynamic molecular events required to main-

tain nucleolar organization. It is possible that a component of the infecting virus or an early viral or cell-coded protein (i) adversely affects the nucleolar organizer genes, (ii) affects the transport of some substances (e.g., proteins or nucleotides) which are essential for nucleolar integrity, or (iii) directly deletes, competes with, or blocks substances in the nucleolus which normally help maintain the intact nucleolar function and/or structure. Since fragmentation occurred early and proceeded rapidly, components of the infecting virus or early viral proteins which are apparently not common to all alphaviruses would appear to be responsible for the observed nucleolar fragmentation. It is noteworthy that no other RNA viruses have been reported to cause this type of fragmentation. Studies are currently being pursued in this laboratory to elucidate the responsible mechanism(s).

Summary. It was observed that nucleoli of chick embryo cells infected with certain alphaviruses fragmented before the cells themselves showed any other cytopathic effects. The nucleoli in cells infected with SFV or SIN virus were fragmented 2 hr after inoculation, but fragmentation was first observed 4 hr postinoculation in cells infected with EEE or Chikungunya viruses. No fragmentation was observed with VEE or any of three strains of WEE virus. These results were obtained with electron and/or light microscopy. Fragmentation was observed in a variety of other cells and cell lines infected with SIN virus. The fragmentation was independent of the multiplicity of infection (MOI) at MOIs greater than 10. No other RNA viruses have been shown to cause this type of fragmentation. Since fragmentation occurred early and proceeded rapidly, components of the infecting virus or early viral proteins which apparently are not common to all members of the alphavirus group appear to be responsible for this fragmentation.

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