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Detection of individual fluorescently labeled reovirions in living cells

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ABSTRACT **Reovirus serotype 1 (Lang) can be conjugated with rhodamine B or fluorescein isothiocyanate in a way that preserves viral infectivity. We have used epifluorescence microscopy to detect individual virions bound to the surface of cells and to follow in real time the early stages of reovirus infection in living cells. Following uptake of the virus into endocytic vesicles, the movement of these vesicles can be observed readily. The vesicle movement is inhibited by nocodazole or colchicine, consistent with previous findings that the movement of intracellular vesicles is often microtubule-based.**

Epifluorescence microscopy has become one of the essential tools in biology. A major attribute of this technique is its sensitivity: as few as 50 fluorescent molecules in a cubic micrometer can be detected (1). In contrast to electron microscopy, epifluorescence microscopy can be readily adapted to experiments with living cells (1). With the advent of highly sensitive detection systems including improved photographic film and video cameras that can image aggregates of fewer than 20 fluorescent molecules, the time seemed ripe for an attempt to detect a single animal virus particle by light microscopy.

Fluorescently labeled viruses have been used in other ways to study virus–host interactions. Epstein–Barr virus conjugated with fluorescein isothiocyanate (FITC) has been used to examine the specificity of binding to cellular receptors (2), and FITC diphosphate-conjugated influenza virus has been used to study viral uncoating in endosomes (3). However, it has not been established whether each individual fluorescent particle represents a single virion or an aggregate of several virions; nor have fluorescently labeled viruses been used for studies in a living cell system.

Based on the size of most animal viruses and the detection systems available, we estimated that ≈500 molecules of rhodamine B per virion might be sufficient for detection. Reovirus serotype 1 (Lang) was chosen for these studies because it grows to high titer in cell culture, is relatively stable at high pH, and is therefore more likely to survive the conditions of dye conjugation (2). We report here that individual virions of reovirus conjugated with either rhodamine or fluorescein can be observed on the surface of infected L cells at early times after inoculation and that endocytic vesicles containing small clusters of virions can be seen moving inside cells in a manner consistent with the translocation of organelles along microtubules.

MATERIALS AND METHODS

Cells and Virus. Mouse L cells (fibroblasts) were grown on glass coverslips or in spinner cultures in "completed MEM" containing Joklik's modified Eagle's medium (Irvine Scientific) supplemented with 2.5% fetal bovine serum (HyClone), 2.5% neonatal bovine serum (Biocell Laboratories), 0.3% glutamine, and 1% penicillin/streptomycin solution (Irvine Scientific).

Plaque-purified reovirus serotype 1 (Lang) was used for conjugation. The virus was purified by sonication and Freon extraction (4, 5). The virus was stored in CsCl solution following the collection of the virus bands from CsCl gradients.

Antibodies. Monoclonal antibodies to the type 1 σ1 protein (5C6; unpublished data) and the type 3 σ1 protein (G5; ref. 6) were purified as described (7).

Conjugation of Virus. The pH of the purified virus suspension was raised to 9.3 in two steps by dialysis against 50 mM sodium bicarbonate-buffered 0.85% saline (3 hr vs. pH 8.5 followed by 3 hr vs. pH 9.3) at 4°C.

Conjugation reaction mixtures contained virus at 1 mg/ml and FITC (Sigma) or rhodamine B isothiocyanate (RITC; Sigma) at 1 mg/ml in 50 mM sodium bicarbonate buffer (pH 9.5). Reactions were carried out in the dark for 1 hr at room temperature. The conjugated virus was purified by gel filtration on a Sephadex G-50 column and recovered in 10 mM Tris·HCl (pH 7.4). The final concentration of the virus was determined by A_{260} (UVcon spectrophotometer, Kontron Instruments, Everett, MA). The standard conversion factor used to calculate the concentration of the virus was that 1 OD unit is equivalent to 2.1×10^{12} particles or 185 μg of protein per ml (8). Similarly, the concentration of rhodamine B was determined by A_{545} and of fluorescein by A_{490}. Rhodamine B has a second minor absorbance peak at 260 nm; therefore its effect on the absorbance of the virus at this wavelength was considered in the calculation of virus concentration. The contribution of rhodamine B at 260 nm ranged from 3–4% up to as much as 17–18% depending on the concentration of rhodamine in the preparation. The conjugations yielded 300–500 fluorescent molecules per virion. Conjugated virus was tested for infectivity by plaque assay (4).

SDS/PAGE. Discontinuous SDS/PAGE gradient gels (4–16%) were run using the method of Laemmli (9, 10). Fluorescent bands were visualized under UV light and photographed on Polaroid 667 black-and-white film. The gels were subsequently fixed and silver-stained by the method of Merril *et al.* (11). Alternatively, the gels were fixed in 30% 2-propanol/10% acetic acid and stained in fixative with 0.5% Coomassie brilliant blue R-250 (Sigma) (10). The gels were destained in 10% methanol/10% acetic acid for several hours.

Inoculation of Cells with Fluorescently Labeled Virus. Circular 12-mm no. 1 glass coverslips (Dynalab/Rochester Scientific) were placed into 24-well tissue culture plates (Costar or Linbro, Flow Laboratories). L cells were seeded at 10^5 per

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Abbreviations: FITC, fluorescein isothiocyanate; RITC, rhodamine B isothiocyanate.

well in 1 ml of completed MEM and allowed to attach overnight. Coverslips were chilled on ice briefly, the medium was removed, and the cells washed with ice-cold phosphate-buffered saline (PBS) prior to addition of fluorescently labeled virus (2–3 × 10^4 particles per cell). Coverslips were kept at 4°C for 1 hr to permit surface binding but not internalization of the virus. Coverslips used for video recording were mounted on a living-cell chamber made of 0.7-mm-thick silicon rubber (12) (N. A. Reiss, Belle Mead, NJ) containing completed MEM medium. A 37°C temperature was maintained during microscopy with a Zeiss air curtain. Alternatively, coverslips were left in the incubator at 37°C and fixed at various times for microscopy. Coverslips were washed three times in cold PBS to rinse off unbound virus and then transferred to wells containing 1 ml of 3% formaldehyde in PBS for 10–20 min and mounted on a microscope slide with glycerol/gelatin (Sigma).

For antibody experiments, the conjugated virus was mixed with either 5C6 or G5 antibody at concentrations of 0.1 mg/ml and 2 mg/ml, respectively, and incubated on ice for 1 hr before inoculation. All subsequent manipulations were performed as described above.

Treatment with Nocodazole or Colchicine. L cells were plated on coverslips and infected as described above except that cells were pretreated with nocodazole (Sigma; ref. 12) at 1 μg/ml or colchicine (Sigma; ref. 13) at 10 μg/ml in completed MEM for 2 hr. Coverslips were either mounted on a living-cell chamber or fixed at various time points after infection as described above.

To examine the recovery from the effects of 2 hr of nocodazole treatment, cells were washed with PBS and placed in fresh MEM for 20 min. The cells were then examined at 37°C by epifluorescence microscopy.

Fluorescence Microscopy and Video Microscopy. Fluorescent virus was visualized using a Zeiss Axiophot or Photomicroscope III equipped with a 100-W mercury bulb and Planapo or Neofluar objective lenses (×63 or ×100) as described (14). Filters for visualizing fluorescein or rhodamine were described previously (12, 14). Video recording was made with a Hamamatsu C2400-08 video camera and a JVC Super VHS recorder. Image processing was performed with an IBM AT equipped with an Image Pro 100 processor. Fluorescence micrographs were made with Kodak T-Max 400 film exposed at E.I. 1600 and developed in Kodak T-Max developer.

Fluorescent and Electron Microscopic Images of the Same Field. A stock solution of rhodamine B-conjugated reovirus stabilized in 0.5% gelatin/saline (136 mM NaCl/2.7 mM $CaCl_2$/0.08 mM $MgCl_2$/19 mM H_3BO_3/0.13 mM $Na_2B_4O_7$/3% gelatin) was diluted 1:300 in PBS. A 10-μl aliquot was placed on a grid doubly coated with Formvar followed by carbon. A drop of 0.5% uranyl acetate was added and drained with a piece of filter paper. This was repeated three times. The grid was then placed on Parafilm that had been pressed onto a light microscopy slide. A drop of water was added and a coverslip was mounted. Fluorescent images of reovirus were visualized by a Zeiss Axiophot microscope and recorded as described above. After dehydration, the virus was examined with a Philips 300 electron microscope.

Ultrastructural Examination of Infected Cell Monolayers. Virus-infected monolayers were washed with cold PBS and fixed at various times postinfection by addition of cold 2% glutaraldehyde in 0.08 M cacodylate buffer (pH 7.4) to the culture wells. Monolayers were postfixed in buffered 1% osmium tetroxide, dehydrated in increasing percentages of ethanol from 25% to 100% and propylene oxide, and then embedded in epoxy resin. Coverslips were removed from embedded cells by brief immersion in liquid nitrogen, and 80-nm sections were cut on a Sorvall MT 6000 ultramicrotome using a diamond knife. Sections were stained with uranyl acetate and lead citrate before examination with a Phillips 300 electron microscope.

RESULTS

Conjugation of Reovirus Type 1 with Rhodamine B or Fluorescein. Reovirus type 1 (Lang) was conjugated with RITC or FITC, purified by gel filtration on Sephadex G-50, and assayed for infectivity by plaque assay in L cells. Titers for stock virus were about 1 plaque-forming unit per 400 particles whereas the conjugated virus had average titers of 1 plaque-forming unit per 2000 particles in four different experiments.

The conjugated virus was examined by epifluorescence microscopy. Remarkably, even at low magnification, what appeared to be individual particles could be readily discerned without electron microscopy (Fig. 1*B*). To investigate whether the individual fluorescent foci visualized by light microscopy corresponded to single virions, electron micrographs (Fig. 1*A*) were made of the same field as the fluorescent images (Fig. 1*B*). Additionally, higher-magnification images (Fig. 1*C*) were made of the same preparation of virus as was used for Fig. 1 *A* and *B*. The lower-magnification images were compared with the fluorescent images from exactly the same field to show that there was virtually a 1:1 correspondence between the virions in Fig. 1*A* and the fluorescent images in Fig. 1*B*. By counting numbers of particles in the two micrographs from which Fig. 1 *A* and *B*

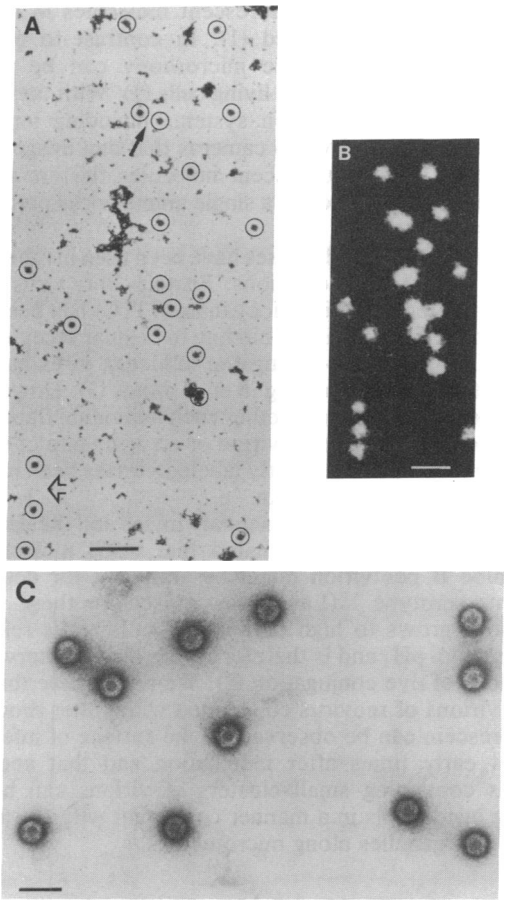

FIG. 1. Individual viral particles detected by electron microscopy or epifluorescence microscopy. (*A*) Electron micrograph of the negatively stained rhodamine B-conjugated virus. (Bar = 0.48 μm.) (*B*) Epifluorescence micrograph of the same field. Individual rhodamine B-conjugated reovirus particles are unambiguously visualized. (Bar = 1 μm.) (*C*) Electron micrograph of the same preparation of virus shown in *A* and *B*. (Bar = 77 nm.)

were taken, we can say that >90% of the virions identified by electron microscopy were fluorescently labeled; of the 286 particles identified by electron microscopy, 271 of them were also identified by fluorescence microscopy. Furthermore, it appeared that the fluorescent intensity of the virions was fairly uniform, and when a single fluorescent spot appeared to show increased fluorescent intensity, examination of the same field by electron microscopy indicated that the apparent increase in intensity was actually caused by the close proximity of two virions (Fig. 1*A*, solid arrow). For example, when two particles were 403 nm apart as measured in an electron micrograph (Fig. 1*A*, open arrow), two fluorescent objects could be detected; however, when the particles were as close as 183 nm apart (solid arrow) they were detected as a single fluorescent object. The high-magnification micrographs (Fig. 1*C*) were used to confirm that the particles identified in the low-magnification micrographs were indeed individual virions and that conjugation of the virions with RITC or FITC did not appear to affect their morphology.

Analysis of Viral Capsid Proteins. SDS/PAGE analysis of the type 1 conjugated viral proteins (Fig. 2) showed that the outer capsid proteins σ3, μ1c, and λ1 or λ2 (possibly both) were fluorescently labeled, whereas σ2, a known core protein, did not appear to be labeled. The doublet bands of μ1c and λ, which were seen consistently with conjugated virus, suggest that conjugation with the fluorescent markers (especially rhodamine B) may cause some conformational changes in the dissociated viral proteins.

Use of Conjugated Virus to Study the Early Events of Reovirus Infection in Living Cells. Reovirus type 1 (Lang) conjugated with RITC or FITC was used to study the early events of reovirus infection in cultured mouse L cells. The fluorescent virus was followed in living cells in real time by epifluorescence video microscopy. To avoid photobleaching of the fluorescent label, brief exposures at different time points were used to follow the fate of the rhodamine-conjugated virus until the outer capsid proteins were removed, 2–3 hr after infection, at which point the fluorescence gradually became diffuse and more difficult to detect.

Observation at different focal planes indicated that at the very early time points (0–10 min), the fluorescence was associated exclusively with the cell surface and had a definite punctate appearance; that is, individual virions could be distinguished on the surface of the cell (Figs. 3 and 4*A*).

When the conjugated virus was preincubated with 5C6, a neutralizing monoclonal antibody directed against the reovirus serotype 1 σ1 protein, at concentrations as low as 0.1 mg/ml for 1 hr prior to inoculation of the cells, binding of the virus to the cell surface was prevented (data not shown). However, preincubation with a 20-fold higher concentration of G5, a monoclonal antibody directed against the σ1 protein of reovirus serotype 3, did not appear to affect binding of the conjugated type 1 virus to the cells (data not shown). Since σ1 is the viral attachment protein (15, 16), these results suggest that the normal binding of the virus to the cell surface via σ1 is probably unaffected by conjugation.

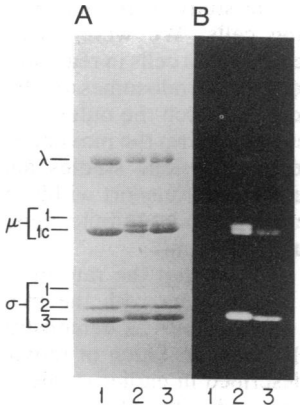

FIG. 2. SDS/PAGE analysis of fluorescein- or rhodamine B-labeled reovirus type 1 (Lang). (*A*) Coomassie blue-stained gel. Lane 1, control unconjugated virus; lane 2, rhodamine B-conjugated virus; lane 3, fluorescein-conjugated virus. (*B*) Same gel illuminated with UV light prior to staining with Coomassie blue.

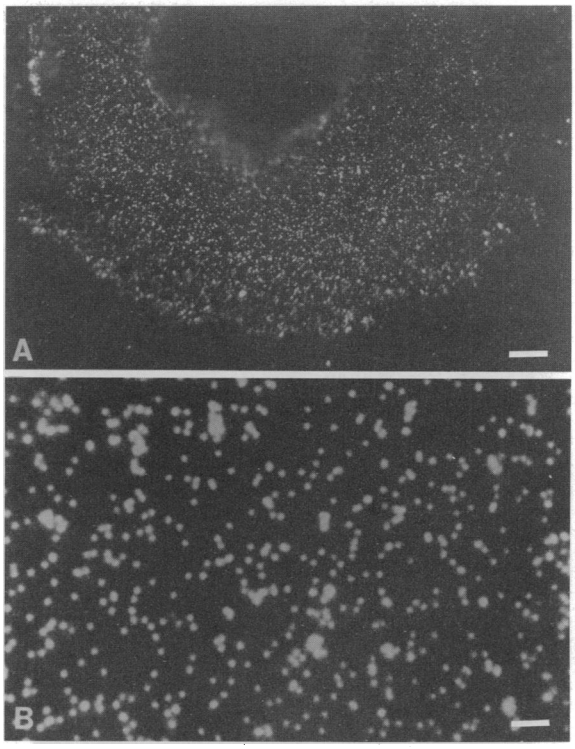

FIG. 3. (*A*) Epifluorescence micrograph of the surface of an L cell infected with rhodamine B-conjugated reovirus. Cells were plated on coverslips overnight, inoculated with $\approx 2 \times 10^4$ fluorescent virus particles per cell at 4°C for 1 hr, and fixed in 3% formaldehyde for 10–20 min. (Bar = 5 μm.) (*B*) Higher magnification of one area of *A*. (Bar = 2 μm.)

By 10–15 min after transfer to 37°C, the fluorescence was no longer found solely on the surface of the cell, signaling the start of internalization of the virus particles (Fig. 4*B*). As previously described for receptor-mediated entry of ligands (including viruses) into cells (2, 17–19), the formation of endocytic vesicles was clearly indicated by the clustering of individual virions into larger and more intensely fluorescent spots now found intracellularly. At about this time, distinct and dramatic movement of the fluorescent vesicles in the cells was seen. The vesicular movement led to the perinuclear localization of the fluorescent vesicles by about 60 min after transfer to 37°C (Fig. 4*C*). The clear shift in fluorescent pattern from surface association to vesicle internalization and eventual perinuclear localization was also shown with cells that were grown and infected on coverslips and then were fixed at various times after infection for fluorescence microscopy (data not shown).

Vesicular movement was particularly striking in elongated portions of the cell body but could be observed throughout the cytoplasm (Fig. 5). When the vesicular movement occurred, it was with successive rapid shifts along a straight line, occasionally interrupted by stops of uneven duration. At the maximum, these vesicles moved at a rate of 0.5–1.0 μm/sec. Motion appeared to take place in both the anterograde and retrograde mode, and frequently a single vesicle seemed to reverse the direction of movement along an

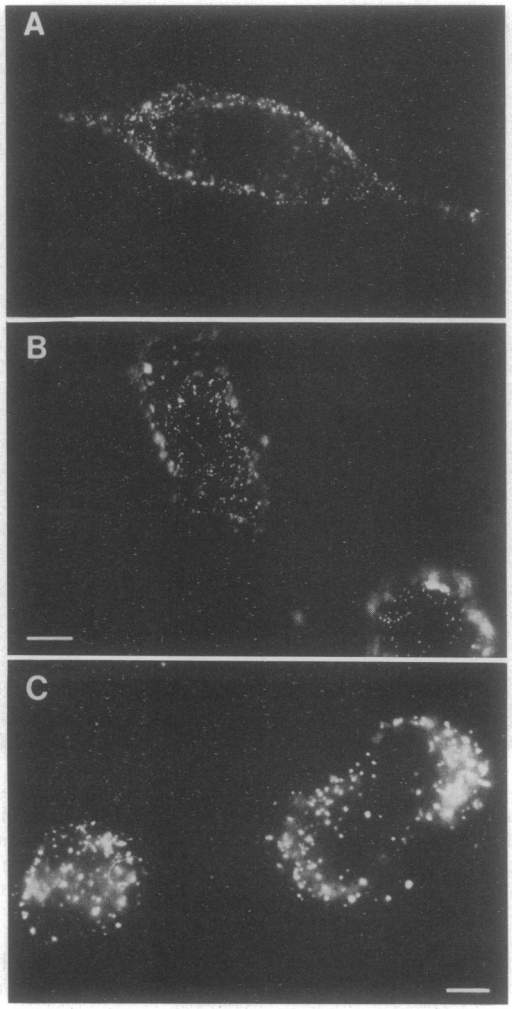

FIG. 4. Time course of infected L cells inoculated with rhodamine-conjugated type 1 reovirus. Points were taken at 0–5 min (*A*), 15 min (*B*), and 60 min (*C*). (Bar = 5 μm in *A* and *B*; bar = 4 μm in *C*.)

established straight path. This movement is similar to the motion of organelles translocating along microtubules (14, 20, 21) (Fig. 5).

The distribution of reovirus antigen differs in cells in which the microtubules have been depolymerized with colchicine, although viral replication is not affected in these cells (22–25). Furthermore, the neural spread of reovirus type 3 in neonatal mice is inhibited in animals that have been treated with colchicine (26). Similarly, nocodazole inhibits neuritic transport of herpes simplex virus in rat sensory neurons (27). We therefore treated L cells with either colchicine or nocodazole to disrupt the microtubules prior to infection with fluorescently labeled virus. In the treated cells, vesicle movement

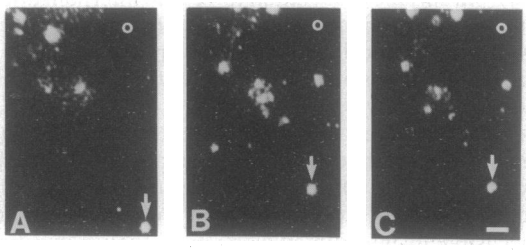

FIG. 5. Epifluorescence video microscopy of movement of vesicles containing internalized reovirus. (*A*) Time 0. (*B*) Plus 10 sec. (*C*) Plus 14 sec. White circle denotes same point in all three panels. The same vesicle is indicated by an arrow. (Bar = 2.4 μm.)

was completely inhibited. Virus particles appeared to bind to the cell surface and to be endocytosed normally. Very little difference could be discerned at the earliest time points between control and colchicine- or nocodazole-treated cells. At later time points, however, no vesicle movement was seen; after 60 min vesicles were still located universally throughout the cell (data not shown). Specific perinuclear localization of fluorescent virus did not occur. The effect of colchicine or nocodazole was reversible. By 70 min after removal of the nocodazole, normal vesicle movement was restored. It seems likely that the vesicle movement described here is dependent on the presence of intact microtubules.

Confirmation by Electron Microscopy That Conjugation of the Virus Does Not Alter the Early Events of Infection. Thin sections were prepared from L cells on coverslips infected with conjugated or unconjugated (control) virus. Infections were performed as for the fluorescence microscopy experiments except that the concentration of virus was increased to 2.3×10^5 to ensure that adequate numbers of virus particles would be present in 80-nm thin sections of L cells. Electron micrographs were taken of cells fixed at 0 and 60 min after transfer to completed MEM at 37°C (Fig. 6). As in previous studies done with unlabeled virus, virus particles appeared to be at or near the surface of the cell at 0 min and in vesicles inside the cell at 60 min, although some particles remained bound to the surface of the cell even after 60 min. In addition, three independent experiments indicated that no differences in endocytosis could be seen between unconjugated and conjugated virus.

DISCUSSION

We have developed a method for direct observation of the early stages of reovirus infection in living cells in real time by combining the conjugation of reovirus with fluorescent markers and epifluorescence video microscopy. To determine the efficiency of fluorescent labeling, fluorescent and electron microscopic images of exactly the same viruses were compared. To reconstruct the same field of fluorescent image (which is at a low magnification and not confocal) by electron microscopic images (which are at a high magnification and confocal) is not straightforward. Nonetheless, it was possible to obtain the two images for comparison. By comparing the two images we showed that >90% of the virions were fluorescently labeled and that the intensity of the labeling was quite uniform, indicating that approximately the same number of rhodamine molecules were conjugated to each virus particle. Once we had established that it is possible to detect individual fluorescently labeled virus particles, we applied this technique to the study of the early events of reovirus infection in living cells. We were able to visualize the interaction of the virus with cells in real time and to follow the fate of inoculated virus in endosomes and lysosomes of living cells up to the point at which the outer capsid proteins were removed. The results confirm the movement and localization of the virus seen in previous studies and allowed us to measure the rate of viral transport within the cells. We also examined the effect of microtubule depolymerization on intracellular virus movement.

These studies indicate that the rate of vesicle movement (0.5–1 μm/sec) is consistent with the transport of cellular organelles along microtubules as demonstrated in other systems (14, 20, 21). The association of reovirus with microtubules has been described in earlier studies. Previous experiments with cells treated with colchicine suggested that viral transport was affected by disruption of the microtubules (22–25). Here we were able to show directly a clear difference between colchicine-treated and untreated cells in the movement and distribution of vesicles containing reovirus. In colchicine-treated cells, virus appeared in vesicles but no

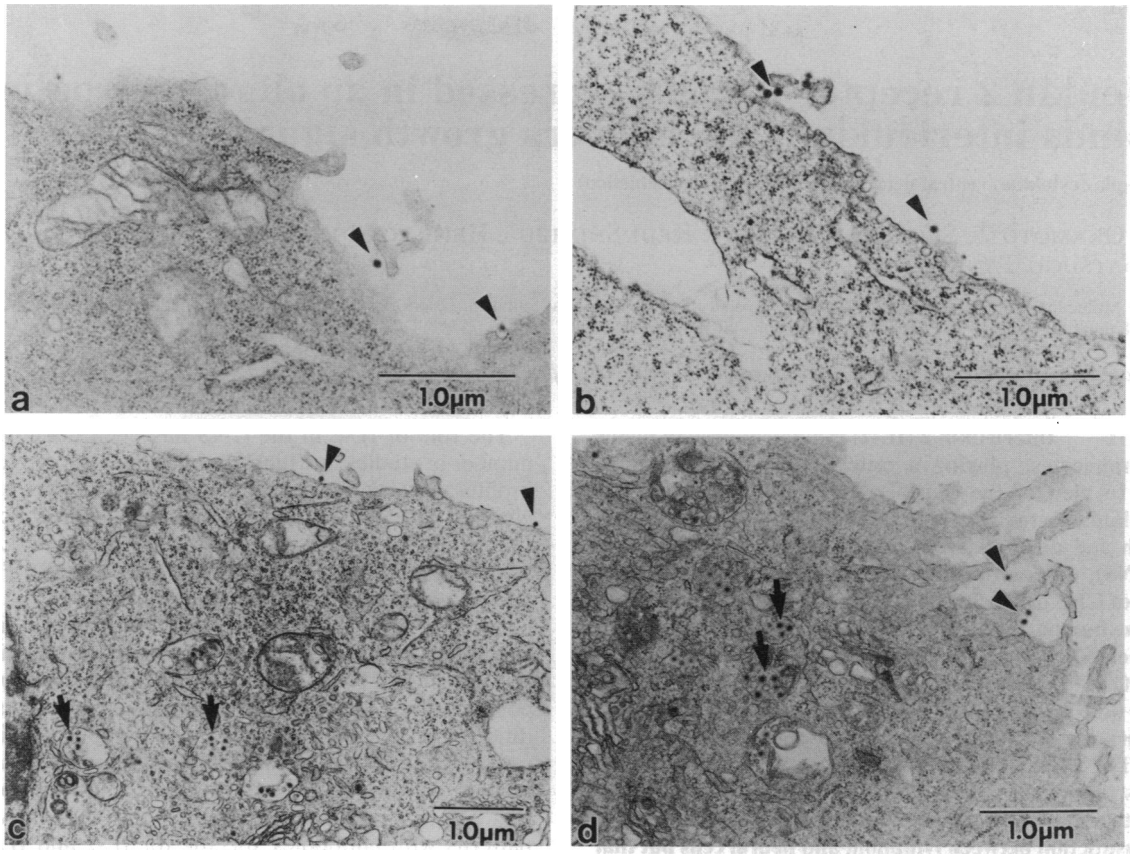

FIG. 6. Electron micrograph of L cells infected with unconjugated (*a* and *c*) or rhodamine B-conjugated (*b* and *d*) virus. Cells were infected as for Fig. 4 except that the amount of virus was increased about 10-fold, to $\approx 2 \times 10^5$ particles per cell. Individual virions are seen at the surface of the cell (arrowheads). Membrane-enclosed viral particles (arrows) are seen inside the cell (*c* and *d*).

vesicle movement was seen. Similarly, in cells treated with nocodazole, no vesicle movement occurred; vesicles containing fluorescent virus remained near the surface of the cells. When nocodazole was removed, however, new microtubules were allowed to form and vesicle movement was restored.

The development of high-resolution epifluorescence video microscopy combined with the ability to fluorescently label virus particles in a way that only partially affects infectivity has provided us with a method for studying virus–host interactions in greater detail. We have shown that it is possible to observe and follow the fate of individual virions from the time they are bound to the cell surface until the fluorescently labeled outer capsid proteins are removed. With this method it is now possible to study the dynamics between various cellular components and virus particles during the early stages of virus infection.

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