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Detection of Dengue Cell-Surface Antigens by Peroxidase-Labeled Antibodies and Immune Cytolysis

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The appearance of dengue-specific plasma membrane (DSPM) antigens in infected LLC-MK_2 cell cultures was studied by ^{51}Cr release in immune cytolysis and at an ultrastructural level using peroxidase-labeled antibodies. DSPM antigen was first detected at 36 h with electron microscopy in approximately 30% of the cells, and this percentage did not increase with time. However, both surface staining with peroxidase-labeled antibodies and ^{51}Cr release indicated that the amount of DSPM antigen per cell increases with time. The appearance of ^{51}Cr release in immune cytolysis experiments with dengue-infected cells occurred much later than the peak of infectious virus release. This was in sharp contrast to immune cytolysis with a group A arbovirus, Eastern equine encephalitis, in which the kinetics of release of infectious virus and ^{51}Cr release were identical. This suggests different mechanisms of insertion of viral plasma membrane antigens in Eastern equine encephalitis and dengue-infected LLC-MK_2 cells.

The presence of dengue antigens in the plasma membrane of infected cells may be important in the pathogenesis of this disease (9). Previous studies from this laboratory utilizing fluorescent antibody on dengue-infected cell cultures demonstrated perinuclear and cytoplasmic fluorescence but not membrane fluorescence (6). It might be concluded from these experiments that dengue-specific plasma membrane (DSPM) antigens were not incorporated into the membranes of infected cells. However, these results were compatible with a DSPM antigen density which was below the resolution limits of fluorescence microscopy. Accordingly, we chose to investigate these alternatives further with techniques of higher resolution. One such technique utilized peroxidase-labeled antibodies to dengue and thus permitted identification of viral antigens at an ultrastructural level. It was also possible to detect cell-surface antigens by immune cytolysis of infected cells with specific antiviral antibodies and complement. Since this latter procedure is quantitative, we felt it would provide a useful correlate for the data obtained by electron microscopy, which is for the most part qualitative.

MATERIALS AND METHODS

Cell cultures. An established line of rhesus monkey kidney cells (LLC-MK_2), susceptible to dengue-2 virus (10), was grown in stationary glass bottles (946 ml) or in plastic flasks (30 ml; Falcon Plastics, Oxnard, Calif.). These were maintained as a monolayer in medium 199 (M-199; Microbiological Ass., Bethesda, Md.) which contained 20% heat-inactivated (56 C for 30 min) fetal bovine serum (Flow Laboratories, Rockville, Md.), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Infection of cultures. The LLC-MK_2 cells were infected with a suspension of suckling mouse brain passage (SMBP) of either dengue 2 (New Guinea C strain; SMBP 35) or Eastern equine encephalitis (EEE; Cambridge strain, SMBP 4) at multiplicities of 2 and 10, respectively. Control cultures were mock-infected with a 20% (wt/vol) normal suckling mouse brain suspension. Both viral and control cultures were incubated for 90 min at 36 C. The inoculum was then decanted and the monolayers were washed three times with Earle balanced salt solution (Microbiological Ass., Bethesda, Md.). Finally, M199 containing 2% fetal bovine serum (maintenance medium) was added, and the culture was incubated at 36 C until the times selected for the studies detailed below.

Preparation of antibodies. The preparation of antibodies in hyperimmune mouse ascitic fluid (HMAF) was described elsewhere in detail (5, 6). Hereafter, the terms antibody and HMAF will be used interchangeably. Normal ascitic fluid (NAF) was prepared in a similar fashion in unimmunized mice.

Preparation of peroxidase-conjugated antibody to dengue-2. The undiluted peritoneal fluid was chromatographed on a Sephadex G-200 column and the immunoglobulin G (IgG)-rich (second peak) fractions were pooled. Horseradish peroxidase (type VI;

Sigma Chemical Co., St. Louis, Mo.) was coupled to these IgG fractions with glutaraldehyde by the method of Avrameas and Ternynck (2).

Exposure of cells to peroxidase conjugated antibody. The adherent monolayers were trypsinized (0.25% trypsin; GIBCO, Grand Island, N.Y.), centrifuged, and washed with M199 (Ca^{2+}, Mg^{2+} free). After centrifugation, infected cells were first incubated for 30 min at room temperature with 0.9 ml of NAF or non-peroxidase-conjugated HMAF. Uninfected cells were incubated with 0.9 ml of NAF under similar conditions. At the end of this time, we added 0.1 ml of peroxidase-conjugated HMAF to each tube. The suspensions were gently agitated and allowed to incubate for an additional hour at room temperature.

Electron microscopy. At the end of the incubation period, each tube received 5 ml of M199 at room temperature which contained 2% bovine plasma albumin (Calbiochem, San Diego, Calif.). The suspensions were washed three times in the same medium. Finally, the cells were washed in ice-cold M199, centrifuged, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 min at 4 C. After three washes in cacodylate buffer, the suspensions were maintained overnight in this buffer containing 0.25 M sucrose in the cold. The suspensions were reacted for peroxidase activity by the method of Graham and Karnovsky (8). The reaction was stopped by the addition of excess ice-cold phosphate buffer (0.1 M, pH 7.4), washed twice in this buffer, and postfixed for 1 h in ice-cold, phosphate-buffered 2% OsO_4. After three washes in phosphate buffer, the pellets were resuspended in hot (60 C) 2% Noble agar (Difco), centrifuged into a pellet, and rapidly chilled. The cell-rich portion of the agar was diced with a razor blade into small blocks, dehydrated in a series of graded alcohols and propylene oxide, and embedded in Epon. Silver to gray sections were cut with a Porter Blum MT 1 microtome, using a diamond knife. The sections were mounted on collodion-coated copper grids and examined either unstained or lightly stained with lead tartrate in an Hitachi 11 UB electron microscope at 75 kV.

Immune cytolysis. The specific interaction of antibody with viral antigens in the plasma membrane of infected cells in the presence of complement (C) results in membrane damage and eventual cytolysis (3). This process is referred to as immune cytolysis and is usually measured by the release of ^{51}Cr from damaged cells. Thus, we added 2 to 3 mCi of ^{51}Cr (New England Nuclear Corp., Boston, Mass) in 5 ml of maintenance medium to the adherent monolayers of LLC-MK_2 cells in 30-ml plastic flasks. After 16 h at 36 C, the monolayers were washed four times with 10 to 15 ml of 0.02 M phosphate-buffered saline and once with triethanolamine-buffered saline containing Ca^{2+} and Mg^{2+} (7). Optimal conditions for immune cytolysis were determined in preliminary experiments using antibodies against the LLC-MK_2 cells themselves. It was found that fresh guinea pig serum diluted in triethanolamine-buffered saline was a superior source of complement. Further, we observed that the lytic ability of an antibody preparation was directly proportional to its complement fixation titer. For this reason, we adjusted all HMAF preparations to a dilution 16 times more concentrated than its complement fixation titer. Thus, antibody concentration should not be a limiting factor.

The following controls were included in these experiments: infected cells were incubated with C alone, HMAF alone, and with a mixture of NAF and C; uninfected cells were incubated with HMAF and C. After incubation with the appropriate mixture, the fluid was carefully aspirated from the monolayer and clarified by centrifugation. Both supernatant and pellet were assayed for radioactivity. The cell monolayers were dissolved in 2 ml of 2 N NaOH and also counted. The percent of ^{51}Cr release was calculated as follows: % lysis = [counts/min of supernatant/counts/min (supernatant + pellet + dissolved cells)] $\times$ 100. We arbitrarily considered nonspecific release to be the highest of all the control values and refer to this in the text and the figures as "background."

RESULTS

LLC-MK_2 morphology. Uninfected cells maintained under identical conditions as infected cells exhibited no degenerative changes nor viral-like particles during the entire culture period. If fixed, embedded, and sectioned in situ, the confluent monolayer consisted of flattened interdigitating epithelioid cells with a central elevation containing the nucleus. In suspension after trypsinization, the cells assumed a spherical configuration and the plasma membrane exhibited a variety of filliform processes.

The sequence of cytological changes after infection of LLC-MK_2 cells by dengue-2 was identical to that already described (6). Initially, we attempted to perform all experimental manipulations with dengue-infected and uninfected monolayers in situ. However, conjugated antibody accumulated between cell junctions and also between the undersurface of the cell and the surface of the culture flask in both infected and control cultures. This accumulation of conjugated antibody could not be reversed by repeated washings and made precise localization of DSPM antigens in situ quite difficult. For this reason, we performed all the experiments detailed below with freshly trypsinized cell suspensions with which such nonspecific accumulations of peroxidase conjugated antibodies did not occur.

Controls. Peroxidase is localized by the identification of the insoluble electron-dense reaction product formed by its enzymatic activity (8). There were two types of controls. (i) Uninfected cells, which presumably did not contain DSPM antigens were incubated with peroxidase-labeled antbody. (ii) Infected cells, which might contain DSPM antigens, were first in-

cubated with excess unconjugated antibody, followed by incubation with the conjugated antibody (specific inhibition). In neither case, at any time was reaction product localized on the plasma membrane of LLC-MK_2 cells. However, the conjugate was actively endocytosed and could be identified in cytoplasmic vacuoles in both infected and uninfected cells. This was not surprising in view of the fact that these cells are endocytically active and all experimental procedures were carried out at room temperature.

Appearance of DSPM antigens in LLC-MK_2 cells. Freshly trypsinized cells were examined for the presence of DSPM antigen at 18, 36, and 72 h postinfection. Although reaction product could not be identified on the plasma membrane of infected cells at 18 h, it was observed in a variety of endocytic vacuoles (Fig. 1, 2). DSPM antigen was first observed at 36 h (Fig. 3, 4). By examining a large number of low-power photomicrographs, it was determined that approximately 30% of the cells bore DSPM antigen. Considering the extreme thinness of the section, this estimate may be much lower than the actual percentage. The distribution of the electron-dense RP was never circumferential; rather it was focal and localized on either flattened runs of plasma membrane or on filliform processes (Fig. 4). The cellular distribution of DSPM antigen at 72 h was similar to that at 36 h (Fig. 5, 6). At this time, it was determined that 30% of the cells bore DSPM antigens; i.e., the same percentage which was antigen positive at 36 h. Despite the fact that there was no increase in the number of DSPM antigen bearing cells with time, there appeared to be an increase in the amount of DSPM antigens borne by positive cells with time. Finally, at both 36 h and 72 h, it was noted that cells containing numerous cytopathic vacuoles filled with virions usually possessed little DSPM antigen and vice versa (Fig. 5).

Viral-specific plasma membrane antigens measured by immune cytolysis. Significant release of ^{51}Cr over background levels was first noted at 60 h postinfection and increased with time in a linear fashion (Fig. 7). Cytopathic effects did not alter background release of ^{51}Cr. Thus, although minimal cytopathic effect was detectable at 60 h postinfection, there was no change in background release of ^{51}Cr from 36 h on (Fig. 7). We did not demonstrate a plateau for immune cytolysis; at later times, i.e., at 84 h, the specific release of ^{51}Cr was slightly higher than at 72 h. At this time, many cells had rounded up and detached from the monolayers and the experiments were terminated. However, this does not imply that immune cytolysis increased after that time; in other studies by J. M. Dalrymple in our laboratory, a chronic carrier state was established from the surviving cells and we could not demonstrate immune cytolysis up to 8 days after each subcultivation. When ^{51}Cr release is compared to the release of infectious virus in acutely infected cells, it is seen that ^{51}Cr release occurs well after the peak of viral release (Fig. 7).

We included for comparison a study of immune cytolysis of LLC-MK_2 cells infected with EEE, a group A arbovirus. In this case, the curves for the release of infectious virus and ^{51}Cr release are essentially identical (Fig. 8). Thus, although both EEE and dengue-2 arbovirus antigens are inserted into the plasma membranes of infected LLC-MK_2 cells, the kinetics of their appearance with respect to the individual growth curves are quite different.

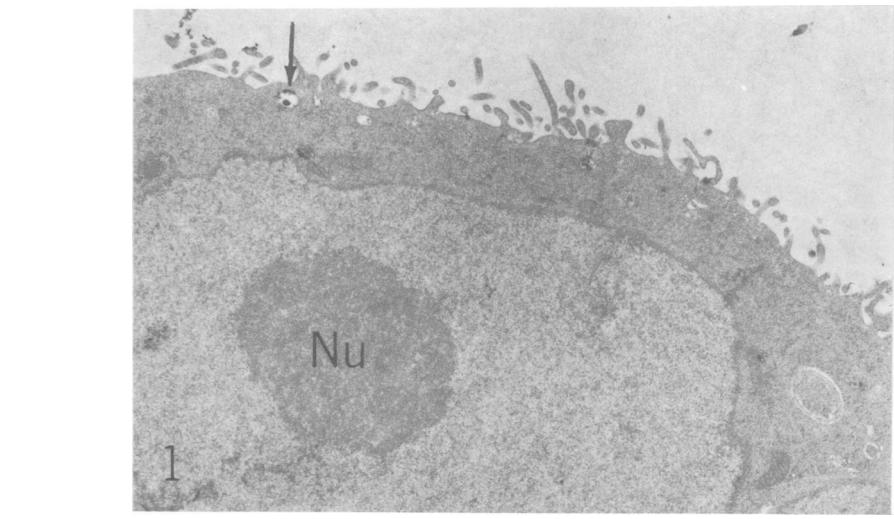
DISCUSSION

We have demonstrated by ultrastructural immunocytochemistry and immune cytolysis that dengue-2 antigens are inserted into the plasma membranes of infected LLC-MK_2 cells. It was observed that ^{51}Cr release from dengue-infected cells by immune cytolysis increased with time. This could be explained by an increase in the number of DSPM antigen-bearing cells with time or an increase in antigenic density per cell with time, or both. Data obtained by electron microscopy suggested that the number of DSPM antigen-bearing cells did not vary with time. Taken together, the data from these two techniques suggest that there is an increase in the amount of DSPM antigen per cell with time. Clearly, DSPM antigen was detected much earlier by means of peroxidase-labeled antibodies than by immune cytolysis. This discrepancy between the two methods may be explained by the requirement for a certain density of viral antigens before immune cytolysis occurs. The observation lends additional support to our suggestion that the amount of DSPM antigen actually increases with time.

It was of interest that the number of DSPM antigen-bearing cells represented a distinct minority of the population. It was shown by Cardiff et al. (6) that most of the LLC-MK_2 cells were not initially infected. The reasons for this are not well understood. Perhaps the fact that LLC-MK_2 cells represent an uncloned population of cells is a factor. Indeed, even when a chronic carrier state was established by continuously subcultivating the infected cells, less than 30% of the cells exhibited dengue-specific fluorescence, and generally no more

than 1,000 plaque-forming units per ml were found in the culture medium (J. M. Dalrymple, personal communication).

Elsewhere, it was shown that group A-specific plasma membrane antigens could be detected by the fluorescent antibody technique, whereas DSPM antigens could not be so identified (6).



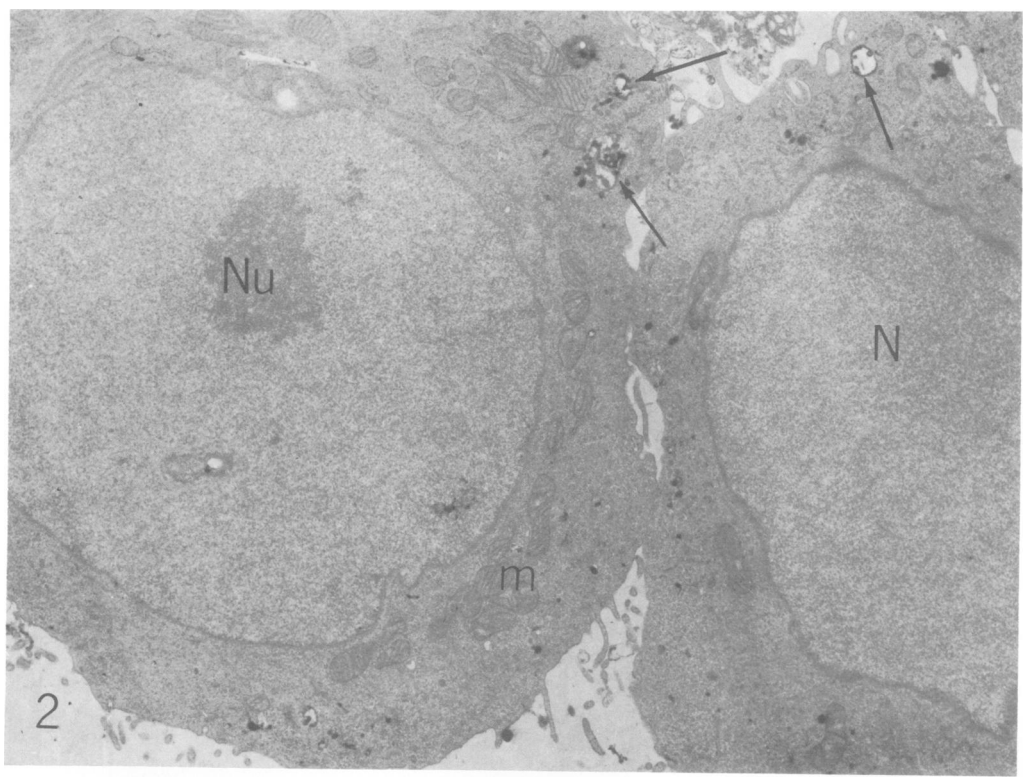


FIG. 1 and 2. *LLC-MK$_2$ cells 18 h postinfection. Note the absence of plasma membrane reaction product. Arrows indicate pinocytic vacuoles-labeled conjugate. N, Nucleus; M, mitochondria. Magnifications ×7,500 (1), ×11,800 (2); unstained.*

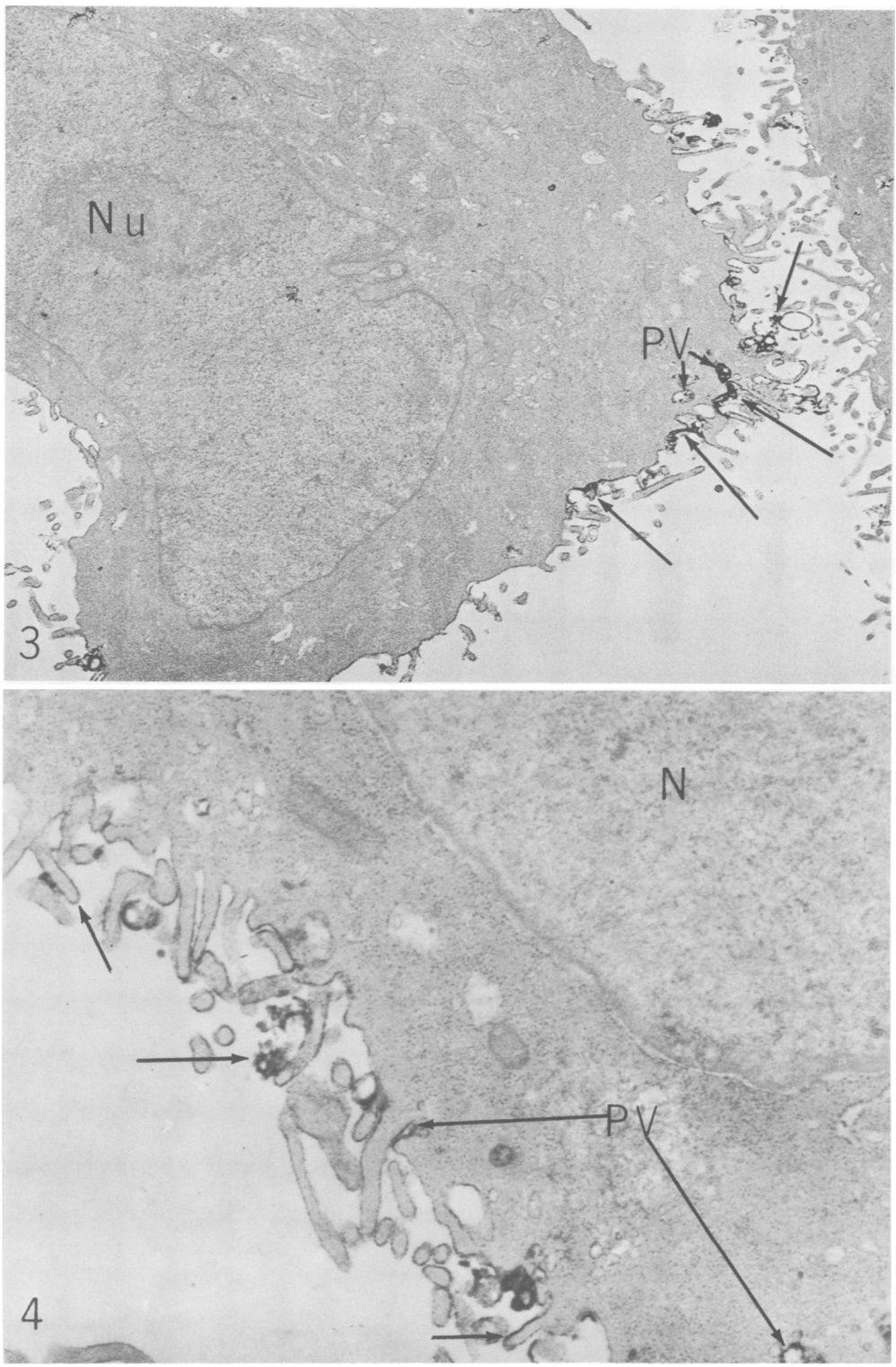


FIG. 3 and 4. *Thirty-six hours postinfection. Arrows indicate electron-dense reaction product marking DSPM antigen. Note the distribution on both flat runs of membranes and slender filliform processes. A variety of pinocytic vesicles (PV) containing peroxidase-labeled conjugate are present. N, Nucleus; Nu, nucleolus. Magnifications ×8,700 (1), ×13,600 (2); unstained.*

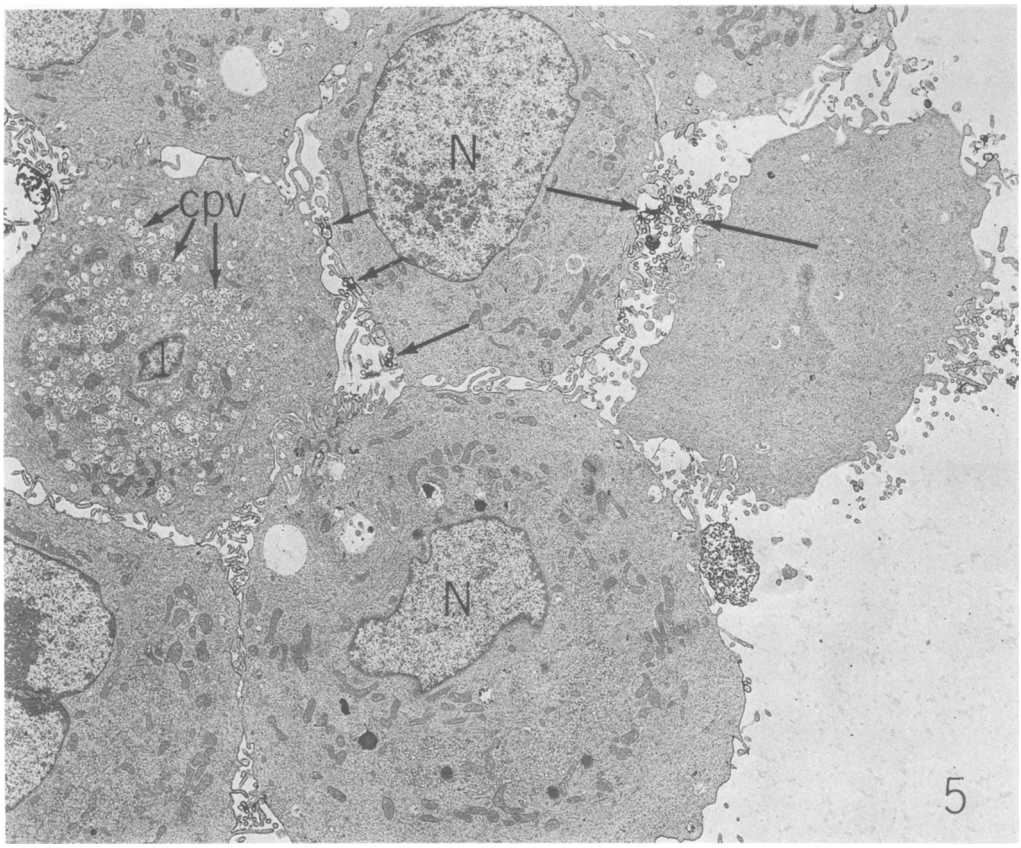


FIG. 5. *Low-power photomicrograph; 72 h postinfection. Note the relative absence of peroxidatic reaction product in cell marked "1" which contains numerous virus-filled cytopathic vacuoles (CPV). Arrows indicate focal collections of reaction product. N, Nucleus. Magnification ×3,400; lead tartrate.*

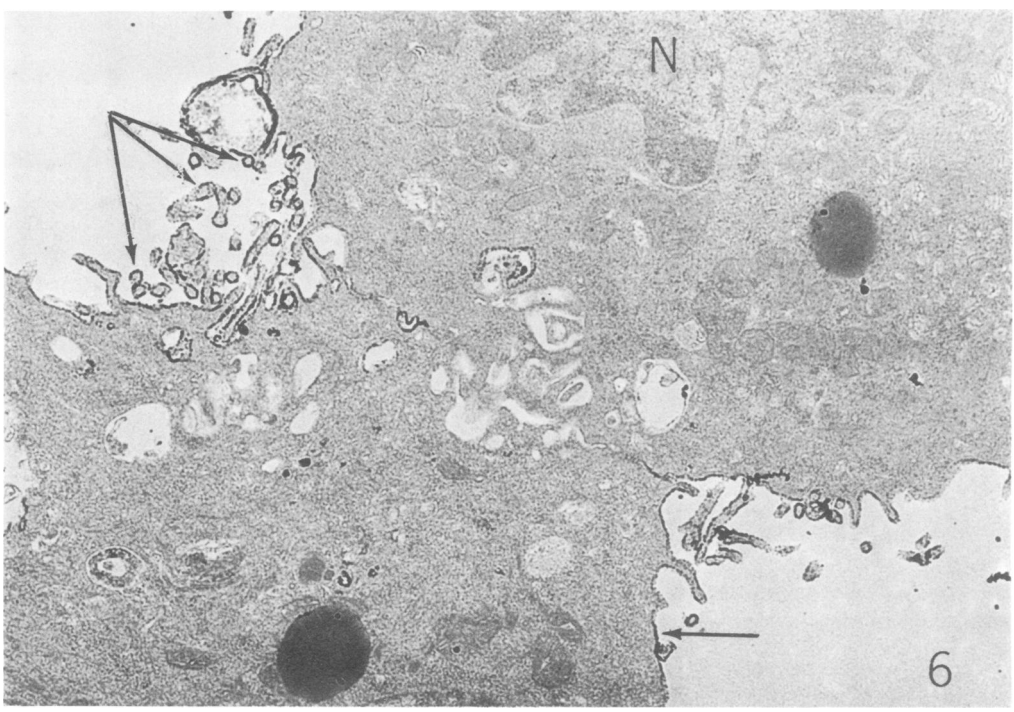


FIG. 6. *Arrows indicate association of DSPM antigen with flat runs of membranes and slender processes; 72 h postinfection. N, Nucleus. Magnification ×7,400; lead tartrate.*

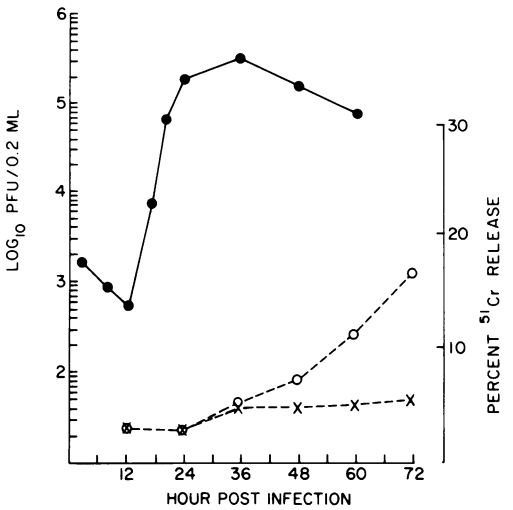


FIG. 7. *Appearance of virus-specific cell surface antigens and infectious virus from LLC-MK$_2$ cells infected with dengue-2 virus. At the indicated times, culture fluids were assayed for infectious virus by plaque formation (●), and the monolayers containing ^{51}Cr were washed and treated with guinea pig complement and antiviral antibodies to produce cell injury as indicated by ^{51}Cr release (○). Background or non-specific ^{51}Cr release (×) produced by complement alone on infected cells; antibody alone produced a slightly lower background in all experiments.*

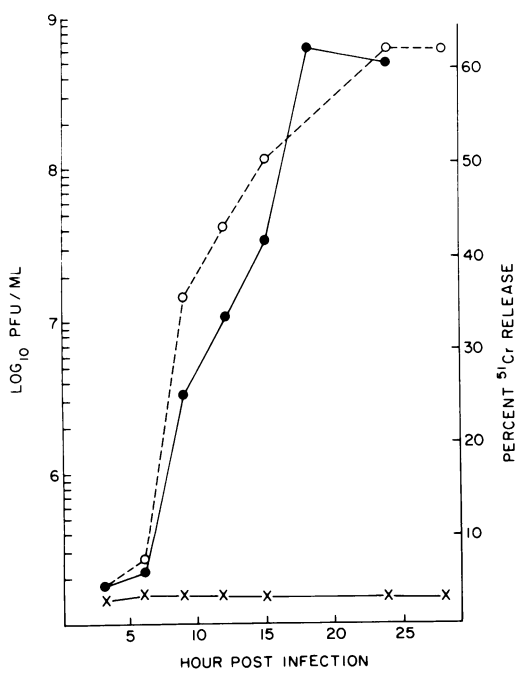


FIG. 8. *Appearance of virus-specific cell surface antigen and infectious virus from LLC-MK$_2$ cells infected with Eastern equine encephalomyelitis virus, a group A arbovirus. Legend same as in Fig. 7.*

From this we may conclude that the amount of DSPM antigen per cell is less than that of group A arboviruses such as EEE and Sindbis. Further differences between these group A arboviruses and dengue-2 virus can be seen by comparing the kinetics of ^{51}Cr release to the viral growth curve. Clearly, the appearance of EEE plasma membrane antigens is closely related to viral release (Fig. 8). This is not surprising since it is known that group A arboviruses undergo final assembly at the surface of infected cells and bud through the plasma membrane (1, 4). This results in the expression of viral-specific antigens in the membranes of infected cells. The peak of ^{51}Cr release with dengue-infected cells occurs much later than the peak of viral release (Fig. 7). For this reason and the fact that the process of dengue release is not precisely known, the mechanism whereby dengue antigens are inserted in host-cell membranes is not clearly understood. However, it has been suggested by others (6) that release of dengue is by exocytosis or reverse pinocytosis of virion-containing vacuoles. We have demonstrated elsewhere (manuscript in preparation) that the membranes of dengue-containing vacuoles contain DSPM antigens. We suggest that these antigen-positive membranes are inserted, in the process of exocytosis, into the plasma membrane.

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