Volume and surface changes in vero cell and its nucleus after infection with measles virus: a stereological study

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Abstract

Measles virus has no or indistinctive cytopathic effects (CPE) in cell culture system. Employment of some detecting methods like plaque assay or stereologic experiments, as a method of detecting viral infection in the cells would be applicable. The aim of this study was investigating the early changes in quantitative parameters of measles virus infected Vero cells. Stereological methods using invarian, were applied for the first time to estimate cell and nucleus volume and surface of the infected Vero cell line with the measles virus. This method can be applied on other cultured cells. Vero cells grown in tissue culture plates for 48 hours at 36°C were infected with 100TCID50 of AIK strain of measles virus. Volume and surface of the infected Vero cells were studied at 4, 9 and 25 hours post infection along with uninfected control cells. The mean cell volume and surface of the cells infected with measles virus, increased ~87% and ~50%, respectively, 4 hours post-infection, as compared with the uninfected control. The nuclei did not show any differences. The mean parameters of infected cells in other time intervals showed no significant difference comparing with the control cells. Although there are other specific methods, stereology may be used as an integrated protocol to detect cytopathic changes of the measles virus infected cells early in the permissive cell culture system.

Introduction

Measles virus is a member of genus morbilivirus, subfamily paramyxovirinae, family paramyxoviridae and the order mononegavirales. It causes measles, a classical childhood disease, in humans either typical or atypical. Some complications are encephalitis (1:1000) with 25% of permanent neurologic impairments and subacute sclerosing panencephalitis (SSPE), occurring in individuals under 20 years of age, also caused by the measles virus (1 per 1 to 3 million) with progressive neurologic deterioration, failing memory and myoclonus.1,6

Although typical measles is diagnosed on clinical grounds, its laboratory diagnosis is necessary in modified or atypical cases. In cell culture, as a classical method of virus isolation, measles virus grows slowly and the isolation is a difficult process in this system.4,7 The typical cytopathic effect (CPE) of measles virus is giant cells formation with multiple nuclei along with both intra-nuclear and intra-cyttoplasmic inclusion body in the infected Vero monolayer.5,6 Intranuclear Cowdry type A inclusion bodies, which are characteristic of morbilivirus infections, occur late in infection.6 However such a CPE takes at least 7-10 days to be produced during which time the general condition of inoculated monolayers and even uninfected control monolayers may deteriorate unless fresh medium is maintained. Sometimes CPE appears after several passages.5,6 Lacking or indistinctive CPE of measles virus has led to the employment of some detecting methods like plaque assay. Stereologic evidence of CPE appearance of Herpes Simplex virus (HSV) as a method of detecting CPE has been reported.7 Therefore the aim was to investigate early changes in quantitative parameters of measles infected cell line before marked changes that may be observed later in the infection. Continuing the pervious study of presenting stereological data for HSV for the first time, measles virus infected Vero cells with late production of obvious CPE. The present method also might be useful to study any early changes in stereological parameters of normal cells before marked changes observed later in the infection with measles virus as a slow producer of CPE. No data is available about any quantitative parameters for measles virus and herein, a method is a new stereological principle for volume and surface estimation of the measles infected Vero cell line. Invariatior as the method of volume and surface estimation was introduced by Cruz-Orive in 2005 and has not been used in biological research widely, but the method is an unbiased estimator of single particle volume and surface.10,11 Although there are methods of cell volume estimation like nucletor and rotator,12,13 a local practical estimator of cell surface had not been presented. Here the method of Cruz-Orive,10,11 using pivotal points, intercepts and intersects has been applied on Vero cell lines.

Materials and Methods

Cell cultures

The Vero cell culture obtained from the Cell Bank of Pasteur Institute, Tehran, Iran, was grown in Dulbecco Minimal Essential Medium (DMEM), supplemented with 5% fetal bovine serum (FBS) from GIBCO. The maintenance medium (MM) was similar but with 2% FBS. All media contained 100 IU/mL of penicillin and 100 μg/mL of streptomycin sulphate.

Virus stock

Measles virus (AIK strain) purchased from Razi institute, Tehran, Iran, was grown in Vero cell culture to a titer of 102.5 TCID50 which was determined by the method of Kärber, 1931.14

Vero cell infection with measles virus

Vero cells grown in tissue culture plates (Nunc, Denmark) in triplicates under 5% CO2 for 48 hours at 36°C were infected with 100 TCID50 measles virus, followed by 45 minutes adsorption at room temperature before addition of maintenance to the cell monolayers. At 4, 9 and 25 hours following the incubation period, tripsinized contents of a triplicate of plates of control and test experiments, were pooled in 1.5 ml Eppendorf tubes and centrifuged at 3000 RPM for 10 minutes and fixed with neutral buffered formaldehyde for one week. Controls of the experiment included uninfected or normal cell monolayers which were harvested in the time intervals as mentioned above.

Stereolar study

The cells were processed by pouring ascend-
ing grades of ethylic alcohol (70%, 80%, 96% and two steps of 100% ethylic alcohol, respectively) into the tube containing the cells. Then xylol and warm paraffin were subsequently poured into the tube. Each step took 15 min. After cooling the tube in a refrigerator, the small paraffin block was pulled out from the tube. To estimate the cell and nucleus volume and surface, isotropic uniform random (IUR) sectioning of the cells is required. We used Isector to achieve IUR sections. Briefly, the small pulled out paraffin block was trimmed to make a nearly spherical model.

Then it was re-embedded randomly in a rectangular mold such that the whole specimen would end up in an isotropic orientation. Uniform random sections through the rectangular block represented IUR sections through the object. Using a micrometre, the sections with 15 μm thickness were obtained consequently. The sections were stained with Heidenhain’s trichrom azan. Usual hematoxillin and eosin (H&E) also can be applied. Microscopic fields were selected in each section in a systematic random manner. The position of the first area was selected randomly outside of the section and the other areas were selected by moving the microscope stage in an equal interval along the X- and Y-direction of stage using a stage micrometer. A high numerical aperture, 100x magnification (NA=1.4) oil immersion lens was used. A lens with this numerical aperture provided better image quality. Estimation of the nucleus and cell volume was performed at the final magnification of x600.

The method is an estimator of number-weighted mean particle volume and surface. It means selection of cells according to their numerical density in a three-dimensional space. To do this, the cells must be selected with uniform random probability using the disector principle. The optical disector is a method in which the cells are sampled in thick sections observed with a light microscope. A microcator (MT12, Heidenhain, Germany) mounted on the microscope was used to measure the movement of the stage in the z-axis. An optical disector design was used to sample the tissue in conjunction with an unbiased sampling frame. At each step, the first 3 μm was traversed as a guard area against cutting artifacts and then the nuclear profiles of the cells were counted in the next 10 μm of the 15 μm section. Using the disector, the particle is selected according to their numerical density and not according to their shape, size, and volume.

In the 15 μm thick sections, the optical section was set at a random depth. A computer and a monitor were connected to a color video camera mounted on top of the microscope. By means of a stereological software (designed at Shiraz University of Medical Sciences), a test system of quadrangles was superimposed on the images of the tissue sections viewed on the sections observed with a light microscope. Briefly, the cells whose nucleolus did not touch the left and inferior borders of the each quadrangle of the test system, was qualified to estimate the number-weighted mean volume and surface. Each sampled nucleolus was adopted as the pivotal point.

The uniform random test system of quadrangles, with predetermined area was laid on the image of the cell.

Through each pivotal point, a test line (blue lines in Figure 1) was drawn perpendicular to the axis joining the points of the test system with pivotal point (red lines in Figure 1). It was needed only to generate those point-sampled test lines that would hit the sampled cell and nucleus.

The intercepts lengths and number of intersections (filled red points in Figure 1) with cells (or nucleus) were estimated. The equations $V = aL$, and $S = 2aI$ were used to estimate volume ($V$) and surface ($S$) of the cells, where $a$ is area per test point (here 36 μ²), $L$ is the sum of the intercept lengths and $I$ is sum of the number of intersections of the vertices with the cell or nucleus borders. Nucleator method as described previously can also be applied for estimating cell volume. Nucleator is quicker than invariator but not applicable for surface estimation.

### Statistical analysis

Experiments were performed at least three times in triplicates. Analysis of variances and Dunnett T-test were used for comparison of test results with those of controls in the other experiments. Results were considered significant when $P < 0.05$.

### Results

The mean cell and nucleus volumes and surface of measles virus infected in different time intervals are shown in Table 1. Mean cell volume and the cell surface of the infected cells, only 4 hours post infection increased significantly ($P < 0.001$) as compared with the unininfected control. Such increase was −87% and −50%, for cell volume and surface respectively. The nuclei did not show any changes in their volume or surface.

The mean parameters of infected cells in other time intervals showed no significant difference comparing with the control cells.

### Discussion

In this study stereological data showed the significant changes in the cell surface and volume of the Vero cells infected with the measles virus as early as 4 hours after infection. At a later time no significant changes were detected.

Figure 1. Estimation of the surface and volume of the Vero cell which is sampled by an optical disector. A quadrangular test system with determined area per point (a) was overlaid on the image of the sampled cell. Through the pivotal point (nucleolus), a test line (blue lines) was drowning perpendicular to the axis joining the points of the test system with pivotal point (red lines). The intercepts lengths (L) and number of intersections (I) (filled red points) with cells (or nucleus) were estimated. The volume ($V$) and surface ($S$) of the cell and nucleus were estimated by $V = aL$, and $S = 2aI$.

<table>
<thead>
<tr>
<th>Table 1: Mean (standard deviation) cell and nucleus volume ($\mu^3$) and surface ($\mu^2$) of vero cells 4, 9, 25 hours after infection (HrAl) with measles virus in control and experimental groups.</th>
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<tbody>
<tr>
<td>Groups</td>
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<tr>
<td>Control 4HrAl</td>
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<td>Control 9HrAl</td>
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<td>Control 25HrAl</td>
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<td>Experimental 4HrAl</td>
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<td>Experimental 9HrAl</td>
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<td>Experimental 25HrAl</td>
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*P < 0.001 Experimental 4HrAl vs. Control 4HrAl.
ed stereologically. Paradoxically, as stated before, marked morphologic changes in the measles virus infected cell, if observable, would be obviously observed after 7-10 days after infection in cell cultures. So, similar to the previous stereological study on HSV infected cells, early diagnosis of infection using cell volume can be considered as a stereological criterion for the early diagnosis of measles virus. Similar to the previous study on HSV, maximum increase was observed in the volume of infected cells early after infection with measles virus, i.e. 4 hours post-infection and interestingly, in contrast, in this study no increase was observed in the later time intervals. Also a 50% cell surface increase was observed significantly in measles virus infected Vero cells.

The increase in cell surface and volume and not in the nuclear volume can be explained with the fact that the measles virus replicates in the cytoplasm of the host cell and produce syncytia in the infected monolayers as a characteristic CPE for this virus. It would be an interesting stereological early finding of infection with virus strains with no CPE, or when CPE is not observable, for example according to long time required for observing measles CPE.

There are a number of laboratory methods for the diagnosis of measles, of which detection of measles virus specific serum IgM antibodies remains the gold standard. Virus detection is an equally valid method, but measles virus has limited sensitivity. There are some specific methods for rapid measles virus detection such as shell vial cell culture using indirect immunofluorescence which is completed in 2-3 days or RT-PCR to detect virus replication. In the present study, stereological parameters were obtained using this method which is inexpensive and easier compared to molecular methods. Measles Virus isolation using tissue culture techniques is complicated, however transferring the infected cells on a microscopic slide and estimation of cell volume and surface enable the investigator to detect CPE changes, just 4 hours post-infection.

It should be noted that this novel method was applied for cell surface estimation. The method of volume and surface estimation was introduced by Cruz-Orive in 2005, and has not been used in biological research widely. The method is an unbiased estimator of particle volume and surface. Using isotropic uniform random section, pivotal point surface estimation of a single object is also possible.

The nucleator method was applied in the previous research, that technique is a good estimator of object volume and not the surface. The present technique makes the surface estimation of single objects also possible.

Conclusions

The stereological methods in this study showed that the mean cell and surface volume of the measles infected cells increased very soon (at 4 hours) after the infection as compared with uninfected controls. This finding may be applied to the early diagnosis of measles virus infection of cell cultures which appear as CPE so slowly, or in absence of other means of rapid viral diagnostics with no significant CPE in cell culture such as the rubella virus.

Although there are some other specific methods, stereology might be used as an integrated protocol to detect CPE changes in the measles infected Vero cells in culture systems at early stages even if CPE can not be detectable by inverted microscope finally.

References