Viral Identification by Scanning Electron Microscopy of Preparations Stained with Fluorescein-Labeled Antibody

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A new technique combines the specificity of fluorescent-antibody labeling and the resolution of the scanning electron microscope to identify and distinguish between viruses. Hemagglutination of chicken erythrocytes by influenza virus was used as a model system to demonstrate the technique.

Microscopy, as a tool for the biologist, is improved when independent but parallel means to identify the structures visualized are available. Fluorescence microscopy utilizes fluorescein-labeled, specific antibodies to detect cell-associated antigens (4, 11), but interpretations are limited by the relatively low magnification and resolution obtainable. The transmission electron microscope provides adequate magnification and resolution, and elegant immunological techniques have been developed to identify cellular structures, metabolic products, and viruses. Ferritin-labeled antibodies (12) and the anti-hapten antibody bridge methods (L. Wolf-sy, P. C. Baker, K. Thompson, J. Goodman, J. Kimura, and C. Henry, *J. Exp. Med.*, in press) have been successfully used to localize and identify antigens on the cell surface. However, the small sample size that can be observed and the need to use ultrathin sections make it difficult to determine the distribution, organization, and numbers of specific antigens on a single cell as well as within the cell population. Heterogeneity of cell type, metabolic and cyclic states all contribute to problems in statistical analysis of data obtained from the transmission electron microscope.

Resolutions of 7 nm can be obtained with the scanning electron microscope, and specimen preparative methods which prevent artifact production have been developed (9, 10). This instrument readily scans large numbers of cells for surface characteristics and produces a three-dimensional picture with great detail (10). However, the means to identify the structures observed have been lacking. Recently, Nemanic and co-workers have tried to solve this problem by utilizing the hapten sandwich method with tobacco mosaic virus as a marker (M. K. Nemanic and D. P. Carter, *Fed. Proc.* **33**:754, 1974; M. K. Nemanic, D. P. Carter, D. R.

Pitelka, and L. Wolf-sy, *J. Cell Biol.*, in press). The cell-associated viral particles are clearly identified in their work, but the technique is cumbersome and there is no independent way to visually check the specificity and activity of the reagents. We have developed a technique for visual identification of specific antigens complexed with fluorescein-labeled antibodies on the surface of cells by means of the scanning electron microscope.

The images formed by the scanning electron microscope result from the release of secondary electrons, and the difference in brightness may be a consequence of differential ionization potentials of the structural elements. A bright image from the conjugated fluorescein residues can be anticipated because of the low ionization potential of aromatic compounds (7 to 8 eV) relative to the ionization potential of aliphatic compounds (10 to 11 eV) (2). Similar theoretical considerations of the photoelectric effect have recently been shown to have promise as a new method of mapping the positions of biological surface components (1, 5, 6). Utilizing these principles in conjunction with the magnification power of the electron microscope and with the specificity of fluorescent antibody labeling, we were able to identify and distinguish between two viruses, parainfluenza 1 and influenza A.

The hemagglutination test was used because it offers a means to demonstrate adsorption of virus to cell surfaces under well-controlled conditions, and the chicken erythrocyte membrane surface is devoid of microvilli, filopodia, and secretory products that might be confused with or obscure the virus under investigation.

The viruses used were the standard reagents for diagnostic identification at the Viral and Rickettsial Disease Laboratory, (California State Department of Health, Berkeley, Calif.).

FIG. 1. Identification of influenza type-A virus adsorbed to chicken erythrocytes. (a) Chicken erythrocyte, normal, untreated ($\times 7,500$); (b) influenza virus, type A adsorbed to surface of chicken erythrocyte ($\times 7,500$); (c) influenza virus, type A adsorbed to chicken cell and treated with specific fluorescein-conjugated antibody ($\times 7,500$); (d) influenza virus, type A adsorbed to chicken cell and treated with specific antibody, without fluorescein ($\times 15,000$); (e) influenza virus type A adsorbed to erythrocyte and treated with labeled antibody to influenza virus type B ($\times 7,500$); and (f) parainfluenza 1 virus and influenza virus type A adsorbed to erythrocytes, treated with labeled antibody to influenza virus, type A ($\times 15,000$).

Parainfluenza 1 (C-35) and influenza A virus (A/Eng/42/72) were grown in monkey kidney tissue cultures; influenza B (B/Mass/3/66), used in this study only for production of anti-

sera, was grown in embryonated chicken eggs. Sucrose gradient-banded virus served as antigen for preparation of specific antiserum in rabbits. The antisera were conjugated with

FIG. 2. *Influenza virus type A, labeled with specific antibody, adsorbed to erythrocytes ($\times 2,000$).*

fluorescein and shown to be specific by fluorescent microscope techniques (11).

The adsorption of influenza and parainfluenza 1 viruses to the surfaces of chicken erythrocytes was done according to the protocol of Salk (8). Briefly, the virus dilutions (to 1:40) were added to 0.25% suspension of freshly prepared chicken erythrocytes, and the mixture was held at room temperature for 2 h. The agglutinated cells were collected on a 12.5-mm membrane filter (0.45 μm ; Millipore Corp., Bedford, Mass.) in a Swinex unit. The virus-erythrocyte complex was washed by passing two changes of phosphate-buffered saline (0.5 ml) through the filter. The appropriate antiserum (0.2 ml) was then layered over the filter, and adsorption was allowed to proceed at room temperature for 2 h. The excess antiserum was removed with two consecutive phosphate-buffered saline washes. The antibody-virus-erythrocyte complex was then prepared for the scanning electron microscope; fixation in 0.2 ml of osmium tetroxide (4% in 0.1 M cacodylate buffer, pH 7.3) was overnight at 4 C; dehydration through graded ethanol series was followed by a Freon series. The specimens were critical-point dried with a Freon vehicle (3). Silver conducting paint was used to attach the filter to the specimen holder. The specimens were examined at a 40° angle with a Kent-Cambridge Stereoscan S4-10 scanning electron microscope operating at 5 and 10 kV.

A smooth-surfaced chicken erythrocyte is shown in Fig. 1a. Figure 1b shows indistinct particles with diffuse edges approximately 100 nm in diameter on the surface of a cell that has

been exposed to influenza A virus. The virus-cell complex treated with fluorescein-conjugated anti-influenza A antibody shows bright, discrete particles (800 to 1,000 nm) on the cell surface (Fig. 1c), whereas specific antibody without fluorescein does not enhance the contrast of the viruses adsorbed to the erythrocyte surface (Fig. 1d). Labeled antibody to influenza virus type B does not enhance the contrast of type-A influenza virus (Fig. 1e). A mixture of parainfluenza 1 and influenza type A viruses was adsorbed to erythrocytes and treated with fluorescein-labeled antibody to influenza virus only. Bright particles contrasting with discernible but indistinct particles are seen on the cell surface (Fig. 1f). We conclude that the labeled antibody distinguishes the influenza virus from the parainfluenza virus and that the bright particles are the result of interaction of fluorescein with the electron beam.

Labeled virus particles on the erythrocyte surface are readily distinguished at low magnification ($\times 2,000$) in the scanning electron microscope (Fig. 2). The detection of virus at this magnification is possible because the apparent diameter is increased 8- to 10-fold by adsorption of labeled antibody. The unlabeled virus measures 100 nm (Fig. 1b), whereas labeled virus appears to be 800 to 1,000 nm in diameter (Fig. 1c).

The physical aspects of these results can at this time be only speculative. Degrees of brightness observed can be influenced by interaction of the electron beam with molecular structures, and this will be determined by the work function (i.e., ability to release energy) of the

molecule. The higher the work function of the molecule, the brighter the image. The fluorescein molecule has a different ionization potential than the molecular components of the antibody, viral envelope, or erythrocyte membranes. Experiments to define the parameters of the increased brightness in the presence of fluorescein are in progress, but preliminary results suggest that the reason for the brightness of the antigen-antibody complex is due to the clustering of fluorescein molecules and the interaction of secondary electrons in response to the electron beam. This could account for the apparent increase in diameter of the virus particles. Studies are in progress to identify oncornaviruses budding from cell surfaces as well as other membrane-associated antigens and cell products. There are obvious applications to studies on the quantitative aspects of virus-cell interactions, identification and topographical distribution of antigenic sites on cell surfaces, cell-to-cell interaction, and in a broader sense applications in the fields of cell biology, virology, and immunology.

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