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Intracellular Localization of Core and Envelope Proteins of Venezuelan Encephalitis Virus by Immunofluorescence (40888)[1]

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Abstract. Envelope and core proteins of Venezuelan encephalitis virus were detected by indirect fluorescent antibody staining in cultured primary hamster embryonic cells infected under single-cycle conditions. Antibody to purified core protein stained the entire cytoplasm at 7 hr postinfection, but never the outer surface of the plasma membrane. By 24 hr postinfection, when cytopathic effects were extensive, core protein was present as cytoplasmic granules. Antibody to purified envelope proteins stained a single bright perinuclear area in less brightly fluorescing cytoplasm at 7 hr postinfection, and plasma membranes at 8 hr postinfection. The perinuclear location of Venezuelan encephalitis virus envelope glycoproteins was interpreted as involving the Golgi apparatus which is a known location of intracellular glycoprotein synthesis. Thus, the Golgi apparatus is apparently a source of the smooth membranes with which α-virus envelope proteins have been associated after cell fractionation.

Studies aimed at defining the cellular organelles with which alphavirus proteins are associated have employed either fractionation of α-virus-infected cells in sucrose gradients or cytologic observations after immunofluorescent staining. Cell fractionations have associated radioactivity in viral envelope proteins with rough and smooth cellular membranes (1, 2). The rough membranes were morphologically rough endoplasmic reticulum (RER) but the origin of the smooth membranes has not been established, although they were assumed to derive from smooth ER and plasma membrane (2). Viral core proteins associate with 60 S ribosomal subunits and are rapidly incorporated into nucleocapsids (3).

Cytologic studies have usually employed antisera made to mixtures of antigens present in intact α-virions (4–6). Therefore, cytologic experiments have not distinguished viral core and envelope proteins in infected cells. When antisera to separated proteins of a vaccine strain of Venezuelan encephalitis (VE) virus were used, envelope protein antiserum did not localize fluorescence to any particular site within Vero monkey kidney cells and no virus-specific fluorescence was seen with core protein antiserum (7).

Thus, further immunofluorescent studies were done to localize envelope and core proteins of an α-virus, VE, and, in particular, to attempt to identify the smooth-membraned organelle(s) with which VE envelope proteins are associated. We present evidence that this smooth-membraned organelle is the Golgi apparatus, that VE envelope proteins accumulate there prior to their insertion into plasma membrane, and that core protein in nucleocapsids is not exposed on the extracellular surface of infected cells.

Materials and methods. Cell cultures and virus. Primary cultures of hamster embryonic cells (HEC) were prepared on 11 × 22-mm coverslips in 8-cm² wells of plastic plates as described previously for chicken embryonic cells (CEC) (8). VE strain 63U2 was used after six passages in suckling mouse brain and two in CEC (9). Virus was titrated as plaque-forming units (PFU) in CEC (10).

Preparation of separated viral proteins. Virus, grown in CEC after infection with MOI of about 1 PFU/cell and purified as described (11), was used to prepare sepa-

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rated core and envelope proteins. The 400 μl of purified virus suspension (containing 400 μg of protein measured by a Lowry assay (12) using bovine albumin as a standard) was incubated with 2 ml of 1% Triton X-100 in 0.2 *M* Tris, pH 8.0 (1 hr, 37°C, frequent stirring), to solubilize viral envelope proteins. After standing overnight at 5°C, the mixture was centrifuged to pellet viral nucleocapsids (175,000 *g*, 2.5 hr, 5°C), supernatant was stored at −65°C, and the pellet resuspended in TNE buffer (11) and centrifuged in a 10−30% (w/v) sucrose/TNE gradient (90,000 *g*, 3 hr, 5°C). Fractions were collected from the bottom of the centrifuge tube and the OD_{260} of each fraction was determined with a spectrophotometer. A sharp peak occurred in fractions 12−15. Pooled fractions 12−15 were diluted to 5 ml with TNE buffer, treated with ultraviolet irradiation in an uncovered 60-mm petri dish 4.5 in. from a Westinghouse Sterilamp (Type WL 782-30) for 12 min at room temperature to inactivate infectivity, and stored at −65°C. Pooled, irradiated material from these fractions formed no plaques in CEC at $10^{-1.3}-10^{-6.3}$ dilutions. Supernatant of the Triton X-100-treated virus was analyzed on SDS−polyacrylamide slab gels (11) and contained only two proteins with the same electrophoretic mobilities as the two envelope proteins of this strain of VE virus; a pool of fractions 12−15 contained only a protein that migrated in the gel like VE core protein.

Preparation of antisera to viral proteins. Antisera to core and envelope proteins were produced in female English short-hair guinea pigs 3−4 months of age, caged singly. The core preparation (0.25 ml containing about 5 μg of protein) and the envelope preparation (0.4 ml containing about 30 μg of protein) were diluted with an equal volume of complete Freund's adjuvant and each inoculated intraperitoneally into individual guinea pigs on Days 0 and 9. Another inoculation without adjuvant was made on Day 16, and guinea pigs were bled on Days 27 and 29. Neither the envelope nor core protein preparation initiated viral replication in guinea pigs since no viremia was detected at 42 hr after inoculation.

Antisera from three guinea pigs immunized with envelope proteins had high VE complement-fixation (CF), hemagglutination-inhibition (HI), and plaque reduction neutralization (N) antibody titers when tested as described elsewhere (13). Reciprocal titers using strain 63U2 as antigen were: CF 256, 512, 512; HI 1280, 5120, 40960; N 50, 40, 800. Sera from two guinea pigs inoculated with core protein preparation had antibody detectable by

TABLE I. RESULTS OF IMMUNOFLUORESCENT STAINING OF HAMSTER EMBRYONIC CELLS INFECTED WITH VE VIRUS

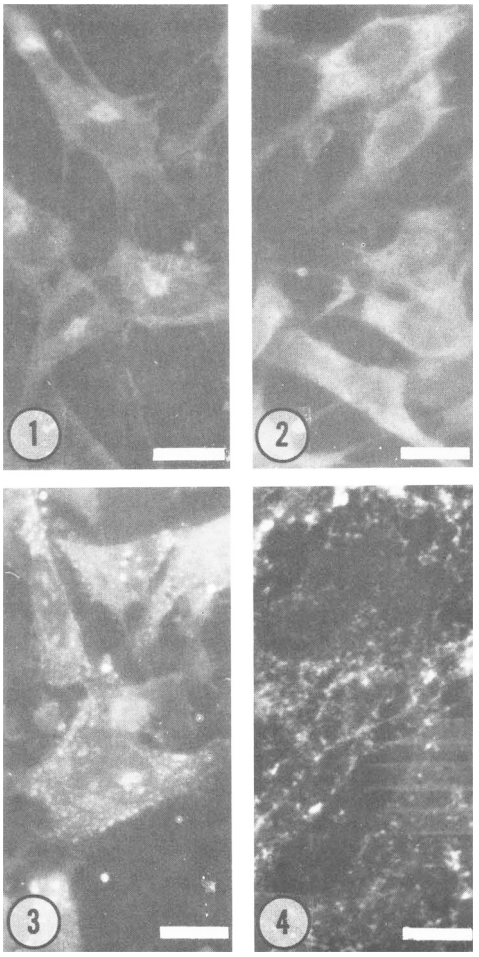
Types of fixation[a] and antibody	Fluorescence observations by cellular location		
	Perinuclear region	Cytoplasm	Plasma membrane
Acetone-fixed cells			
Anti-envelope	Bright spot 7−12 hr, gone by 24 hr	Moderately bright by 7−12 hr,	Bright 8−12 hr, dull by 24 hr
Anti-core	Bright by 7−12 hr but not localized to a spot and no brighter than rest of cytoplasm	Bright by 7−12 hr, granules by 24 hr	0
Unfixed cells			
Anti-envelope	0	0	Bright granular by 8−12 hr, dull by 24 hr
Anti-core	0	0	0

[a] Intracellular proteins were stained in acetone-fixed cells; cell surface proteins in unfixed cells.

CF test, but only barely detectable or undetectable by HI and N tests (CF 32, 128; HI < 10, 10; N < 8, <8).

Immunofluorescent staining of infected cells. Infected HEC on coverslips were placed in 0.1 *M* phosphate-buffered saline, pH 7.0 (PBS), for 10 min, acetone for 30 sec, and PBS for 10 min. A 1:10 dilution of anti-core or anti-envelope serum was applied; anti-core serum was from the guinea pig with VE CF titer of 128, HI 10, and N < 8, and anti-envelope serum was from the guinea pig with VE CF titer of 512, HI 40960, and N 800. Coverslips were incubated in moist chambers for 30 min at 37°C, washed in three changes of PBS, covered with fluorescein isothiocyanate-conjugated goat anti-guinea pig IgG (1:5 in PBS) which was absorbed before use for 30 min with hamster liver powder (100 mg/ml), and incubated for 30 min at 37°C. Coverslips were again washed in three changes of PBS, rinsed in glass distilled water, and mounted in Gelvatol 20–30. Cells were observed using a Leitz Ortholux 1 fluorescence microscope equipped with an HBO-200 mercury arc lamp, BG-3 exciter filter, OG-1 barrier filter, dry darkfield condenser (D = 0.80), and achromatic objectives. Photographs were taken using Tri-X film. Controls included uninfected cells plus guinea pig antiserum, infected cells plus preimmune guinea pig serum, and infected cells plus antiserum to VE strain 63U2 produced in rabbits.

Results. At 2, 6, 7, 8, 9, 12, and 24 hr postinfection (p.i.) with VE virus (2.5 PFU/cell), HEC were stained with anti-envelope or anti-core antibody by the indirect fluorescent antibody technique (Table I). No fluorescence was seen in infected cells at 2 or 6 hr p.i. or in the nucleus at any time p.i. At 7 hr, there was a single, bright, perinuclear spot and moderately bright diffuse staining throughout cytoplasm in acetone-fixed cells stained with anti-envelope serum (Table I, Fig. 1). This anti-envelope serum had antibody titers of CF 512, HI 40960, and N 800. The entire cytoplasm of acetone-fixed cells fluoresced brightly with anti-core serum (Table I, Fig. 2). The antibody titers of this anti-core serum were CF 128, HI 10, N < 8. This pattern of cytoplasmic fluorescence was maintained to 12 hr, but by 24 hr, anti-envelope serum failed to stain cytoplasm, and cytoplasmic granules were seen in cells stained with anti-core serum (Table I, Fig. 3). Plasma membrane fluorescence was first observed in fixed and unfixed cells stained with anti-envelope serum at 8 hr after infection; this was maintained until 12 hr and decreased by 24 hr (Table I, Fig. 4). Anti-



Bars equal 5 μm.

FIG. 1–3. Acetone-fixed VE-infected HEC. 1. stained with anti-VE envelope serum at 8 hr p.i. Note perinuclear fluorescence. 2. stained with anti-VE core serum at 8 hr p.i. Note homogeneous cytoplasmic fluorescence. 3. stained with anti-VE core serum at 24 hr p.i. Note cytoplasmic granules.

FIG. 4. Unfixed VE-infected HEC stained with anti-VE envelope serum at 8 hr p.i. Note granular fluorescence of plasma membranes.

core antibody failed to stain unfixed cells. Controls for nonspecific fluorescence (see Methods) were negative. In these experiments, the maximal extracellular virus concentration ($10^{8.9}$ PFU/ml) was reached by 8 hr and was maintained through 24 hr. Cytopathic effects were seen in 5–10% of cells by 9 hr, 30% by 12 hr, and 90% by 24 hr.

Thus, these immunofluorescence observations of cultured hamster cells infected with VE α-virus localized envelope protein antigens first as a bright spot in the perinuclear region and as moderately bright diffuse staining throughout cytoplasm. Subsequently, envelope proteins were stained in a granular distribution at the plasma membrane. Core proteins occurred throughout the cytoplasm of fixed cells but were not seen at the plasma membrane of fixed or unfixed cells.

Discussion. The observation of a perinuclear location of VE envelope glycoproteins provides direct morphologic evidence which suggests that the Golgi apparatus is a source of the smooth membranes with which α-virus envelope proteins are associated in cell fractionation studies (1, 2). The Golgi apparatus is perinuclear (14) and is involved with addition of terminal carbohydrate moieties to and packaging of cellular glycoproteins prior to their insertion into cell plasma membranes (15). The observation of VE envelope proteins first in the perinuclear region at 7 hr p.i. but not in plasma membrane until 8 hr p.i., is consistent with this sequence of protein migration. The greater intensity of fluorescent staining of VE envelope proteins in the perinuclear region than in other cellular locations suggests that VE envelope proteins accumulate in the Golgi apparatus prior to their insertion into plasma membrane. Observations that α-virus envelope glycoproteins contain terminal sugars that require transferases present in the Golgi apparatus (16, 17) are also consistent with the concept that α-virus envelope proteins associate with the Golgi apparatus.

The diffuse cytoplasmic staining of VE envelope proteins probably represented newly synthesized envelope proteins on polysomes bound to endoplasmic reticulum or inserted into membranes of rough and smooth endoplasmic reticulum as seen with Sindbis virus (1). In addition, these diffuse envelope proteins could be in membranes of vesicles transporting these proteins from Golgi apparatus to plasma membrane, as has been proposed for cellular (15) and α-virus envelope (17) glycoproteins.

The even distribution of core protein throughout cytoplasm based on staining with antibody to core protein indicated core protein that was probably in viral nucleocapsids migrating to the plasma membrane (18, 19). The fluorescent cytoplasmic granules stained with anti-core protein antibody at 24 hr after infection, when cell damage was extensive, may represent alphavirus cytopathic vacuoles of type II (membranous cytoplasmic structures surrounded by electron-dense spheres with the diameter of nucleocapsids some of which appear to bud into the lumen of the vacuole) (20).

Nonspecific staining of nucleoli with anti-core serum was not observed in HEC as reported by Pedersen and Eddy in infected and uninfected Vero cells (7). Evidently, the specificity of their anti-core serum differed from that of the antiserum used in the present report since it failed to detect cytoplasmic core protein in VE-infected cells in any of the patterns reported here.

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