JOURNAL OF VIROLOGY, Sept. 1996, p. 5916–5921 Vol. 70, No. 9
0022-538X/96/$04.00+0

Directional Budding of Human Immunodeficiency Virus from Monocytes

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Received 11 January 1996/Accepted 6 June 1996

Time-lapse cinematography revealed that activated human immunodeficiency virus (HIV)-infected monocytes crawl along surfaces, putting forward a leading pseudopod. Scanning electron micrographs showed monocyte pseudopods associated with spherical structures the size of HIV virions, and transmission electron micrographs revealed HIV virions budding from pseudopods. Filamentous actin (F-actin) was localized by electron microscopy in the pseudopod by heavy meromyosin decoration. Colocalization of F-actin and p24 viral antigen by light microscopy immunofluorescence indicated that F-actin and virus were present on the same pseudopod. These observations indicate that monocytes produce virus from a leading pseudopod. We suggest that HIV secretion at the leading edges of donor monocytes/macrophages may be an efficient way for HIV to infect target cells.

Activated monocytes and macrophages are motile cells; they extend a leading pseudopod as they move forward (12). Considerable evidence suggests that in both mononuclear and polymorphonuclear blood cells, movement entails redistribution of filamentous actin (F-actin) into the leading pseudopods (5, 7, 20, 43, 45, 46). Rearrangement of F-actin in cells of the immune system may also be associated with secretion. For example, in helper T cells, cytotoxic T cells, and natural killer cells, evidence indicates that F-actin accumulates at the point of contact with an antigen-presenting cell or with a target cell. Actin polarization is followed by reorientation of microtubules and the Golgi apparatus toward the area of specific cell-cell contact to ensure that cytokines or cytotoxic or lytic proteins are secreted specifically toward the antigen-presenting cell (36) or the target cell (4).

Secretion of virions may also be related to actin redistribution. In a previous study we induced F-actin to concentrate on one end of human immunodeficiency virus (HIV)-infected T cells either by treating them with colchicine or by causing cells to adhere to a solid substrate. In these cells, budding of virus occurred at the positions where F-actin concentrated (32).

In the present study, we examined HIV production by primary monocytes. We theorized that progressing monocytes secrete virus from a leading pseudopod where F-actin is concentrated. To test this hypothesis, we used microscopic and cytochemical techniques to examine motile, HIV-infected, primary activated monocytes. The observations presented here may be relevant to the role of HIV-infected monocytes/macrophages in the transmission and dissemination of HIV.

MATERIALS AND METHODS

Preparation of HIV-1-infected monocytes. Semipurified leukocytes (10 ml) from normal, HIV-seronegative donors (obtained from the Greater New York Blood Center, New York, N.Y.) were diluted in 30 ml of RPMI 1640 over 15 ml of Histopaque-1077 solution (Sigma, St. Louis, Mo.) and centrifuged for 30 min at 400 × *g*. The mononuclear cell fraction (peripheral blood mononuclear cells [PBMC]) was seeded at 10^7 cells per ml in RPMI 1640 containing 10% fetal bovine serum and 5 μg of phytohemagglutinin (GIBCO, Grand Island, N.Y.) per ml. After 48 h, the PBMC were infected either with primary isolates of HIV type 1 (HIV-1) P1-2 or O/S (gifts from Ruth Connor and David Ho) or with a monocyte-tropic strain, AdaM (ARRRP). Seven days later, infected PBMC were mixed 1:5 with freshly isolated noninfected PBMC. Cells were seeded at 10^7/ml. After 1 week, nonadherent cells were removed by washing with phosphate-buffered saline (PBS). Virus production was monitored by p24 enzyme-linked immunosorbent assay of the medium after 24 h of culture. The cells were used if the p24 production was greater than 50 ng/10^6 cells. It was previously determined that the cultures reached peak HIV production at about 7 days postinfection. HIV production remained at about this level for a few days and then fell sharply.

Time-lapse cinematography. For time-lapse cinematography, 25-ml T flasks (Corning, Corning, N.Y.) were coated with rat tail collagen type 1 (lot 900874; Collaborative Biomedical Products, Bedford, Mass.) according to the manufacturer's instructions. HIV-infected monocytes were added (10^6/ml) to the flasks, the top was screwed onto the flask tightly, and the culture was transferred to a Nikon inverted microscope equipped with a 37°C chamber and Sage-Boulex 16-mm time-lapse recording system. Cells were photographed on Kodak Plus X reversal film with a ×20 or ×40 phase-contrast-objective lens at a frame rate of one frame per 10 s. Films were viewed with a Kodak photo optical data analyzer.

Scanning electron microscopy. For scanning electron microscopy, cells were washed in RPMI 1640 (without serum) and fixed in 2.5% phosphate-buffered glutaraldehyde. The following day, cells were washed in water and attached to a poly-L-lysine-coated coverslip, critical point dried, and sputter coated as described previously (33).

Transmission electron microscopy. For transmission electron microscopy, 0.5 ml of fixative (2.5% glutaraldehyde in 0.2 M phosphate buffer at pH 7.4) was added to 10^6 cells in 0.5 ml of medium in a 1.5-ml Eppendorf tube. The suspension of cells in fixative was mixed by inversion. One minute after the addition of fixative, cells were centrifuged in a microcentrifuge for 2 min at 4,000 × *g*. The supernate was carefully removed and replaced with fresh fixative; the tube was refrigerated overnight. The next day the pellet of cells was processed for transmission electron microscopy as described previously (32).

Ultrastructural visualization of F-actin. Actin filaments were visualized by decoration with heavy meromyosin (HMM) by a modification of the formaldehyde fixation-saponin permeabilization method (31). The modification consisted of the addition of tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin (Sigma) to all the solutions used for the treatments preceding decoration, because phalloidin has been shown to stabilize actin filaments during preparative procedures prior to HMM decoration or fluorescent staining assays (25, 48). We used TRITC-phalloidin because it binds F-actin with higher affinity than unconjugated phalloidin (3, 11). Cells were centrifuged at 300 × *g* for 5 min, rinsed briefly first with PBS, pH 7.2, and then with cytoskeleton-stabilizing (CS) buffer {137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO4, 0.4 mM $K_2H_2PO_4$, 5.5 mM glucose, 5 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 1 mM $MgCl_2$, 2 mM EGTA (pH 6.9)} (41), and fixed for 10 min at 4°C with freshly prepared 1% paraformaldehyde in CS buffer containing 1 μg of TRITC-phalloidin per ml. The cells were then centrifuged, resuspended, and thoroughly washed with buffer containing TRITC-phalloidin for 30 min at 4°C. Cells were subsequently centrifuged, resuspended, and permeabilized in 0.5% saponin in buffer–TRITC-phalloidin for 10 min at room temperature (RT).

After incubation in saponin, HMM (lot 9014; Sigma) was added to the cell

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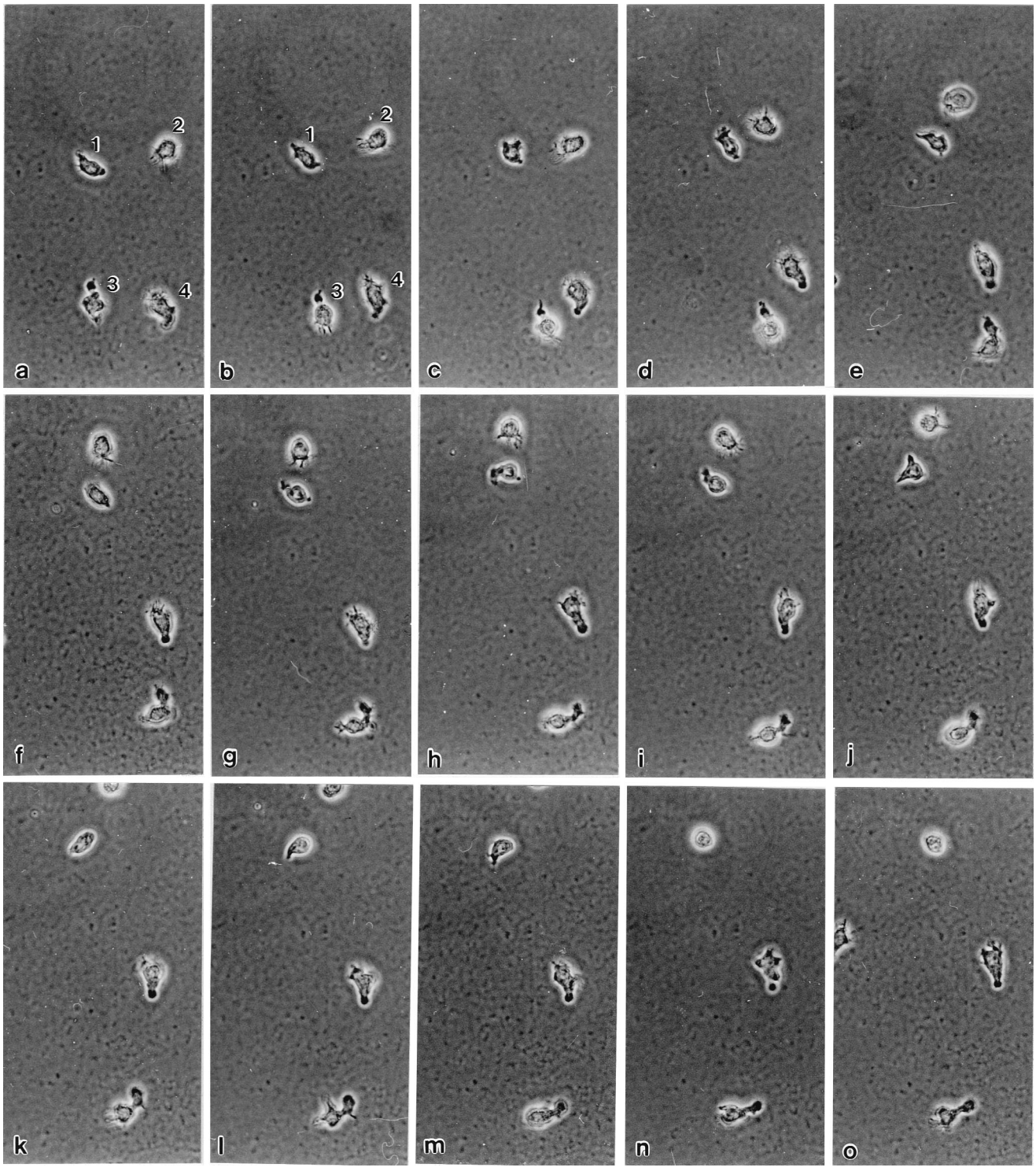

FIG. 1. Successive phase-contrast photomicrographs of motile monocytes taken at 30-s intervals. Cells undergo rapid activity and changes in shape. Some cells (e.g., cell 1) remain in place, whereas others (e.g., cell 2) move the length of a cell in 1 or 2 min. Other cells (e.g., cells 3 and 4) move more slowly. Magnification, ×233.

suspension to a final concentration of 3 μg/ml in buffer and incubated for 4 h at 4°C. The cell suspension was then diluted 10-fold with buffer to remove unbound HMM. After 30 min, cells were postfixed for 30 min at RT with glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in buffer containing tannic acid (Polysciences, Inc.) at a final concentration of 2% glutaraldehyde and 0.2% tannic acid (1). Tannic acid was added to the fixative immediately before use. The cells were then centrifuged and washed in phosphate buffer. The pellet was removed from the tube, postfixed with 1% OsO_4 in buffer for 30 min at 4°C, washed in buffer, and stained en bloc with 1.5% uranyl acetate. The pellet was subsequently embedded in Epon (Polysciences, Inc.). Thin sections were stained with 4% aqueous uranyl acetate and viewed in a Philips 300 microscope.

Localization of F-actin and p24/25 by immunofluorescence. The following primary and secondary antibodies were used: rabbit polyclonal antiserum to HIV-1 p25/24 *gag* (National Institutes of Health AIDS Research and Reference

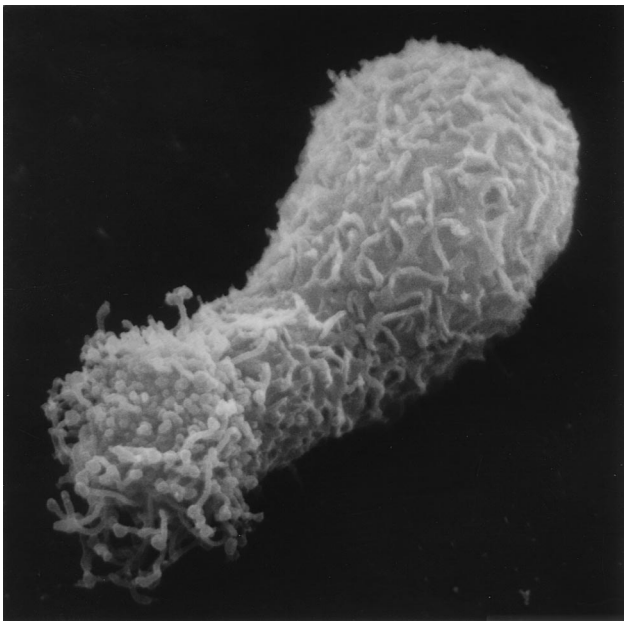

FIG. 2. Scanning electron micrograph of an HIV-infected (strain P1-2), activated monocyte. The surface of most of the monocyte is characterized by irregular microvilli. However, the monocyte's pseudopod (lower left) is covered with small spherical structures which appear to be HIV virions. Magnification, ×4,650.

Reagent Program) diluted 1:250 and monoclonal, fluorescein isothiocyanate-conjugated, anti-rabbit immunoglobulin G (Sigma) diluted 1:20.

Before staining, monocytes were centrifuged at 300 × *g* for 5 min, rinsed briefly with PBS and CS buffer, and fixed in suspension for 20 min at 4°C with a freshly prepared solution of 4% paraformaldehyde in buffer. The cells were thoroughly rinsed with the same buffer and treated with 10 mg of NH_4Cl per ml in 0.1 M phosphate buffer, pH 7.2, for 30 min at RT to quench free aldehyde groups. After two final rinses, single drops of cell suspension were placed on glass slides precoated with 0.04% polylysine (molecular weight, >300,000; Sigma) and the cells were allowed to settle and adhere to the slide for 30 min. The cells were then permeabilized with 0.1% Triton X-100 in CS buffer for 7 min at RT, washed three times with PBS, and double-labelled for F-actin and p24 as described below.

To block specific binding of antibodies, the slides were preincubated for 30 min with 10% normal mouse serum (Sigma) in PBS for p25/24. The slides were then drained, incubated for 1 h in the primary antiserum, washed three times for 5 min each time in PBS, incubated in the appropriate secondary antiserum for 1 h at RT in the dark, washed three times for 5 min each time, and mounted in Vectashield antifade (Vector Laboratories, Burlingham, Calif.). Preadsorbed antisera were centrifuged at 15,000 × *g* for 10 min to remove aggregates. Sera and antisera were diluted in PBS containing 0.2% bovine serum albumin Fraction V (Sigma). Incubations were performed in a humidified chamber at RT. Controls were prepared by omitting the primary antisera and substituting the primary antisera with the nonimmune sera from the same species. For F-actin labelling, a stock solution of TRITC-conjugated phalloidin was prepared as described by Wallace et al. (47), and TRITC-phalloidin was added to the secondary antisera solutions at a final concentration of 0.7 μg/ml. Cells were observed with a Nikon epifluorescence microscope. For fluorescein visualization, a 520 to 546 (Zeiss) selective barrier filter was used in addition to the normal 520 fluorescein barrier filter to eliminate all the red fluorescence from rhodamine.

RESULTS

Time-lapse cinematography. When 16-mm films were viewed, monocytes were observed to change shape rapidly and continuously. In many cells, small changes in contour were detected between sequential frames (10-s intervals). Many cells remained stationary for some time while changing shape, while others showed ameboid movements, with a leading pseudopod. Often a pseudopod was withdrawn and a new one rapidly protruded on another side of the cell, resulting in a random locomotor activity. The shapes of the leading pseudopods varied among cells and within individual cells during progression. Broad, pointed, and rounded pseudopods were observed (Fig. 1). A consistent feature of cells that progressed in one direction was that the cell nuclei were observed in the trailing regions of the cells, opposite the leading pseudopods.

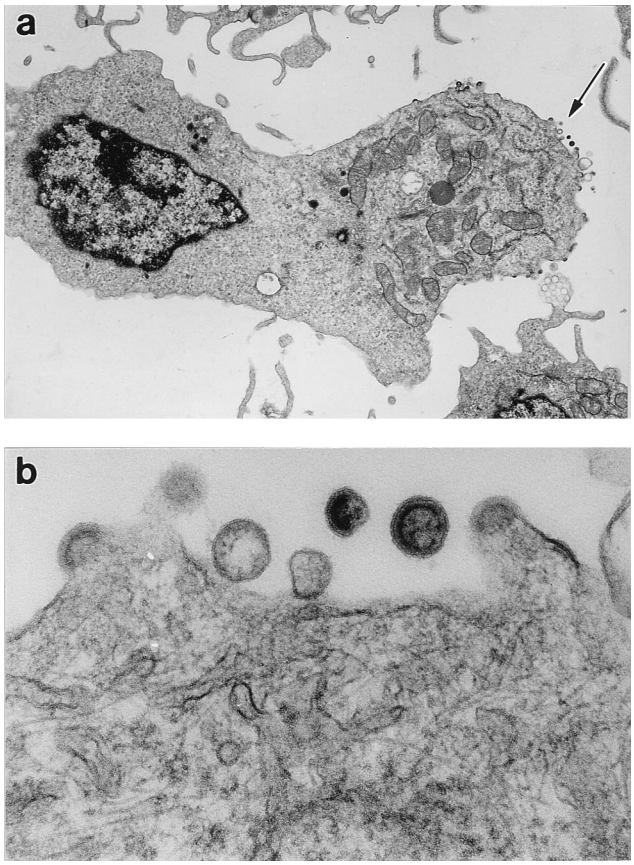

FIG. 3. (a) Transmission electron micrograph of an activated monocyte showing a long pseudopod with budding virus (strain O/S) (arrow). (b) Higher magnification of the cell shown in panel a showing budding and immature HIV. Some virions appear to be forming on microvilli. Magnification, ×3,069 (a); ×38,130 (b).

Morphology. Viewed by scanning electron microscopy, most monocytes displayed a variety of forms, ranging from very distended to nearly spherical. The predominant shapes were irregular and elongate. Monocytes were characterized by irregular broad folds and frequently displayed a pseudopod or, in some cases, two pseudopodia. In many cells the surface architecture of the pseudopod was dissimilar to that of the rest of the cell. Instead of thin folds, the surface of the pseudopod was covered with spheres the size of an HIV virion (Fig. 2). These pseudopods often had several long tubular microvilli with a small spherical bulge at the tip of each microvillus.

We examined monocytes by transmission electron microscopy for the presence of HIV virions. Many of the cells were associated with both immature and mature virions. In each instance, viruses were situated at the tip of a pseudopod. The nuclei of these cells were situated on the side of the cell opposite the pseudopod. Mitochondria, centrioles, and other cellular organelles were usually positioned between the nucleus and the pseudopod (Fig. 3a and 4a). In some cells we observed only budding and immature virions (Fig. 3b), whereas in other cells mature virions were associated with the pseudopods (Fig. 4b).

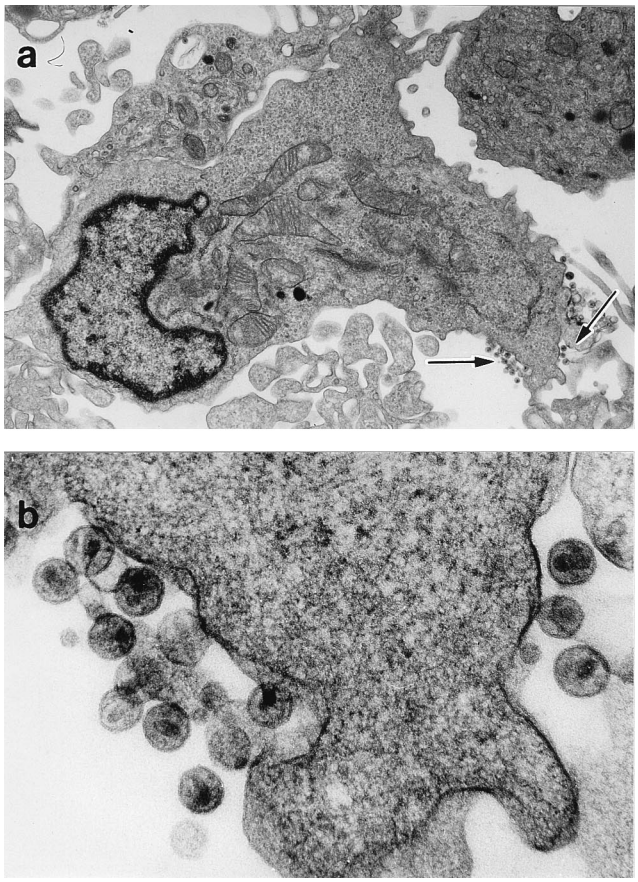

FIG. 4. (a) Transmission electron micrograph of an activated monocyte showing a pseudopod with associated (strain O/S) virions (arrows). (b) Higher magnification of the cell shown in panel a showing mature HIV virions associated with the pseudopod. Magnification, ×3,135 (a); ×38,950 (b).

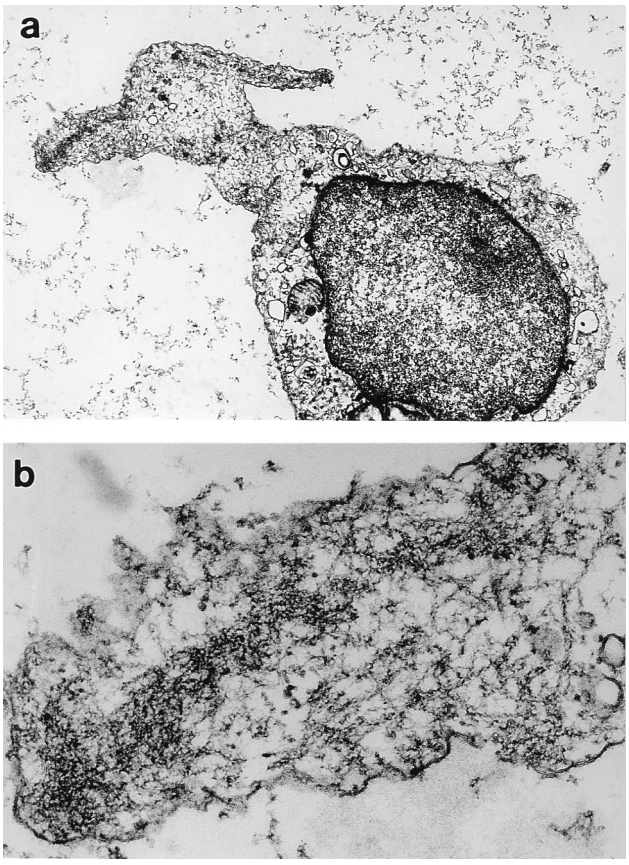

FIG. 5. (a) Actin distribution in an HIV-infected monocyte as revealed by HMM staining. The concentration of HMM is greatest in the pseudopod. (b) Higher magnification of the monocyte in panel a showing the typical arrowhead appearance of HMM-decorated F-actin. Magnification, ×3,720 (a); ×43,710 (b).

Cytochemistry. We used immunocytochemistry to confirm our premise that HIV secretion occurs from the leading portion of a motile monocyte. It has previously been established that in a number of types of motile cells, movement involves redistribution of F-actin into the leading pseudopods. Using HMM to decorate actin, we determined that pseudopods were filled with an actin network denser than that in other parts of a cell (Fig. 5a and 6a). Most filaments had a discrete length, and their continuity through the network could often be traced. At the rear of a cell, the density of the actin filaments was much lower than in the pseudopod, and the filaments were shorter and organized in loose arrays throughout the cytoplasm. The front of a pseudopod often showed thin, stiff-looking projections, where actin microfilaments were organized in bundles parallel to the direction of the pseudopod, or protrusions containing a very dense actin meshwork (Fig. 5b and 6b). These protrusions presumably correspond to the so-called microspikes and ribs observed at the leading edges of a variety of motile cells, which are thought to indicate very dynamic rearrangements of actin cytoskeletons in rapidly advancing lamellipodia and pseudopodia (15, 39, 40). Unfortunately, the procedure for decorating F-actin involves a detergent treatment which removes most or all of a plasma membrane and any virions that might be associated with it. We therefore employed immunofluorescence to colocalize F-actin and p24 *gag*-encoded protein. In almost every elongated cell, actin was concentrated primarily at the tip of the pseudopod as expected. Not all monocytes stained with the p24 antibody. However, in those that did, both actin and p24 were found on the same pseudopod (Fig. 6a and b and 7a and b).

DISCUSSION

We have presented evidence that HIV-infected, activated monocytes secrete HIV from the tip of their leading pseudopods. F-actin localization was employed to confirm that HIV buds from the front of a cell. The asymmetric distribution of the actin meshwork and the gradient density of the meshwork observed from the rear to the leading edge of a monocyte are comparable to those observed in other motile cells (43, 45, 46). Furthermore, the organization of F-actin in monocyte pseudopods is basically similar to that seen in leading pseudopods of a variety of motile cells types (6, 9, 21, 40, 42), including macrophages (35).

Although the precise role of F-actin in HIV budding is not clear, multiple lines of evidence suggest that a close relationship exists between enveloped viruses and the actin of a host cell. Actin filaments are involved in mediating interactions between measles virus envelope proteins in a plasma membrane and the cytoplasmic nucleocapsid (2). In addition, membrane proteins of paramyxoviruses have been shown to interact with actin (14) and nucleocapsids of a murine retrovirus have been shown to bind to actin filaments (29). Many retroviruses, pox viruses, measles viruses, and FV3 frog viruses bud from infected cells at the apices of virus-induced membrane protru-

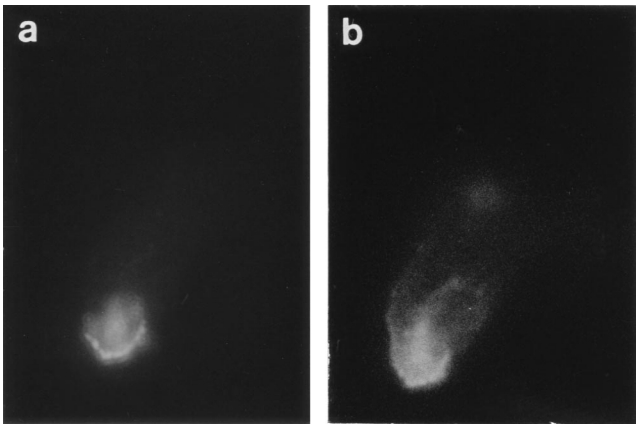

FIG. 6. (a) Phalloidin staining for F-actin. Actin is localized to the pseudopod. (b) p24 staining of the cell shown in panel a. HIV p24 colocalizes with F-actin. Magnification, ×1,581 (a and b).

sions resembling microvilli or filopodia (2, 8, 17, 28, 30, 51). These cellular extensions have been shown to contain actin filaments, and the virions have often been seen closely associated with the filaments (2, 17, 30). Furthermore, a structural polypeptide of simian virus 40 has been shown to be attached to the actin cytoskeletal networks of infected cells (24).

In the case of HIV it has been demonstrated that interactions between actin and myosin at a plasma membrane are involved in HIV-1 budding from infected T lymphocytes and monocytes (37) and that HIV-1 reverse transcriptase interacts with β-actin (18), an actin isoform that is present in leukocytes (9). We have previously demonstrated that when actin is induced to concentrate on one end of an HIV-infected T cell, budding of virus occurs at the same position (32).

In retroviruses, including HIV, the transport of envelope glycoproteins with a nucleocapsid to the site of assembly at the cell surface follows the secretory pathway (22). However, the pathways of transport to a plasma membrane and the assembly of the nucleocapsid components of HIV are ill defined (22). The *gag* and *gag-pol* precursors are synthesized on free polyribosomes and directed by still-unclear mechanisms to the site of assembly. Capsid assembly and virus protrusion occur simultaneously (22). Therefore, it is likely that interactions between envelope components and nucleocapsids in polarized monocytes occur only after they have been separately targeted to the plasma membranes of the leading pseudopods.

The possibility that in directionally secreting monocytes, the envelope glycoproteins are targeted directly to the leading edges of the plasma membranes is unlikely, as it has been demonstrated that in motile cells, vesicles are inserted into plasma membranes posterior to the leading pseudopods (38). It is therefore likely that HIV envelope components are inserted in a plasma membrane behind the front of the pseudopod. They are subsequently moved forward to the leading edge of a motile cell either by passive forward extension of a membrane, which is reshaped by active cytoskeletal processes (38), or forward active movement of transmembrane proteins and glycoproteins that bind through motor proteins to the underlying moving cytoskeleton, which drives protein flow on the plasma membrane (10, 26).

Nucleocapsid components are probably transported forward to the tip of a pseudopod by the actin network and the rapid turnover of actin filaments in a locomoting cell. Intracytoplasmic directional transport along actin filaments involves myosin motors and has so far been demonstrated only for vesicles and membrane-bound organelles (10, 27). However, it has been shown that F-actin is associated not only with different enveloped viruses or viral components but also with mRNA and polysomes (16, 19) and a variety of soluble cytoplasmic proteins (23, 50). The molecular architecture of an actin cytoskeleton is continuously remodelled as the overall shape of a cell changes during locomotion. The rearrangement involves the regulation of movements of subunits and of short fragments of severed filaments towards the advancing pseudopod with net polymerization of actin at the front and net depolymerization elsewhere (6, 9, 45). Viral components of the nucleocapsid which are attached to an actin filament may therefore be passively transported toward the leading edge of the pseudopod.

Secretion from the leading pseudopod might be beneficial for the virus. It is possible that when a motile activated HIV-infected macrophage/monocyte encounters a target cell, the virions, simply by virtue of being secreted from the front of the donor cell, would be in the most advantageous position to infect the target cell. This may be especially relevant to sexual transmission, as there is considerable evidence suggesting that transmission of HIV during sexual contact may be mediated by HIV-infected macrophages as opposed to lymphocytes or free virus. Evidence comes from studies which indicate that transmission may be mediated by mononuclear cells rather than free virus (34, 44). It has been suggested that these cells are monocytes/macrophages rather than lymphocytes, because viruses isolated from patients infected through sexual contact and in the acute phase of infection are primarily macrophage tropic (52) and monocytes/macrophages are more abundant than lymphocytes in semen (49) and more long lived than lymphocytes (13). An interesting feature evidenced by the experimental model we have used in this study is that no external signals, such as a chemical gradient in the environment, chemokines, or receptors on the target cells, are necessary to elicit directional budding of virions. We suggest that HIV may have exploited the cytoskeletal system, which enables motile cells to secrete from the leading pseudopod, in order to facilitate an efficient mechanism for its own dissemination.

ACKNOWLEDGMENTS

This research was supported primarily by grant RO1 AI37793 from the National Institute of Allergy and Infectious Diseases and by grants from the Ministry of University and Scientific Research of Italy.

We are grateful to Christine Burillo, Inna Brizinova, and Vanaja Zacharopoulos for skillful technical assistance. We also thank Ruth Connor and David Ho for the P1-2 and O/S strains of HIV.

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