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Apoptosis as a Cause of Death in Measles Virus-Infected Cells

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To determine the mechanism of measles virus-induced cell death, we studied the infection of Vero cells and monocytic cell lines with wild-type (Chicago-1) and vaccine (Edmonston) strains of measles virus. DNA fragmentation indicative of apoptosis was apparent by flow cytometry, agarose gel electrophoresis, and electron microscopy. Within syncytia, DNA strand breaks were demonstrated by end labeling with terminal transferase and then by visualization.

A number of viruses have recently been shown to cause cell death by induction of apoptosis (10, 15, 18, 21, 24, 31). Often, viruses that cause persistent infection have developed mechanisms to prevent or delay the induction of apoptosis or infect cells resistant to virus-induced cell death (2, 11, 27). Measles virus, a linear minus-stranded RNA virus belonging to the paramyxovirus family, causes both lytic and persistent infections in vivo and in vitro. In contrast to many of the previously studied viruses, the characteristic cytopathic effect of measles virus infections in vitro and in vivo is the production of syncytia, or multinucleated giant cells (8, 12).

In vivo, multinucleated giant cells are most frequently seen in lung tissue but can be seen in other organs as well (8, 28). The primary cells infected by measles virus in vivo are monocytes and endothelial and epithelial cells (9, 22). To determine how measles virus causes cell death and the relationship of syncytium formation to cell death, we have studied the interaction of a vaccine and a wild-type strain of measles virus with monocytic and epithelial cells in vitro. These studies show that measles virus induces apoptosis of infected cells and that nuclei of cells recruited into syncytia are initially normal and then develop evidence of endonucleolytic cleavage of chromosomal DNA.

This endonucleolytic cleavage and condensation of nuclear chromosomal DNA can be observed by flow cytometric analysis of the cells (4). Vero cells (American Type Culture Collection, Rockville, Md.) were inoculated with either Edmonston (American Type Culture Collection), the standard laboratory strain from which current measles virus vaccines are derived, or Chicago-1 (multiplicity of infection [MOI], 0.01 to 0.1 PFU per cell), a recently isolated wild-type strain of measles virus (29). At 24-h intervals, 10^6 cells were harvested, resuspended in media, and stained with DNA-binding Hoechst 33342 (Molecular Probes, Eugene, Oreg.; 10 μM) fluorochrome for 30 min at 37°C. Cells were analyzed with a Becton Dickinson FACstar^{PLUS} flow cytometer with UV excitation at 360 nm. Forward and side light scatter were analyzed at a cell throughput rate of 200 cells per s. Data from 30,000 cells were collected in ungated form and analyzed in a gated form for viable cells. Histograms measuring fluorescence on the *x* axis and cell count on the *y* axis were generated. Cell viability was determined by trypan blue exclusion at each time point.

Uninfected Vero cells stained at 24-h intervals with Hoechst 33342 showed the typical pattern of DNA in dividing cells with two distinct peaks of G_0/G_1 and G_2 nuclei and an intervening S phase (Fig. 1). Measles virus-infected Vero cells showed sub-G_0/G_1 peaks on day 4 (Edmonston) and day 6 (Chicago-1) after infection (Fig. 1). At these times, more than 90% of the cells continued to exclude trypan blue (data not shown). The presence of this distinct cell cycle region has been shown previously to represent DNA fragmentation in cells undergoing apoptosis (4, 5, 20). Fewer than 4% of control cells were present in this region of the histogram, while in measles virus-infected cells 25 to 30% of the total events counted occurred in this region.

Infected and uninfected Vero cells were studied by electron microscopy. Vero cells were grown to subconfluence on 35-mm-diameter petri dishes and infected at an MOI of 0.01 to 0.1 PFU per cell. At 24-h intervals, aliquots of cells were stained with trypan blue and subjected to flow cytometric analysis as stated above. At the times represented in Fig. 1, when all cells even at low MOIs should have been infected, cells were fixed, rinsed, and postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate. En bloc staining was performed with 2% uranyl acetate. Sections (80 nm thick) were cut and stained with lead citrate and were then viewed on a Zeiss EM10 transmission electron microscope.

The uninfected Vero cells appeared healthy (Fig. 2A), while measles virus-infected Vero cells showed distinctive condensation and margination of chromatin, with mitochondrial preservation characteristic of apoptosis (Fig. 2B). The helical nucleocapsid structure of measles virus was readily seen in the cytoplasm of some cells which also showed the membrane-associated condensed chromatin (Fig. 2B). Cells fragmented into apoptotic bodies (clusters of membrane-bound segments), some of which contained dense balls of chromatin (data not shown).

Classically, nuclei with condensed chromatin on morphologic observation contain low-molecular-weight DNA of 180- to 200-bp oligonucleosomal fragments which can be resolved by agarose gel electrophoresis (35). Vero cells were grown on T75 (75-cm^2) tissue culture flasks (Corning, Corning, N.Y.). Also, since measles virus replicates in monocytes in vivo (9), we examined the effect of measles virus infection on two relevant cell lines: THP-1, a human monocytic cell line (32), and HL-60, a promyelocytic cell line (3) (American Type Culture Collection), which were grown in suspension in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were infected with measles virus at MOIs of 1 to 10 and were harvested at 24-h

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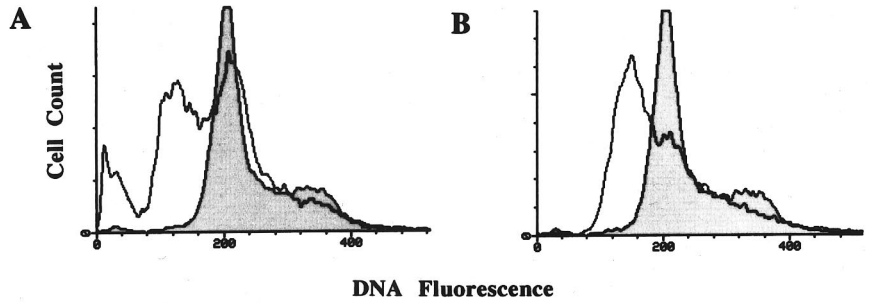

FIG. 1. Cytometric analysis of Vero cells infected with measles virus. The open histograms represent Vero cells infected with Chicago-1 (MOI, 0.01 on day 6 after infection) (A) and Edmonston (MOI, 0.1 on day 4 after infection) (B) strains of measles virus; the shaded histograms represent uninfected Vero cells at the same time points. The sub-G_0/G_1 peak is seen only for the infected cells.

intervals. Prior to gel electrophoresis, cells were stained with trypan blue to ensure greater than 90% viability. Aliquots of 10^6 cells were pelleted prior to analysis by the gel electrophoresis method adapted from that of Eckhardt (7).

DNA fragmentation was seen both in well-differentiated (THP-1) and poorly differentiated (HL-60) monocyte cell lines 24 h after infection with the Chicago-1 strain of measles virus (MOI, 1 to 10) (Fig. 3). Cellular DNA from both Edmonston- and Chicago-1-infected Vero cells also revealed the characteristic cleavage pattern at 48 h after infection with virus at an MOI of 1 (data not shown).

Nuclei undergoing apoptosis can be identified by in situ end labeling of DNA strand breaks with terminal transferase (1). To determine whether any or all of the nuclei within the measles virus-induced syncytia showed evidence of apoptosis, we applied this technique to Vero cells infected at a low multiplicity with Chicago-1 (Fig. 4). Cultures of 2×10^6 Vero cells were grown to subconfluence on two-well Lab-Tek chamber slides (Nunc, Inc.), infected (MOI, 1), and fixed in 4% paraformaldehyde. At 24 and 48 h after infection, 40 μl of DNA end-labeling mixture (30 mM Tris [pH 7.2], 140 mM potassium cacodylate, 1 mM cobalt chloride, digoxigenin-dUTP [0.25 nmol/20 μl], and terminal deoxynucleotidyl transferase [12.5 U/20 μl], all from Boehringer Mannheim) was placed on each side of the chamber slide and incubated for 60 min at 37°C in a humid chamber as previously reported (1). Slides were rinsed in 2× sodium citrate saline (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min, in buffer 1 (100 mM Tris, 150 mM sodium chloride [pH 7.5]) for 1 min, and then in buffer 1 with 2% normal rabbit serum and 0.2% Triton X for 30 min. Portions (100 μl) of the antidigoxigenin antibody (Boehringer Mannheim; 1:500 dilution in buffer 1) were placed on each side of the chamber slides for 30 min at room temperature. The slides were then rinsed in buffer 1 for 10 min and in buffer 2 (100 mM Tris, 100 mM sodium chloride, 50 mM magnesium chloride [pH 9.5]) for 10 min. Portions (500 μl) of the color solution (45 μl of nitroblue tetrazolium, 35 μl of X-phosphate [5-bromo-4-chloro-3-indolyl phosphate; Boehringer Mannheim], 2.4 mg of levamisole [Vector Laboratories, Inc., Burlingame, Calif.] added to 10 ml of buffer 2) were incubated overnight. The slides were washed in buffer 3 (10 mM Tris, 1 mM EDTA [pH 8.0]) for 10 min, counterstained with eosin, dehydrated, and mounted. Uninfected control slides were processed simultaneously.

Nuclei within the center of the syncytium were labeled with terminal transferase, while adjacent nuclei were not (Fig. 4). In larger syncytia, central nuclei showed staining while nuclei at the periphery of the giant cell did not (data not shown). This result suggests that cells which have not begun the process of DNA fragmentation (a relatively early step in apoptotic cell death), and possibly even cells which are not infected, are being actively recruited into these multinucleated giant cells.

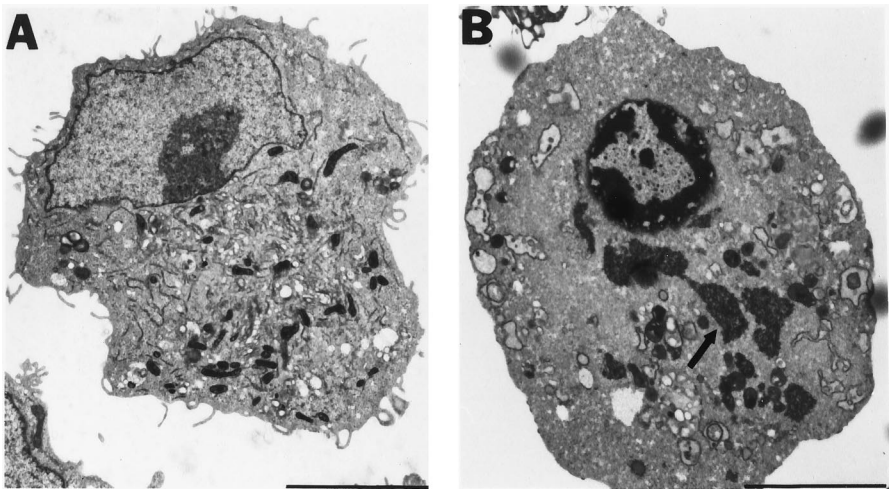

FIG. 2. Electron microscopy of uninfected and infected Vero cells. (A) Normal Vero cell. (B) Chicago-1-infected (MOI, 0.01 on day 4) Vero cell. Margination and condensation of chromatin can be seen in the Chicago-1-infected cell (B), with the helical nucleocapsids of measles virus apparent in the cytoplasm (arrow). Magnification, ×4,000; bar, 5 μm.

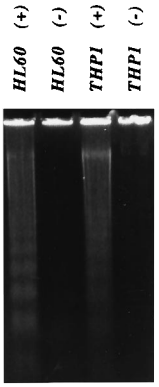

FIG. 3. Agarose gel electrophoresis of Chicago-1-infected (+) and uninfected (−) monocyte cell lines in vitro. Lanes 1 and 2, HL-60 cells; lanes 3 and 4, THP-1 cells. The infected cell lines reveal the characteristic DNA cleavage pattern of apoptosis.

When Enders and Peebles first isolated measles virus in 1954, they described the cytotoxicity as including a "'redistribution of the chromatin,'" which then "assumed a marginal position where it formed a dense ring or crescent" (8). In 1958, examination of autopsy tissue obtained from patients dying with measles revealed condensed peripheral chromatin and shrinking and clumping of the chromatin at the base of the syncytial cytoplasm (30). Additionally, Nichols et al., studied chromosome damage with measles virus in vitro in 1965 and reported pulverization of chromosomes particularly within the syncytia (23). Nearly four decades later, we can verify that these observations are associated with apoptosis (6, 16, 17, 34, 36), now known to be the mode of cell death in a variety of infections (2, 10, 15, 18, 21, 24, 27, 31, 37).

The role of the induction of apoptosis in the outcome of clinical measles is not known. In a previous study, correlation of numbers of monocytes in peripheral blood with numbers of infected monocytes suggested measles virus-induced monocyte death (9). It is not known whether this is the outcome of infection in all individuals or in all monocytes. A variety of immunologic and nutritional factors influence the course of clinical measles (33) and may influence cellular susceptibility to the induction of apoptosis. For instance, the cytokine interleukin-10 induces expression of the antiapoptosis protein bcl-2 in B cells (19), suggesting a role for the immune response in cell survival. Retinoic acid abrogates the induction of apoptosis in hematopoietic cells (25), T-cell hybridomas, and thymocytes (14). Since vitamin A deficiency is associated with an increased severity of measles (26) and vitamin A supplementation decreases measles mortality (13), it is possible that protection of cells from measles virus-induced apoptosis is one mechanism by which vitamin A decreases the severity of the disease.

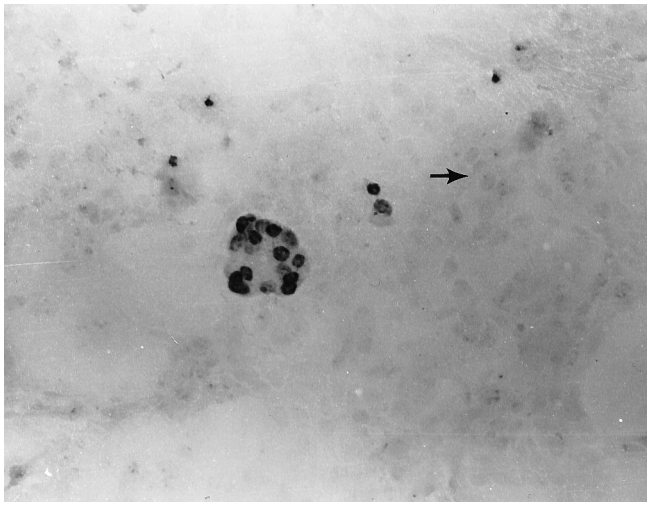

FIG. 4. In situ end labeling of DNA strand breaks within a large syncytium in a Chicago-1-infected Vero cell monolayer. Dense staining is seen in many of the nuclei at the center of the syncytium, while many of the nuclei near the intact monolayer are negative (arrow). Magnification, ×64.

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