NOTES

Use of Antiviral-Antiferritin Hybrid Antibody for Localization of Viral Antigen in Plasma Membrane

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Reduced Fab' fragments of viral antibody hybridized with reduced Fab' fragments of antiferritin immunoglobulin G bind to viral antigenic sites in the plasma membrane of L cells infected with vesicular stomatitis virus. The hybrid antibody reacts specifically with ferritin, which can be identified by electron microscopy, and with fluorescein-conjugated apoferritin, which can be identified by fluorescence microscopy.

Antibody conjugated with ferritin or fluorescein isothiocyanate is commonly used for identification of viral antigens in infected cells examined by electron microscopy (7) or fluorescence microscopy (1; G. Goldstein, Ann. N.Y. Acad. Sci., in press). Each method has a significant drawback: the resolution of fluorescence microscopy is inadequate for ultrastructural analysis, whereas ferritin-antibody conjugates often react nonspecifically with cells. In this report we describe the application of a specific method using hybrid antibody for microscopic determination of the cellular sites and kinetics of viral antigen synthesis. The hybrid-antibody technique is based on the principle first suggested by Nisonoff and Rivers (8) that reduction and reoxidation of a mixture of antibodies, such as antiviral and antiferritin Fab' fragments of immunoglobulin G (IgG), should recombine randomly to form bivalent SS molecules, a considerable proportion of which, in our experiments, should have one viral antigen-combining site and one ferritin antigen-combining site.

Preparation of hybrid antibody was based on the method described by Hammerling et al. (2) as modified by Graziano and Goldstein (in preparation) for studying microbial antigens. A detailed summary of the procedure for preparing hybrid antibody appears in a companion paper by Heine and Schnaitman (submitted for publication) on penetration of vesicular stomatitis (VS) virus. In brief, rabbits were hyperimmunized with VS virus grown in chick embryo cells and purified about 1,000-fold by sequential steps of precipitation with polyethylene glycol (4), chromatography on diethylaminoethyl cellulose, rate zonal centrifugation, and isopycnic centrifugation (5). The antiviral rabbit antiserum diluted 1:256,000 neutralized about 90% of VS virus plaque-forming units. Other rabbits were hyperimmunized with ferritin. IgG was separated from each pool of antiserum by differential ammonium sulfate precipitation and batchwise absorption with Whatman DE-52 cellulose and then concentrated by vacuum dialysis. The purity of each IgG preparation was tested by immunoelectrophoresis, which revealed in each case a single IgG precipitin line with goat antiserum to whole rabbit serum.

The antiviral and antiferritin IgG preparations were separately acidified and cleaved with pepsin into SS bivalent (Fab')2 fragments and small peptides; the digested oligopeptides were removed by dialysis. The antiviral and antiferritin SS antibody globulins were then mixed in a ratio of 1:4 and incubated under N2 at 37°C for 1 hr with 2-mercaptoethamine hydrochloride at a final concentration of 0.015 M. Reduction of disulfide bonds converts (Fab')2 fragments to univalent Fab' fragments (8). The reducing agent was removed by passage through a column of Amberlite IR-120 equilibrated with 0.05 M acetate buffer at pH 5. The peak protein fractions eluting from the Amberlite column were collected, pooled, adjusted to pH 8 with 1 M NaOH, and reoxidized gently by stirring under an O2 atmosphere for 6 hr. The reconstituted antibody was
passed through a column of Sephadex G 100 that had been equilibrated with 0.15 m tris(hydroxymethyl)aminomethane, 0.2 m NaCl (pH 8.0); 47.5% of the protein applied to the Sephadex column was recovered in a peak fraction representing recombined 5S IgG. This fraction contained bivalent hybridized antibody, a proportion of which had one site reactive with viral antigen and the other with ferritin (heterodimer); the rest of the 5S IgG consisted of recombined fragments in which both sites reacted only with virus or ferritin (homodimer) or other extraneous antigens.

Monolayer cultures of L cells (9) were grown to confluence on the surface of cover slips (22 by 22 mm) placed in plastic petri dishes (100 mm in diameter) and incubated at 37 C in medium 199 supplemented with 10% calf serum. Drained cultures were infected with VS virus (9) at a multiplicity of 100 (30 min adsorption). At intervals after infection and incubation at 37 C, cover slips were removed with a forceps, rinsed in phosphate-buffered saline (PBS) containing 0.25 m sucrose, and drained by touching the edge to filter paper. The cell layer on the surface of the cover slip was then overlaid with 50 ml of hybrid antibody and incubated at room temperature for 20 min. The antibody solution was drained and the cells were scraped with the aid of a rubber policeman into a test tube [2 by 0.25 inch (ca. 5.1 by 0.64 cm)]; the cover slip surface was rinsed with 2 to 3 ml of PBS, and the washings were collected in the test tube. The cells were then sedimented into a loose pellet by centrifugation at 1,000 g for 2 min and washed twice with PBS. The pelleted cells (~ 3 x 10^6) were resuspended in 150 ml of ice cold PBS, and 50 ml of PBS was removed for fluorescence microscopy. To the remaining 100 ml of infected, antibody-treated cells was added 100 ml of ferritin suspended in PBS at a concentration of 2.5 mg/ml. The cell-ferritin mixture was incubated with periodic shaking for 20 min at room temperature; the cells were then washed twice by centrifugation to remove excess ferritin. The suspension of cells exposed to ferritin was prepared for electron microscopy, as previously described (3), by transfer to a microfuge tube filled with buffered glutaraldehyde. After pelleting the cells, they were washed in glutaraldehyde, fixed in OsO_4, stained with uranyl acetate, embedded in Epon 812, sectioned, post-stained with 1% uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 1A microscope at an initial magnification of × 40,000.

The other third of each infected cell suspension (~ 10^6 cells), which had been exposed to hybrid antibody and washed, was incubated at 0 C for 20 min with 50 mliters (~ 0.03 mg) of apoferritin (0.7 mg/ml) that had been conjugated with fluorescein isothiocyanate (Graziano and Goldstein, in preparation). The cell suspension was then washed by centrifugation and stored at 4 C after adding 1 drop of 1:1 glycerol-PBS. The unfixed cells were spread on a glass slide and examined by the method of Möller (6) with a Leitz Ortholux microscope equipped with an HBO-200 ultraviolet light source. Photographs were taken at a magnification of × 540 employing a 5840 K2 filter.

Figure 1 shows representative electron micrographs demonstrating progressive degrees of ferritin attachment to plasma membrane of L cells exposed to hybrid antibody at intervals after infection with VS virus. At 2 hr after infection, the plasma membrane was essentially free of ferritin except for small patches (Fig. 1A) which could conceivably represent residual input virion membrane (3). By 5 hr, electron-dense ferritin molecules were adherent over a greater area of the cell surface (Fig. 1B); by 8 hr, the entire surface of the plasma membrane was covered with ferritin (Fig. 1C) in every cell examined, including budding virus particles (Fig. 1D). Controls consisting of uninfected cells exposed to hybrid antibody plus ferritin and of infected cells not treated with hybrid antibody but exposed to ferritin were completely devoid of ferritin in every section of membrane examined.

Figure 2 illustrates the corresponding light microscopic appearance of the same cells infected with VS virus and treated with hybrid antibody prior to exposure to fluorescein-conjugated apoferritin. Specific fluorescent staining was noted in circumscribed patches on the cell surface at 2 hr (Fig. 2A) and completely surrounded every cell at 8 hr after infection at high multiplicity (Fig. 2B). No specific fluorescence could be seen on double-blind examination of uninfected cells exposed to hybrid antibody and fluorescent apoferritin or of infected cells exposed to fluorescent apoferritin alone.

Similar results were obtained on a repeat experiment in which L cells were examined at intervals of 2 hr after infection with VS virus at a multiplicity of 100. In this experiment a single-cycle growth curve was constructed by assaying for plaque-forming virus released into the medium at the same intervals after infection. The data were identical to those previously reported (9), which revealed an eclipse period of 2 hr followed by exponential growth to a peak yield per cell of 200 plaque-forming units at 8 hr. Monitoring of the cells by electron microscopy and fluorescence
Fig. 1. Electron microscopy of L cells infected with VS virus, treated with antiviral-antiferritin hybrid 5S IgG at intervals after infection, exposed to electron-dense ferritin, fixed, and sectioned. (A) Two hours after infection: sparse ferritin granules on plasma membrane. (B) Five hours after infection: increased patchy labeling with ferritin. (C) Eight hours after infection: ferritin granules uniformly distributed over plasma membrane. (D) Eight hours after infection: ferritin labeling of budding VS virus and adjacent plasma membrane. × 105,000.
microscopy after exposure to hybrid antibody and ferritin indicated that viral antigen is progressively inserted into the entire plasma membrane of L cells infected with VS virus at high multiplicity. Mature virions, however, only bud from localized regions of the membrane. Similar generalized distribution of viral antigen in plasma membrane has been noted in cells infected with myxovirus and stained by the more conventional technique of ferritin-labeled antibody (7).

These data are presented as evidence for the feasibility of using antiviral-antiferritin hybrid antibody for studying antigenic conversion of plasma membrane of virus-infected or virus-transformed cells by specific immunological reaction with ferritin.

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LITERATURE CITED


