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Distribution of Dengue-2 Antigens by Electron Immunocytochemistry

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The distribution of dengue-2 antigens was studied in infected monkey kidney cells (LLC MK2) using an indirect, horseradish peroxidase-conjugated immunoglobulin technique. This procedure allowed both light and electron microscopic examination of serial-step sections of individual cells cut in a plane perpendicular to the monolayer. Both virion and nonvirion antigens were identified on the plasma membrane with this technique. A diffuse cytoplasmic reaction product was also present. The intensity and distribution of the cytoplasmic reaction product was related to disruption of the plasma membrane.

The Flaviviruses are important human pathogens. An understanding of the intracellular distribution of Flavivirus antigens has been critical in the interpretation of certain disease states, particularly dengue virus infections and immunologically mediated dengue disease syndromes (10).

Like other Flavivirus, dengue virus matures within the cytoplasm of host cells in association with the endoplasmic reticulum and the Golgi complex. Release of virus appears to be by reverse pinocytosis (6, 7). Maturation by budding through internal or external cell membranes has not been observed. Fluorescent antibody studies have revealed intense staining at the perinuclear region of the cytoplasm, with little or no visible plasma membrane staining (6). These observations implied that infected cells did not carry dengue antigens on the cell membrane.

Recent evidence has indicated, however, that virus-related antigens do exist on the surface of dengue-infected cells. Stohlman et al. found radiolabeled dengue proteins in plasma membrane fractions of dengue virus-infected BHK cells (13). Catanzaro et al. demonstrated dengue-specific plasma membrane antigens by using immunoperoxidase and immune cytolysis techniques on viable LLC MK2 cells (7). Brandt and Russell have confirmed and extended these observations to Japanese encephalitis virus by using immune cytolysis (4). These last two studies have been confined to cell surface antigens and have utilized antibodies against crude extracts of dengue-2-infected mouse brain (anti-DEN-2).

Other antibody preparations have been produced against purified virions (anti-rapidly sedimenting hemagglutinating [RHA] antigen) and against a nonstructural dengue (anti-soluble complement-fixing [SCF]) antigen. All three antibody systems have been previously characterized (5, 6). The purpose of the studies reported here was to determine whether or not both structural and nonstructural antigens appeared at the cell surface and to study the intracellular distribution of these antigens at an ultrastructural level using an indirect immunoperoxidase system.

MATERIALS AND METHODS

HMAF. Three types of immunogens were used to produce hyperimmune mouse ascitic fluid (HMAF): (i) crude 20% (wt/vol) homogenates of dengue-2-infected mouse brain; (ii) virions (RHA) purified from dengue-2-infected mouse brain; and (iii) SCF antigen purified from dengue-2-infected mouse brain. The preparation, storage, and characterization of these HMAF has been reported extensively elsewhere (5, 6). The HMAF used in the experiments reported herein are from the same batches used in previous experiments and were kindly provided by W. E. Brandt, Walter Reed Army Institute of Research, Washington, D.C. (6, 7).

Preparation of anti-mouse globulin (AMG)-HRP conjugates. The glutaraldehyde horseradish peroxidase (HRP) conjugation technique of Avrameas and Ternynck was used (1). A 60-mg amount of HRP, type VI (Sigma Co., St. Louis, Mo.), was dissolved in 0.8 ml of 0.1 M phosphate buffer containing 1.25% (vol/vol) glutaraldehyde. The mixture was gently stirred for 17 h at room temperature and then passed through a Sephadex G-25 column (2 by 26 cm) containing 0.15 M NaCl with ultraviolet monitoring at absorbance at 256 nm. The first of two peaks (28 to 42 ml) was pooled and concentrated to 4 ml by pressure dialysis on an Amicon PM10 membrane at 30 lb/in^2 using nitrogen.

Twenty milligrams of goat AMG (Antibodies Inc., Davis, Calif.), prepared by 3× precipitation with 50% saturated ammonium sulfate and with diethylaminoethyl fractionation, was added to the glutaral-

dehyde-treated HRP. The volume of the reaction mix was increased to 7.2 ml with 0.15 M NaCl, followed by 0.8 ml of 0.04 M carbonate-bicarbonate buffer, pH 9.5. After 24 h of incubation at 4 C, 0.4 ml of 0.2 M lysine was added to block unconjugated aldehyde radicals. Incubation was then continued at 4 C for 2 additional h. After dialysis against three 50-volume changes of phosphate-buffered saline (PBS), the globulins were precipitated with an equal volume of saturated ammonium sulfate. The precipitate was washed 3× with 50% saturated ammonium sulfate. The final precipitate was dissolved in 2 ml of PBS, pH 7.4, and exhaustively dialyzed against PBS. After dialysis, a small amount of insoluble material was cleared from solution by centrifugation at 20,000 × *g* for 20 min. The final conjugate was stored at −20 C.

Characterization of HRP-AMG conjugate. The staining specificity of the conjugate was evaluated by immunodiffusion. Standard 0.6% purified agar plates in 0.001 M PBS were used. After double diffusion of reagents, the plates were washed in PBS for 72 h. The plates were then immersed for 10 min in 0.05 mg of diaminobenzidine tetrahydrochloride per ml in tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.6, containing 0.01% H_2O_2. The plates were washed extensively in PBS and read.

Microtiter system. A microtiter system was modified for use with dengue virus systems (9). LLC MK2 cells (kindly provided by W. E. Brandt, Walter Reed Army Institute of Research) were removed from 75-cm^2 flasks by trypsinization. After washes in media, the cells were adjusted to a concentration of 4×10^5 cells/ml by suspension in 0.08 M tricine buffer, 0.075% $NaHCO_3$, penicillin (100 U/ml), streptomycin (100 mg/ml), mycostatin (10 U/ml), and 10% fetal calf serum. The pH was adjusted to 7.2 with 0.1 M NaOH. A 20% dengue-2-infected mouse brain suspension (kindly provided by W. E. Brandt, Walter Reed Army Institute of Research) was serially diluted in medium 199 without fetal calf serum. For virus infection, equal proportions of each virus dilution and cells were mixed and plated immediately by delivering 0.002 ml into each well of a microtiter plate (Falcon Plastic, Los Angeles, Calif.), using a Hamilton syringe (Hamilton, Whittier, Calif.) with a repeating dispenser. Each well received approximately 1,000 cells. After 18 h, each well received an additional 0.005 ml of complete media. Virus titers in infectious units were established by Poisson analysis using the microtiter system (9). For antisera titers, each microtiter well received 0.5 to 1.0 infectious units of dengue-infected mouse brain seed per cell.

The microtiter plates were incubated at 37 C for 24, 48, or 72 h. The plates were rinsed with PBS and fixed 1 h with 4% paraformaldehyde, rinsed in cold ethanol, and rinsed again with PBS. After fixation, the cells were stained by either indirect immunofluorescent (6) or immunoperoxidase (IP) techniques. Wells were viewed at 40× and 100× on a Zeiss photomicroscope. For Poisson distribution, the wells could be scored as positive or negative. For immunofluorescence or IP studies, the wells were studied for staining pattern and intensity.

Preparation of cells for electron immunocytochemistry. LLC MK2 cells in 35-cm^2 plastic petri dishes were overlaid with 20% dengue mouse brain seed for 2 h at 37 C at a multiplicity of infection of 0.25 to 1.0 infectious units/cell. The mouse brain seed was poured off and washed with vigorous shaking 3× with medium without fetal calf serum. The cells were incubated for varying time periods depending upon the experimental design. At the appropriate time, the cells were washed 3× with medium 199 without fetal calf serum and fixed by four 15-min changes in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, containing 0.005 M $CaCl_2$-H_2O. The cells were thoroughly washed in PBS. The cells were then covered with the appropriate dilution of HMAF (primary antibody) and incubated for 2 h at 37 C, followed by a 12-h wash in PBS. The HRP-AMG (secondary antibody) was overlayed at a 1:640 dilution, and incubation took place at 37 C for 10 to 18 h; then the HRP-AMG was washed with repeated changes of PBS for an additional 18 h. The cells were fixed in 2% glutaraldehyde, 0.1 M phosphate buffer, pH 7.3, for 1 h, washed in PBS, and incubated for 5 to 10 min in the stain reaction mixture consisting of 0.05 mg of diaminobenzidine tetrahydrochloride per ml in tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.6, containing 0.01% H_2O_2. The monolayers were rinsed 3× in PBS and postfixed with 1% OsO_4 in phosphate buffer for 1 h.

After dehydration in a graded series of alcohols and subsequent infiltration, a thin layer (about 2 mm) of an epon-araldite epoxy resin mixture was poured over the cells and polymerized at 60 C. After polymerization, the sides of the petri dish were removed with a pair of pliers. The plastic dish was then carefully bent until it began to separate from the resin. Removal of the plastic sheet containing the cells was then easily accomplished. The embedded monolayers could, at this point, be studied from above by light microscopy. Selected areas were cut out and re-embedded in fresh resin in flat molds so cells could be sectioned in a plane perpendicular to the monolayer (vertical).

Vertical, thick serial-step sections, 1 μm, were cut with glass knives, spread with xylene vapors, and mounted on glass slides. Fifteen to thirty sections were taken from each block, covering a total distance of 40 to 60 μm. The mounted sections were placed on a low-temperature hot plate overnight to assure tight adherence to the slide and then were covered with a cover slip. Even with this protocol, wrinkles often occurred at the interface of the layers of resin. The IP reaction was readily identified by the red-brown reaction product. Photomicrographs were taken on a Zeiss photomicroscope II without benefit of counter stains using a 40× oil Planapochromat objective lens.

Thin sections were alternated with the 1-μm sections, using a diamond knife on a Reichert OMU-2 ultramicrotome, mounted on slotted grids, and viewed in an AEI EM 6B electron microscope. The cells were studied without heavy metal stains, so that contrast was primarily provided by the electron-dense osmicated IP reaction products (1).

Immunocytochemical controls. Several series of controls were examined. In penetration controls, normal mouse ascitic fluid was used on uninfected LLC MK2 cells followed by HRP-AMG, without intervening rinses to document penetration of immunoglobulins under standard conditions. Deletion controls included omission of the primary antibody, the secondary antibody, or the diaminobenzidine hydrochloride system. Substitution controls included exchange of normal mouse ascitic fluid for primary HMAF (complemented the penetration control), substitution of uninfected cells for infected cells, and substitution of uninfected mouse brain for infected mouse brain (mock infection). A blocking control was used with unconjugated AMG before the HRP-AMG conjugate. Zero-hour controls utilized infected cells, which were fixed immediately after the dengue-2 seed was washed off the cells at 2 h postinfection.

RESULTS

Characterization of antibodies. The reactivity of the three primary antibodies has previously been reported (5, 6). The IP system proved to be more sensitive than the immunofluorescent system (Table 1). Both the immunofluorescent and IP systems revealed the same perinuclear distribution of antigen (Fig. 1a and b).

Under most experimental conditions, the controls for the IP system were negative (Fig. 1 and 2). Some reactivity was observed, however, in 0-h and mock infection controls, which suggested a non-dengue reactivity. Immunodiffusion analyses revealed that all three HMAF had reactivity against homogenates of uninfected mouse brain. When the cells were vigorously washed after adsorption, however, the HRP reaction product was reduced to a low or negligible level background in control cells (Fig. 1 and 2).

Vertical serial-step sections. The technique of vertical serial-step sectioning allowed three-dimensional reconstructions of the reaction product staining pattern for each cell.

At a light microscopic level, the IP gave both a cytoplasmic and a membrane stain (Fig. 3). The membrane stain was diffuse rather than focal. It was spread over the entire cell surface exposed to the media but not on the surface against the plastic. The membrane stain pattern was the same for all three primary antibody systems used.

Some cells had a very light, diffuse cytoplasmic stain that could only be detected by comparison with the nucleus, which was negative. Other cells contained very dense stain, which tended to be localized either on one side of the nucleus (polar), or in the region directly above the nucleus, or both. Other cells had no cytoplasmic stain.

The examination of the serial-step thick sections was crucial in interpreting the intensity and pattern of stain in a particular cell. For instance, whereas a given cell might have a light cytoplasmic stain in one plane of sectioning, sections several microns further into the same cell would have an area of intense cytoplasmic stain (Fig. 3).

Electron microscopy. As with light microscopy, electron microscopic examination revealed similar, if not identical, reaction product stain with all three primary antibody systems. The electron-dense stain was found both on the plasma membrane and in the cytoplasm (Fig. 4–6). Utilizing the alternate thin sections, individual cells that exhibited a particular stain pattern, as described in the preceding section, were located and studied (Fig. 3 and 4).

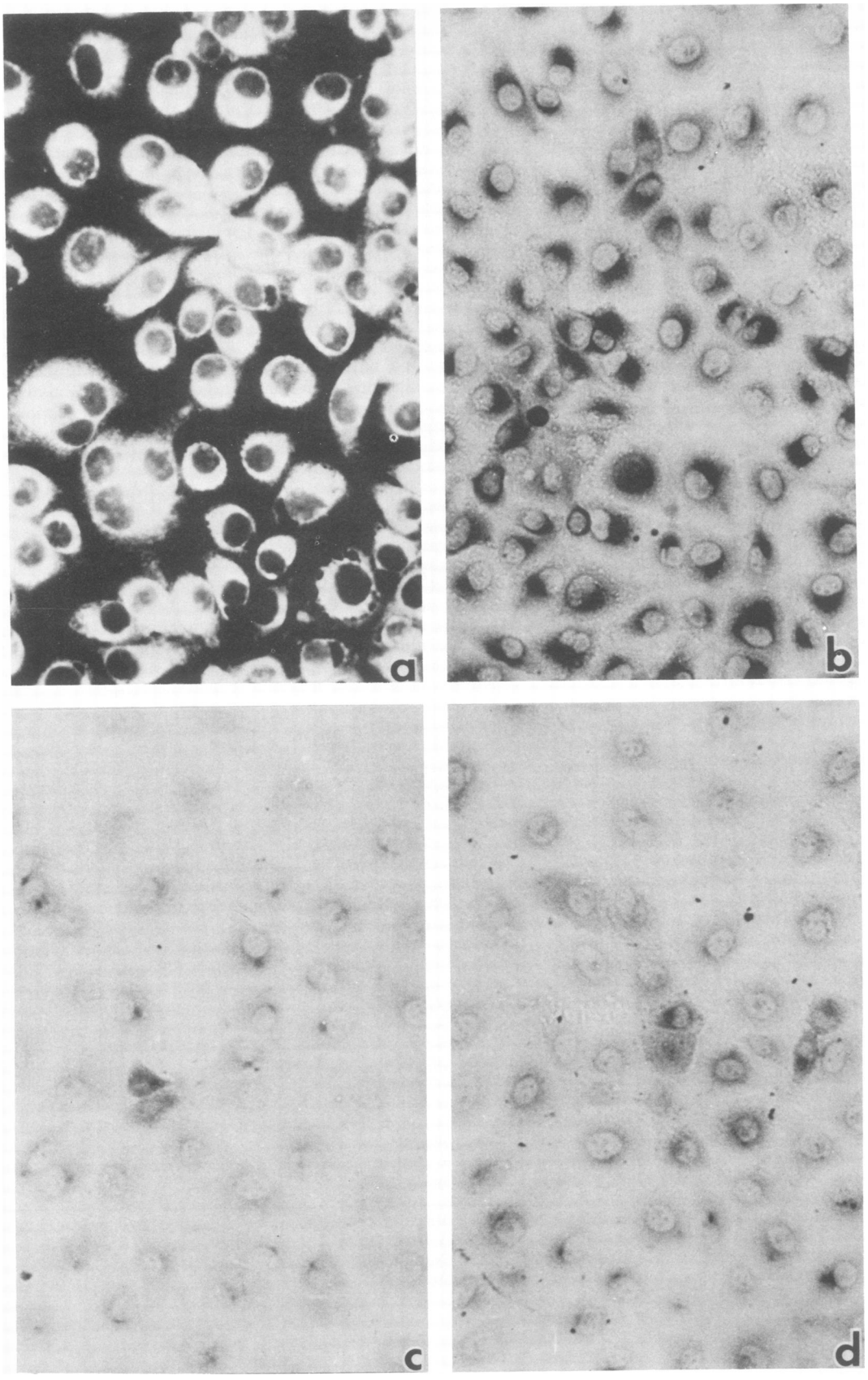
An electron-dense reaction product stain was observed on the outer surface of the plasma membrane using antibodies against either virion (anti-RHA) or nonvirion (anti-SCF) antigens (Fig. 5). The stain diffusely covered the entire cell surface (Fig. 3 and 4) without preferential clustering of the stain (Fig. 5). Some cells with membrane stain did not have identifiable cytoplasmic staining, although the same cells contained both virus particles and cytopathic vacuoles.

The cytoplasmic deposits were also widespread without any discrete localization. The stain in slightly positive cells tended to be particulate or granular and appeared to enhance the electron density of the rough endoplasmic reticulum, suggesting a ribosome-related distribution (Fig. 6a). In these lightly stained cells, the degree of reactivity could best be

TABLE 1. *Characterization of antibody systems*

Antibody	Source	Reactivity	Immunofluorescent titer	IP titer
Anti-DEN-2	HMAF	Broad	1:40	1:640
Anti-RHA	HMAF	Virion	1:40	1:320
Anti-SCF	HMAF	Nonvirion	1:40	1:640
Anti-mouse	Goat	Mouse IgG[a]	1:10	1:512

[a] IgG, Immunoglobulin G.


a
b
c
d

judged by comparing electron density of the cytoplasm to that of the nucleus, which was always negative. The density of the stain in other cells was very electron dense and prevented accurate interpretation of the cytoplasmic reaction (Fig. 6b). Occasionally, deposits were observed along the nuclear envelope. The cytoplasmic membrane system, virus particles, and the characteristic cytopathic vacuoles did not have stain with any of the three primary antibody systems (Fig. 6a).

Cells exhibiting a polar stain pattern were carefully studied. Areas that did not have reaction product stain frequently did have evidence of dengue infection in the form of intracisternal virus particles and cytopathic vacuoles. Careful reconstruction revealed that the areas of cytoplasmic deposits were always adjacent to, but not necessarily directly beneath, disruptions of the surface membrane (Fig. 4 and 6c). Thus, the polar distribution was, in part, a reflection of antibody penetration.

DISCUSSION

The most significant aspect of this study has been the identification of both virion (RHA) and nonvirion (SCF) antigens on the surface of dengue-2-infected LLC MK2 cells. These studies corroborate and extend similar biochemical observations with immunological data (13). The nonvirion SCF is of particular interest since it has been regarded as an antigen that might be associated with dengue hemorrhagic fever or shock syndrome (8, 10, 11). The presence of SCF on the surface of dengue-infected cells increases the likelihood of host response to this antigen. Such an immunological response against SCF has been observed in patients with secondary dengue virus infections (8). The host immune response could be directly against the cell surface RHA or SCF antigens, creating an in vivo immunocytolysis (4, 7). Alternatively, an external position could facilitate antigen release (3). Circulating antigens could combine with antibodies to make immune complexes, which have been discussed in the pathogenesis of dengue shock syndrome (10).

The diffuse nature of the membrane stain appears to conflict with the previous observations of focal stain (7). It should be emphasized that the present study utilized prefixed cells, reacted in situ, whereas the study of Catanzaro et al. (7) used viable cells fixed after the immunological reactions were completed. The distribution of the membrane stain could well reflect the relative mobility of the antigens in the membranes of fixed and unfixed cells (2).

The interpretation of the membrane reaction must be tempered by the recognition that all three primary HMAF had antibodies against mouse brain antigens. The most rigorous means of circumventing this problem would have been extensive adsorption of the HMAF with mouse brain. The limited amount of HMAF available and the many negative controls encouraged us to work with the HMAF at high dilutions. Most of the 0-h postinfection cells and most of the mock-infected cells were negative. As has been illustrated, however, some of the control cells did have a slightly positive membrane reaction. Whereas the majority of negative control cells and the very light stain on the 0-h control cells provide evidence of specificity, a minor contribution from residual mouse brain antigens cannot be excluded in any given positive cell in the dengue-infected cultures.

An intracytoplasmic stain was also found using all three HMAF. The pattern of the stain was clearly dependent upon the extent of penetration of the immunoglobulins into the cytoplasm. The three-dimensional reconstructions demonstrated that the cytoplasmic stain could be found only in cells with disrupted membranes. Penetration must be considered the primary cause of polar stain pattern and negative virions, cytopathic vacuoles, or other nonreactive areas. As a result, areas or organelles without stain must be regarded as inadequately penetrated areas rather than antigen-negative areas.

The areas of the cell that were adequately penetrated with immunoglobulins revealed a diffuse granular cytoplasmic stain. This pattern of stain could be attributed either to antigens soluble in the cytoplasm and/or to membrane-free polyribosomes as sites of protein synthesis. In either case, the dengue-2 antigens were not limited to the membranes of the endoplasmic reticulum, as is implied by biochemical studies of dengue and of other Flaviviruses (12, 13).

These studies established that both virion and nonvirion antigens appear on the plasma membrane of dengue-infected cells. They correlate well with the findings of radiolabeled den-

FIG. 1. *Comparison of the distribution of dengue-2 antigens in LLC MK2 cells using anti-DEN-2 and (a) a fluorescein-conjugated AMG on dengue-infected cells (×320); (b) HRP-conjugated AMG on dengue-infected cells (×240); (c) HRP-conjugated AMG on uninfected cells (×240); (d) HRP-conjugated AMG on 0-h control in which the viral seed was introduced to the cells and immediately washed off (×240). Note the tight perinuclear distribution of stain in both (a) and (b).*

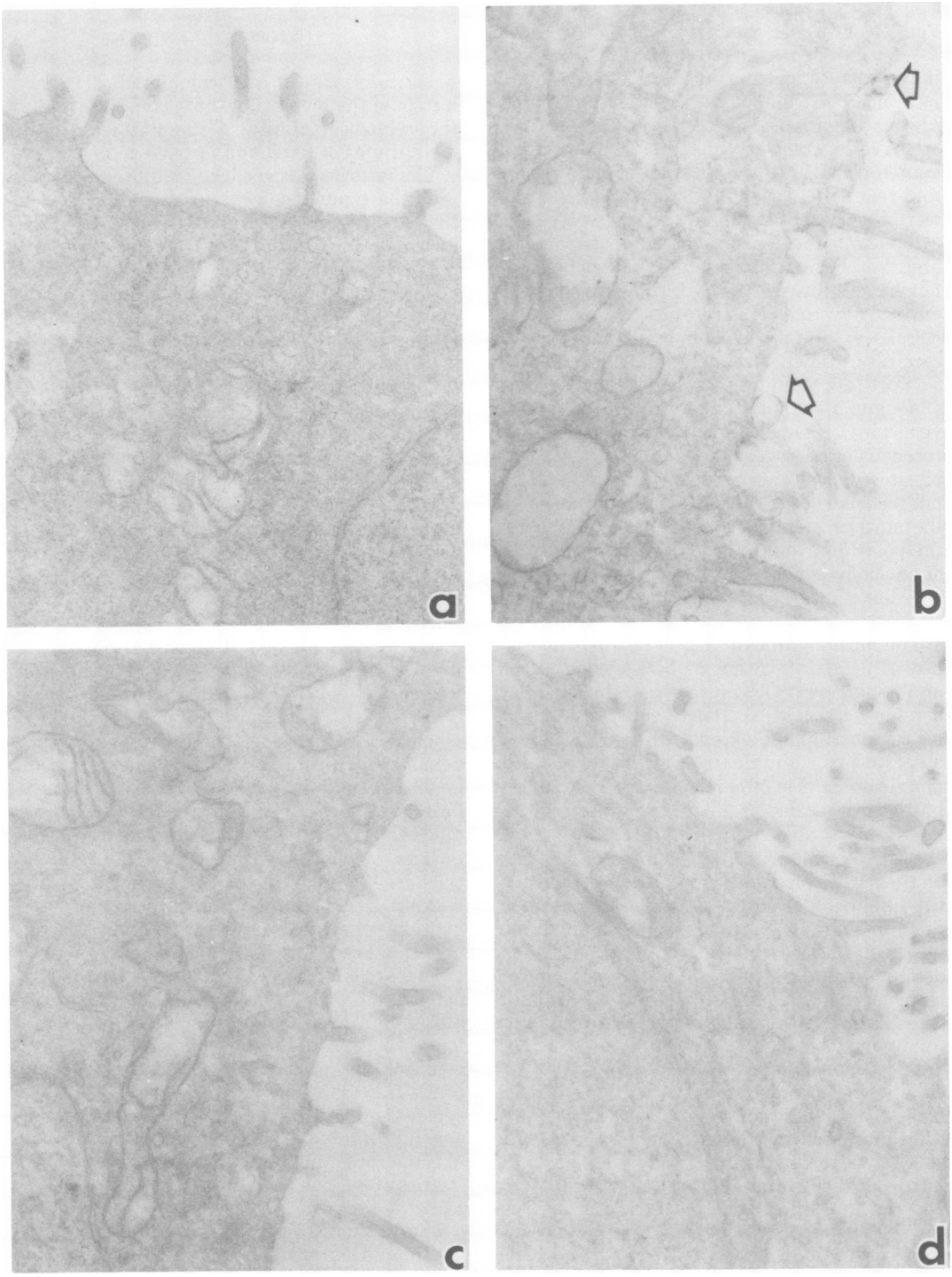


FIG. 2. *Electron micrographs of LLC MK2 control cells. (a) Dengue-2-infected cell exposed to normal mouse ascitic fluid and HRP-conjugated AMG. This substitution control showed no electron-dense reaction product (×22,000). (b) Zero-hour control of dengue-2-infected cell illustrating a relatively light electron-dense reaction product along the cell surface (arrows). This is most likely due to residual dengue-infected mouse brain and demonstrates the maximum level of background reaction product detected in the negative controls (×22,000). (c) Dengue-2-infected cell illustrating HRP-conjugated AMG without anti-DEN-2 (deletion control; ×22,000). (d) The complete anti-DEN-2 HRP-AMG system on an uninfected cell (substitution control; ×22,000). Note that the fixation with paraformaldehyde provided adequate ultrastructural preservation, although some swelling of the mitochondria was present. No heavy metal stains were used to enhance electron density.*

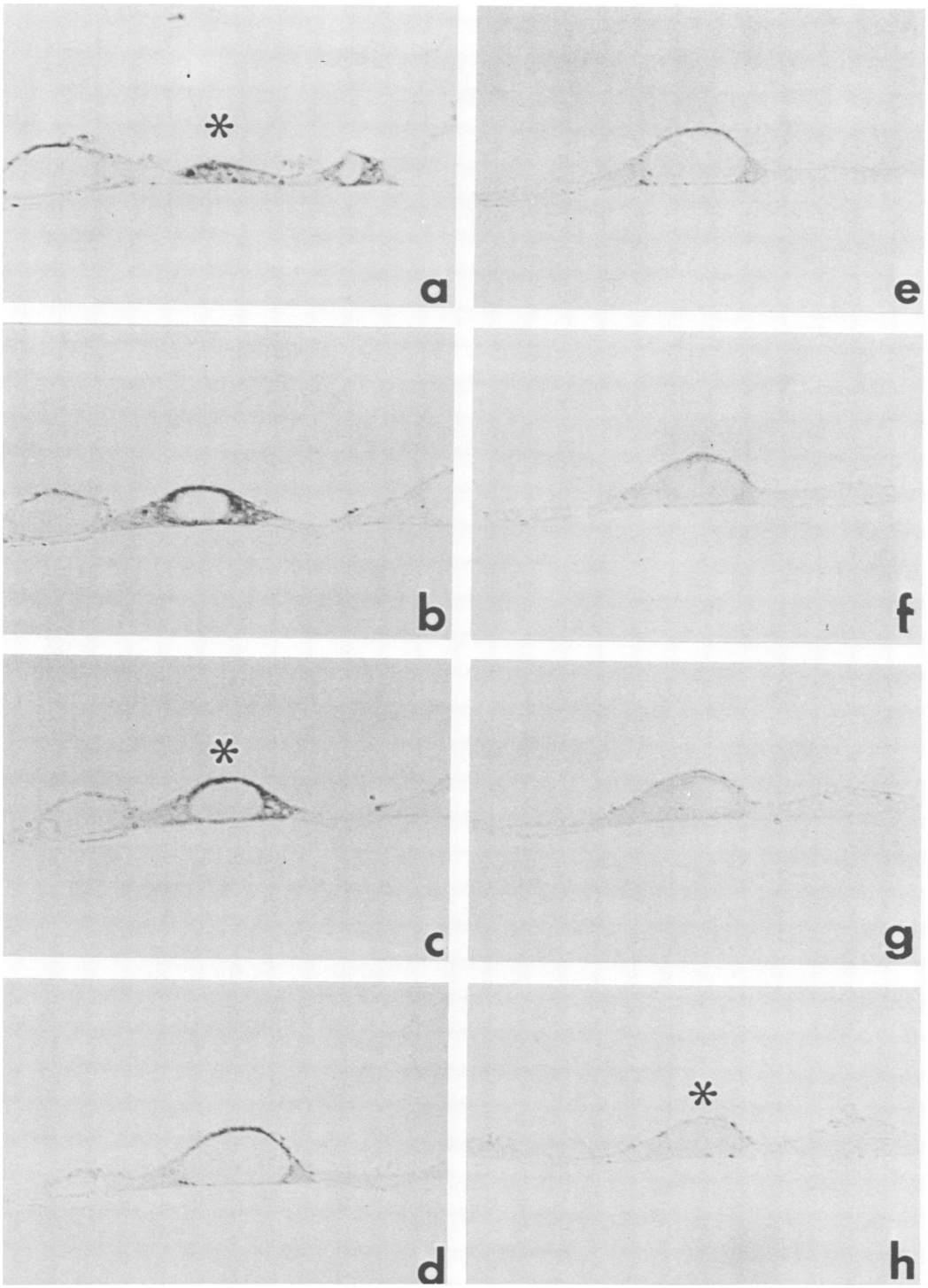


FIG. 3. *Vertical serial-step sections of dengue-2-infected LLC MK2 cells reacted with anti-DEN-2, HRP-conjugated AMG and sectioned in a plane perpendicular to the monolayer. The central cell has membrane and cytoplasmic reaction product (a, b, c) and membrane reaction product without significant cytoplasmic reaction (h). Asterisks indicate approximate level of corresponding thin section illustrated in Fig. 4 (×700).*

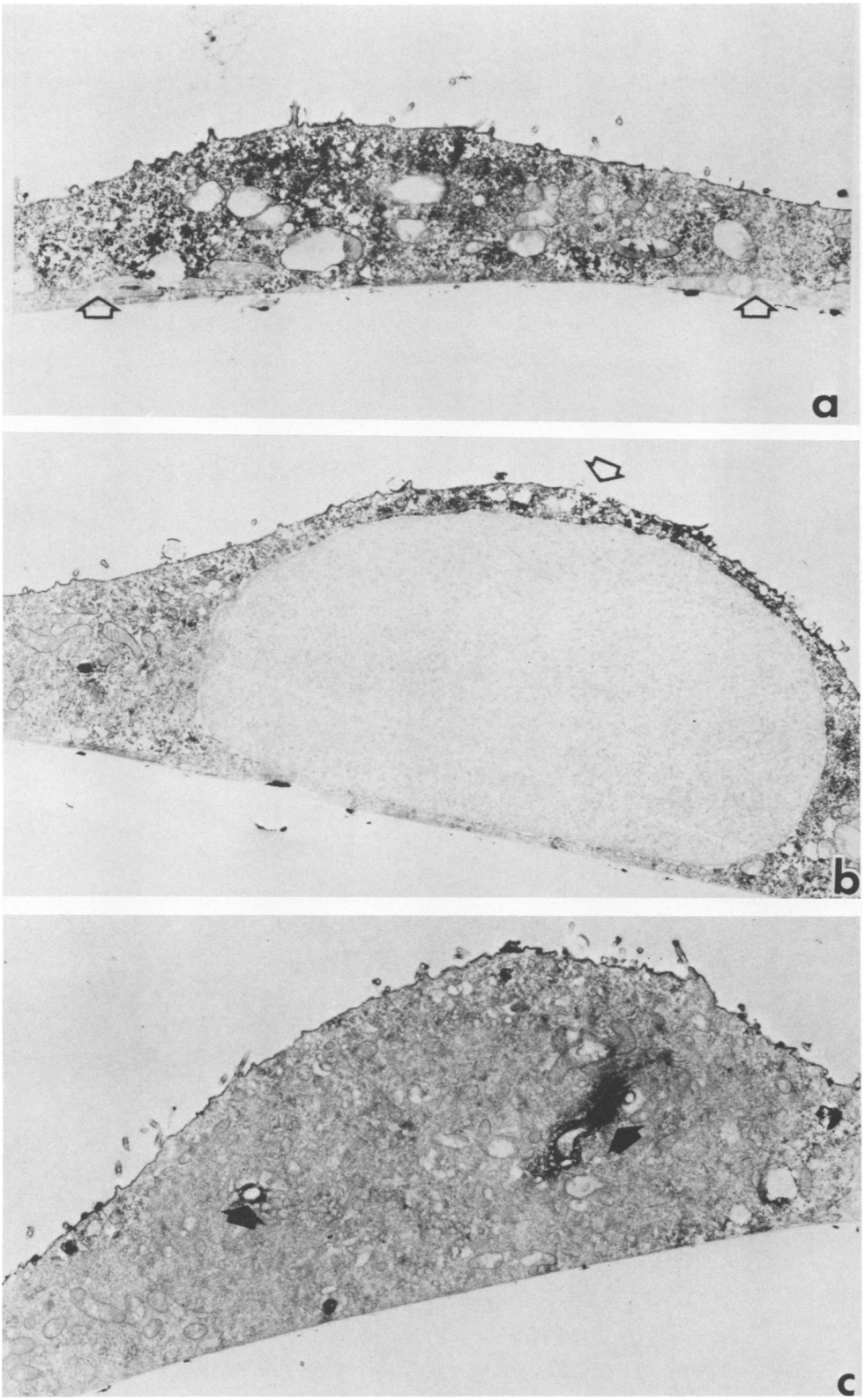


FIG. 4. *Electron micrographs of cell illustrated in Fig. 3. (a) Corresponds to Fig. 3a and demonstrates a dense cytoplasmic reaction with a positive membrane reaction. Note that processes of adjacent cells do not contain reaction product stain (arrows; ×7,200). (b) Corresponds to Fig. 3c and demonstrates disruption of the plasma membrane (arrow) with concomitant penetration of reaction product into the adjacent cytoplasm (×7,200). (c) Corresponds to Fig. 3h and demonstrates a dense membrane reaction product. The underlying cytoplasm does have reaction product, but the product is much less dense than that on the opposite side of the nucleus (Fig. 3a, h and 4a). Discrete electron-dense foci are also present (arrows; ×7,200).*

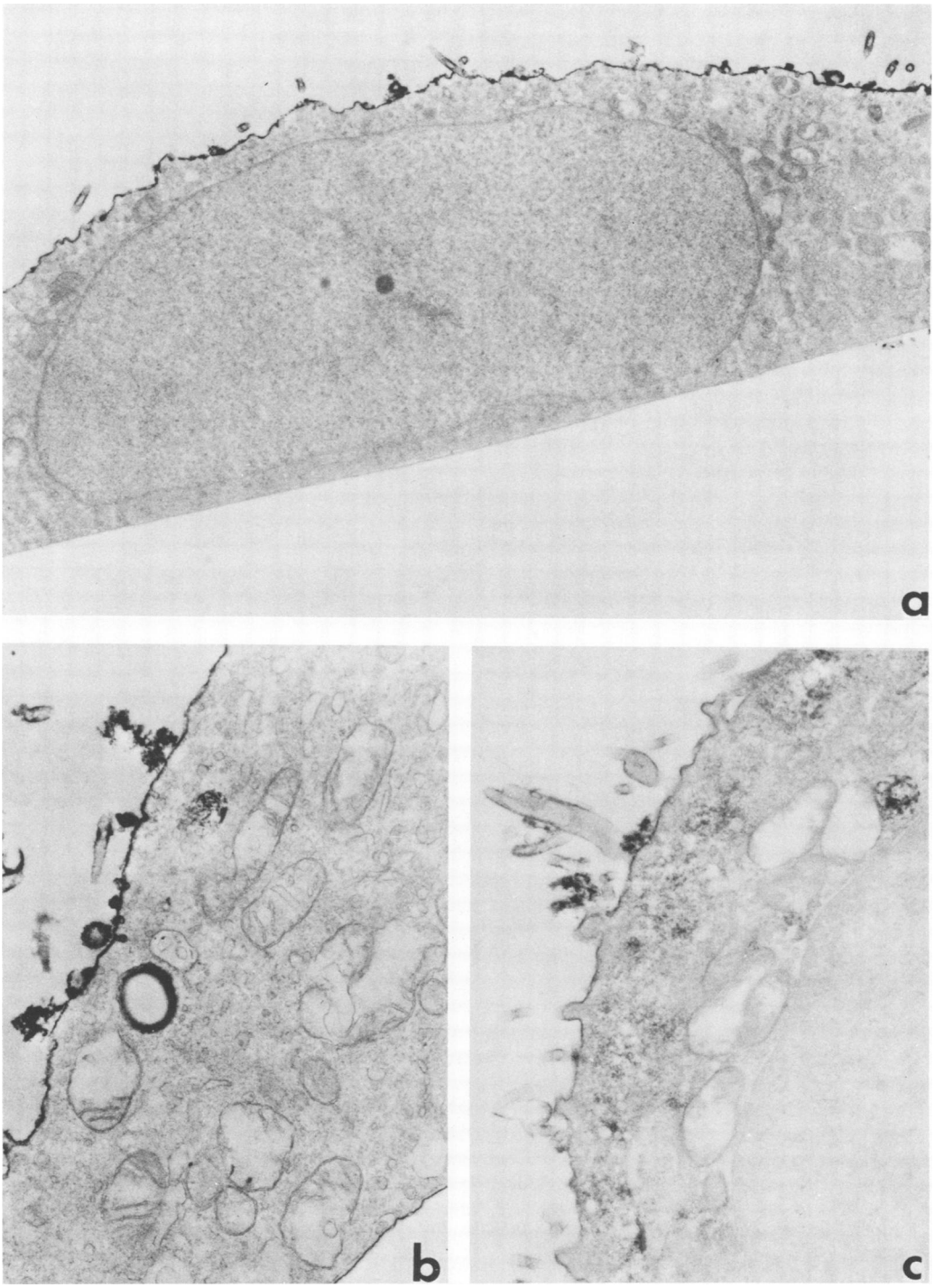


FIG. 5. *Electron micrographs illustrating the distribution of stain on the surface membrane of dengue-2-infected LLC MK2 cells using (a) anti-DEN-2 (×8,000); (b) anti-RHA (×20,000); (c) anti-SCF (×16,000). Compare the density of the reaction product on the surface membrane with that found in controls (Fig. 2).*

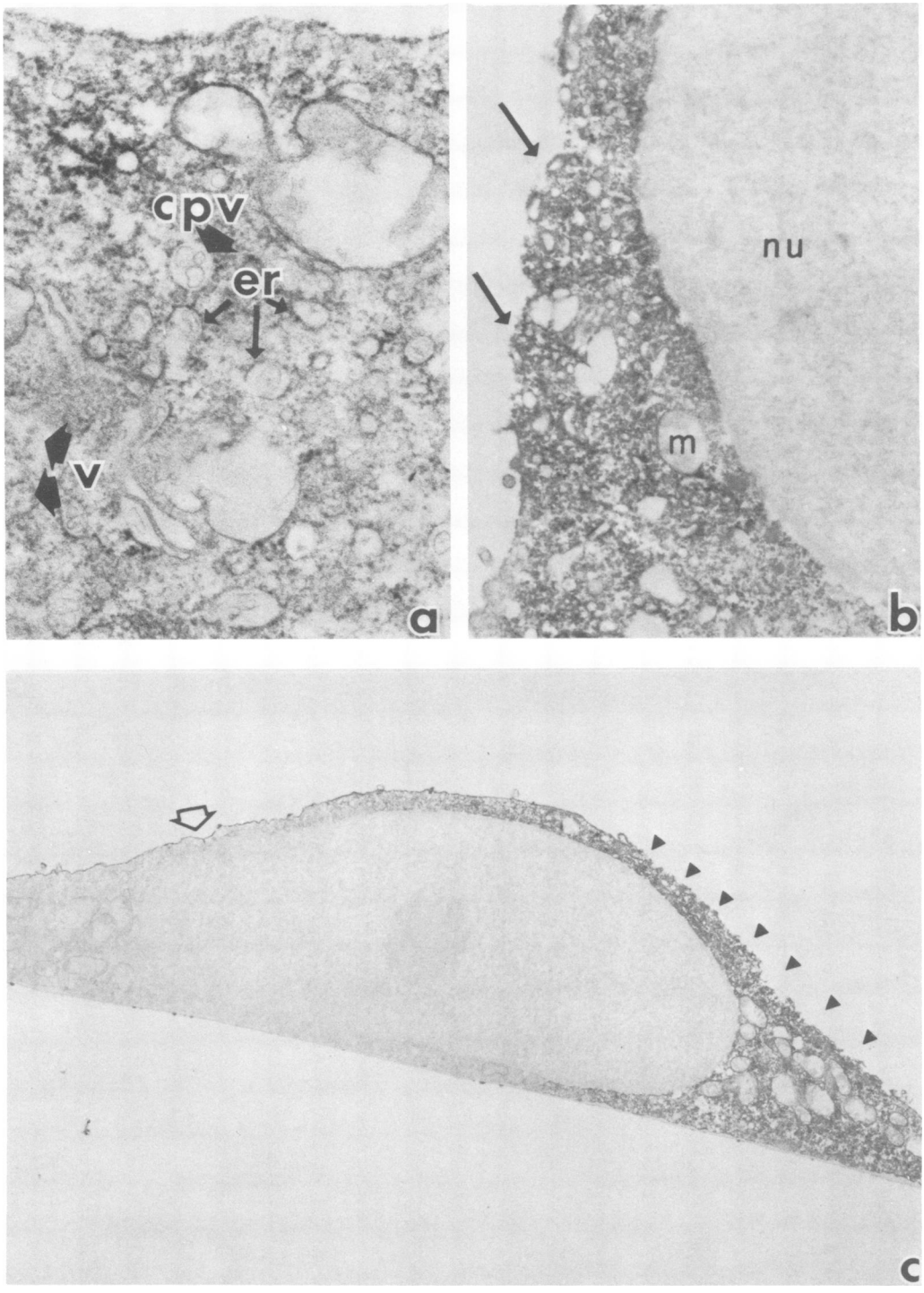


FIG. 6. *Electron micrographs of dengue-2-infected LLC MK2 cells. (a) Using anti-DEN-2 and HRP-conjugated AMG, this micrograph demonstrates the diffuse granular cytoplasmic deposits. The reaction product appears to have affinity for the rough endoplasmic reticulum (er) in some areas and to be free of membranes in others. Cytopathic vacuoles (cpv) and virions (v) do not appear to have deposits (×35,000). (b) Using anti-DEN-2 and HRP-conjugated AMG, this micrograph demonstrates disruption of the plasma membrane (arrows) with subadjacent dense cytoplasmic reaction product. Note that such dense deposits obliterate most cytoplasmic structures, precluding interpretation of precise localization of antigens. m, Mitochondria; nu, nucleus (×20,000). (c) Survey micrograph illustrating the effect of a disrupted plasma membrane (arrowheads) with dense stain beneath the rupture. On the opposite side of the cell, the membrane is intact and the cytoplasm in this area is negative (arrow; ×6,000).*

gue proteins into plasma membranes (13). The hypothesis that these are membrane-bound antigens inserted into the surface membranes during virus expulsion by reverse pinocytosis (exocytosis) (7) would predict that the appearance of the surface antigens would correlate with virus release. The appearance of surface antigens as measured by immune cytolysis does not, however, necessarily correspond with the release of infectious virus (4, 7). These discrepancies might be resolved if not all antigens were bound to the endoplasmic reticulum. The presence of nonmembrane, cytoplasmic, dengue-specific antigens as described above could lead to direct antigen insertion into the plasma membrane quite independent of virus release by exocytosis. Although these experiments provide conceptual alternatives, much more work is required to clarify the molecular biology of Flaviviruses.

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