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Microvesicles Are a Source of Contaminating Cellular Proteins Found in Purified HIV-1 Preparations

JULIAN W. BESS, JR.,[1] ROBERT J. GORELICK, WILLIAM J. BOSCHE, LOUIS E. HENDERSON, and LARRY O. ARTHUR

AIDS Vaccine Program, SAIC, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

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Identification and quantitation of cellular proteins associated with HIV-1 particles are complicated by the presence of nonvirion-associated cellular proteins that copurify with virions. Many cellular proteins are associated with nonviral particles that bud from the surface of cells called microvesicles. Microvesicles band in sucrose gradients in a range of densities that includes the same density as retroviruses. To characterize these microvesicles, HIV-1-infected and uninfected human T-cell lines were propagated and virus and microvesicles were purified from clarified cell culture supernatants by sucrose density gradient centrifugation or centrifugation through 20% sucrose pads. Microvesicles were found to contain various proteins, including HLA DR and β2-M, and a substantial amount of RNA and DNA. The concentrations of HIV-1 $p24^{CA}$, HLA DR, and β2-microglobulin (β2-M) were determined by radioimmunoassay. The ratios of HIV-1 $p24^{CA}$ to HLA DR and β2-M were found to vary with respect to the HIV-1 isolate, host cell, and other factors. Electron microscopic analysis of microvesicles revealed that they consisted of particles of various sizes and morphologies. Although HIV-1 particles are known to contain some cellular proteins, microvesicles from HIV-1 infected H9 cells appeared to contain little or no HIV-1 $gp120^{SU}$.

INTRODUCTION

Certain cellular proteins can associate with retroviruses, including the immunodeficiency viruses HIV-1 and SIV, by incorporation into the viral membrane or interior. Some of the cellular proteins that associate with the membrane of immunodeficiency viruses have been shown to serve as protective immunogens in vaccination experiments. Other cellular proteins, located at the interior of the virus, appear to be necessary for HIV-1 infection. Stott (1991) reported that 2 of 4 macaques immunized with human cells were protected from infection when challenged with SIV propagated in the same human cells, indicating that an immune response to cellular proteins may afford protection from challenge. Immunization and challenge experiments with purified cellular proteins, previously shown to be associated with the membrane of immunodeficiency viruses (Arthur *et al.,* 1992), revealed that monkeys with an immune response to HLA DR or HLA class I were protected from challenge with SIV propagated in human cells (Arthur *et al.,* 1995; Chan *et al.,* 1995). Cyclophilin A has been shown to specifically associate with $p24^{CA}$ in HIV-1 virions and this cellular protein appears to be necessary for HIV-1 infectivity (Franke *et al.,* 1994; Thali *et al.,* 1994; Braaten *et al.,* 1996). While virion-associated cellular proteins located on the virus surface can be identified by immunological techniques (Gelderbloom *et al.,* 1987; Kannagi *et al.,* 1987; Schols *et al.,* 1992; Arthur *et al.,* 1992; Meerloo *et al.,* 1993; Orentas *et al.,* 1993; Gluschankof *et al.,* 1996), identification and analysis of cellular proteins located internal to the virus are complicated by the presence of cellular membrane vesicles which copurify with the virus. We recently reported a proteolytic procedure (Ott *et al.,* 1995b) that effectively removes greater than 95% of proteins associated with these membrane vesicles. This procedure has allowed us to demonstrate that the cytoskeletal proteins, actin, ezrin, moesin, and cofilin are located in the interior of virions (Ott *et al.,* 1996). Although we previously used the term mock virus to refer to the membrane vesicles present in purified viruses (Arthur *et al.,* 1995), the particles are strikingly similar to small budding membrane particles frequently called microvesicles. It therefore appears to be more appropriate to use the term microvesicles when referring to these membrane particles. In this report we identify and partially characterize microvesicles from human T-cell lines and PBL that constitute a significant source of cellular proteins found in purified preparations of immunodeficiency viruses, including HIV-1.

MATERIALS AND METHODS

Cell culture

HIV-1(B)-infected H9 cells [HIV-1(B)/H9] (Popovic *et al.,* 1984), HIV-1(MN) infected H9 cells [HIV-1(MN)/H9] (Reitz *et al.,* 1992), and H9 cells (Popovic *et al.,* 1984; Mann *et al.,* 1989), were obtained from Dr. Robert Gallo (NCI). CEM-SS cells (Nara *et al.,* 1987) were obtained from Dr.

[1] To whom correspondence and reprint requests should be addressed. Fax: (301) 846-5588.

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Peter Nara (NCI-FCRDC), MOLT-3 cells (Minowada *et al.,* 1972) were obtained from Advanced Biotechnologies Laboratories (Columbia, MD), and DAUDI-CD4 cells were obtained from Dr. Paul Clapham (Chester Beatty Laboratories, England). HIV-1(MN)/H9 Clone 4 was prepared by Dr. Steve Nigida (AVP) and is a single-cell clone of HIV-1(MN)/H9 prepared by limiting dilution (Ott *et al.,* 1995a). HIV-1(MN) Clone 4/CEM, HIV-1(MN) Clone 4/MOLT-3, and HIV-1(MN) Clone 4/DAUDI-CD4 were prepared by infecting the respective uninfected parental cell lines with cell free HIV-1(MN) Clone 4 virus. For large-scale production, all cell lines were grown in suspension culture using 850-cm^2 roller bottles (Corning). Cultures were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (Biofluids Inc.), 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Each roller bottle contained 400 ml of medium and was incubated at 37° while rotating at 0.75 rpm on roller racks. Cultures were harvested at 3- to 4-day intervals by decanting 200 ml from each roller bottle and refeeding with 200 ml of fresh medium.

Purification of HIV and microvesicles

Virus and microvesicles were purified as described (Bess *et al.,* 1992). Briefly, virus and microvesicles were purified from clarified cell culture supernatants by two successive ultracentrifugations in sucrose density gradients (double banded). Virus- or microvesicle-containing fractions were identified by UV absorption at 280 and 254 nm. Peak UV absorbing fractions were pooled, diluted to below 20% sucrose with TNE buffer [0.01 *M* Tris–HCl (pH 7.2), 0.1 *M* NaCl, and 1 m*M* EDTA in deionized water], ultracentrifuged to a pellet, and resuspended in TNE. Samples were stored at −70°.

Total protein determinations

Protein concentrations were determined using the Folin phenol reagent (Lowry *et al.,* 1951). Prior to assaying, samples were solubilized by the addition of Sarkosyl detergent to a final concentration of 0.2%. Bovine serum albumin (Sigma) was used as the standard.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Samples were analyzed by discontinuous, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970).

Purified proteins

HIV-1 24^{CA} was purified to homogeneity from sucrose density gradient purified HIV-1(MN)/H9 by immunoaffinity chromatography. A mouse monoclonal antibody against HIV-1 24^{CA} was coupled to cyanogen bromide activated-Sepharose 4B (Sigma) following the manufacturer's recommendations and equilibrated against phosphate-buffered saline (Dulbecco's formulation) containing 0.1% Triton X-100 (PBST). The HIV-1 preparation was treated with a final concentration of 0.5% Triton X-100 to solubilize $p24^{CA}$ and other proteins. After centrifugation at 100,000 *g* to pellet nonsolubilized material, the supernatant was loaded onto the immunoaffinity column. The column was washed to baseline (A_{280}) using PBST and $p24^{CA}$ eluted with 0.1 *M* citrate, pH 2.5. The eluate was titrated to pH 7.0 with 0.1 *M* K_3PO_4. HLA DR4 was similarly purified from H9 cell lysates by immunoaffinity chromatography using a mouse monoclonal antibody prepared against HLA DR1, LB3.1 (provided by Dr. J. Strominger, Harvard University), that cross reacts with HLA DR4. Purified human β2-M was purchased from Valley Biomedical Products and Services, Inc. HIV-1(MN) recombinant $gp120^{SU}$ was obtained from Genentech (San Francisco, CA).

Radioimmunoassays (RIA)

Radioimmunoassays were performed essentially as previously described (Bess *et al.,* 1992).

Electron microscopy

Approximately 5×10^6 cells were removed from a 3-day culture and centrifuged at 1000 rpm for 10 min at 4°. The cell pellet was fixed with 1.25% gluraldehyde. Thin sections were prepared as previously described (Gehle and Smith, 1970), treated with lead citrate, and analyzed by transmission electron microscopy.

DNA and RNA quantitation

Pellets of purified H9-microvesicles, containing 20 mg of protein (measured by the Lowry method), were each disrupted with 2 ml of guanidine isothiocyanate solution as described in Davis *et al.* (1986). To isolate RNA, one dissolved pellet was layered over 2.0 ml of a 96% (wt/vol) CsCl solution that was prepared as described in Davis *et al.* (1986). Another dissolved pellet was layered over a 67% (wt/vol) CsCl cushion for the purpose of isolating DNA and RNA. The 67% cushion had the same sodium acetate concentration and pH as the 96% (wt/vol) CsCl cushion. The tubes were topped off with the guanidine isothiocyanate solution. Samples were centrifuged at 42,000 RPM for 18.5 hr at 20° in an SW60 Rotor (Beckman Instruments, Inc., Fullerton, CA). The tubes were drained, the walls wiped, and the RNA or RNA/DNA pellets were resuspended in 250 μl of diethylpyrocarbonate-treated water.

Single-stranded RNA concentrations were determined using an $OD_{260\,nm}$ of 1.0 being equal to 40 μg/ml. Double-stranded DNA concentrations were determined using an $OD_{260\,nm}$ of 1.0 being equal to 50 μg/ml (Ausubel *et al.,* 1995). DNA levels were determined by subtracting the RNA absorbance of the RNA containing sample, from the absorbance of the RNA/DNA sample. The resulting OD_{260} difference was then used to calculate DNA levels in the pellet. The purity of the isolated nucleic acid was assessed by measuring the A_{260}/A_{280} ratio. At either the 96%

or 67% (wt/vol) CsCl concentrations, proteins remain at or above the guanidine isothionate/CsCl interface. According to the protocol described for the isolation of RNA (Davis *et al.,* 1986), with a 96% (wt/vol) CsCl cushion, RNA pellets through the cushion, and DNA bands within the CsCl cushion. This laboratory has determined that both DNA and RNA pellet through a 67% (wt/vol) CsCl cushion. Additionally, quantitative recoveries of DNA can be obtained for levels of DNA or RNA greater than 1 μg. When 1 μg of *Hin*dIII digested λ DNA is centrifuged through a 67% (wt/vol) CsCl cushion, 100% recoveries of DNA are obtained. This was determined by comparing the intensities of the ethidium stained λ DNA fragments after fractionation on a 1% agarose gel, before and after passing through the 67% (wt/vol) CsCl cushion (data not shown).

Identification of RNA species

RNA samples were fractionated by formaldehyde agarose gel electrophoresis, transferred to nitrocellulose, and probed as described previously (Gorelick *et al.,* 1990). RNA samples were loaded in the presence of 2.5 μg of ethidium bromide. The filter was hybridized in the following order with an 8088 BP AvaI HIV-1 fragment (from pNL4-3) that was ^{32}P-nick translated and a ^{32}P-nick translated human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA probe from Clontech Laboratories, Inc. (Palo Alto, CA). The filter was stripped between hybridizations with boiling water.

Preparation of microvesicles from human PBL

The peripheral blood lymphocytes (PBL) from a lymphapheresis pack were recovered by centrifugation in 1.077 g/liter Ficoll-Hypaque at 900 *g* for 20 min at 20°. The resulting cells (7.5×10^8 cells total) were washed twice with PBS and used to seed 850-cm^2 roller bottles at a density of 1,000,000 viable cells/ml using complete RPMI 1640 containing 2 μg/ml PHA. The cultures were incubated for 2 days at 37° at 0.75 rpm. The PHA was removed by washing with PBS and PBLs were scaled up in complete RPMI 1640 containing 50 units/ml of IL-2. Cultures were harvested twice per week until a total of 10 liters of cell culture was produced. Cells were removed by centrifugation and microvesicles were recovered by continuous flow centrifugation through a 25 to 50% sucrose density gradient. The gradient was monitored for UV absorbance and collected in 25-ml fractions. Each fraction was diluted to less than 20% sucrose and centrifuged at 100,000 *g* for 1 hr. The pellet from each fraction was resuspended in 2 ml of sterile TNE buffer.

RESULTS

Small-scale preparations of HIV-1 produced from various cell lines were prepared to determine the yield and purity of virus. Three liters of 3-day cell culture were obtained from each of the HIV-1-infected cell lines and the uninfected parent lines listed in Table 1. After clarification to remove the cells, the supernatant was centrifuged at 100,000 *g* for 45 min and the pellet was resuspended in 30 ml of sterile buffer. This material was centrifuged through a 20 ml–20% sucrose pad at 100,000 *g* for 1 hr. Visible pellets were found in all preparations, including the material from uninfected cells. Finally, the pellets were resuspended in TNE buffer at 500$\times$ relative to the starting cell culture supernatant. Detergent disrupted samples of each preparation were analyzed by Lowry assay, HIV-1 p24^{CA} RIA, HLA DR RIA, and β2-M RIA. All cell lines, including the uninfected parental lines, produced particulate-proteinaceous material dense enough to penetrate 20% sucrose during centrifugation. As seen in Table 1, the p24^{CA} concentration of the HIV-1(MN)/H9 Clone 4 preparation was substantially higher than those of the two prototypical HIV-1 producing cell lines, HIV-1(B)/H9 and HIV-1(MN)/H9, indicating greater virus yield. The HIV-1(MN) Clone 4-infected CEM-SS, DAUDI, and MOLT-3 cells also provided good virus yields. HLA DR antigens were detected in virus and microvesicle preparations from all cell lines that express HLA DR (H9 and DAUDI). Also, β2-M was found in the virus and microvesicles produced from all the cell lines that express this protein (H9, CEM-SS, and MOLT-3). Notably, the amount of HLA DR exceeded the amount of p24^{CA} in some virus preparations. As an index of relative purity, the ratio of viral p24^{CA} concentration to the sum of the HLA DR and β2-M concentrations was calculated for each preparation. The HIV-1(B)/H9 Clone 4 preparation was significantly more pure in this sense than the two prototypical HIV-1 producing cell lines, HIV-1(B)/H9 and HIV-1(MN)/H9. However, much better ratios were found for the HIV-1(MN) Clone 4/CEM-SS and HIV-1(MN) Clone 4/Daudi.

Sixteen double-banded preparations of H9 microvesicles were prepared by double banding in sucrose gradients as described under Materials and Methods. The primary banding was in a 20 to 60% (1.08 to 1.29 g/ml) sucrose density gradient. UV absorbing material was detected in all portions of these gradients and the peak absorbing material was found at 1.13 g/ml. The average protein yield was 1.36 $\pm$ 0.37 mg per liter of cell culture supernatant ($\pm$1 standard deviation). For comparison, double-banded HIV-1(MN)/H9 and HIV-1(MN)/H9 Clone 4 were also prepared. These and a typical H9 microvesicle preparation were analyzed by SDS–PAGE (Fig. 1). The H9 microvesicle preparation contains a large number of minor protein bands and a single major band near 42 kDa (actin). In addition to the easily seen viral *gag* proteins (p24^{CA}, p17^{MA}, and p6/p7^{NC}), the HIV-1(MN)/H9, and HIV-1(MN)/H9 Clone 4 preparations appear to contain a significant amount of the material observed in the H9 microvesicle preparation. However, HIV-1(MN)/H9 Clone 4 contains slightly less of this material in the 30- to 40-kDa range. Much of the material in this region is HLA DR.

H9 cells were examined by transmission electron mi-

TABLE 1

Analysis of HIV-1 or Microvesicles Recovered from Various Cell Lines

Cell line	Lowry mg/ml	HIV-1 $p24^{CA}$ RIA ng/ml	HLA DR RIA ng/ml	β2-M RIA ng/ml	HIV-1 $p24^{CA}$ to HLA ratio
HIV-1(B)/H9	4.40	11,216	50,540	8,366	0.19:1
HIV-1(MN)/H9	7.84	2,426	113,147	1,542	0.02:1
HIV-1(MN)/H9 Clone 4	4.04	208,348	38,618	6,566	4.61:1
HIV-1(MN) Clone 4/CEM-SS	3.73	125,096	0	830	150:1
HIV-1(MN) Clone 4/DAUDI	6.41	87,154	734	0	119:1
HIV-1(MN) Clone 4/MOLT-3	1.99	25,734	0	3,205	8.03:1
H9	3.83	0	29,832	4,558	NA
CEM-SS	1.49	0	0	519	NA
DAUDI	2.82	0	787	0	NA
MOLT-3	1.87	0	0	1,960	NA

Note. Clarified culture supernatant from each cell line was centrifuged at 100,000 *g* for 45 min. The pellets were resuspended in buffer, centrifuged through 20% sucrose at 100,000 *g* for 1 hr, and resuspended at 500× relative to the starting material. These preparations were analyzed to determine their total protein, HIV-1 $p24^{CA}$, HLA DR, and β2-M concentrations. As an index of relative purity, the ratio of $p24^{CA}$ concentration to HLA concentration (i.e., the sum of the HLA DR and β2-M concentrations) were calculated. Preparations from the uninfected cell lines all contained proteinaceous, particulate material (i.e., microvesicles) that was dense enough to pass through 20% sucrose during centrifugation. As shown by the $p24^{CA}$ concentrations, the HIV-1(MN)/H9 Clone 4 line produced substantially more virus than the two prototypical HIV-1 producing cell lines, HIV-1(B)/H9, and HIV-1(MN)/H9, and had a better ratio of $p24^{Ca}$ to HLA. However, the best ratios were found for the preparations of HIV-1(MN) Clone 4 produced by CEM-SS and MOLT-3 cells.

croscopy and typical cells were seen to have numerous microvesicles of various shape, size, and electron density around their periphery (Fig. 2). Since the cells were centrifuged at low speed to prepare a cell pellet for electron microscopy, it is apparent that many of the microvesicles would have remained in the supernatant. Thus, the number seen here greatly underrepresents the number found in the cell culture supernatant. These amorphous particles range in size from 50 to 1000 nm in diameter and many appear to be budding from the cell's surface. The larger "particles" may be portions of cells sliced out of plane. Most of the microvesicles have an electron density similar to that of the cell's cytoplasm. However, some were significantly less dense with lightly defined margins and, less frequently, others were nearly electron transparent with clearly defined margins (Fig. 2, inset). For comparison, sucrose density gradient purified HIV-1(MN)/H9, HIV-1(MN)/H9 Clone 4, and H9 microvesicles were also examined by electron microscopy (Fig. 3). The HIV-1(MN)/H9 preparation contained many HIV-1 particles as identified by the electron dense cores of the mature particles. However, there were significantly more particles that appear to be microvesicles than viruses. The HIV-1(MN)/H9 Clone 4 preparation consisted primarily of HIV-1 particles and relatively few microvesicles. The purified H9 microvesicle preparation contained the same complement of heterogeneous particles as seen around the cell in Fig. 1, indicating that all of these variously appearing particles had a similar buoyant density in sucrose.

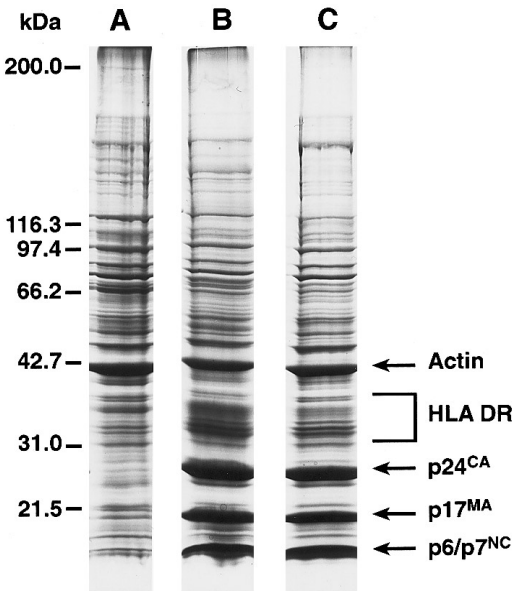

FIG. 1. SDS–polyacrylamide gel electrophoresis of H9 microvesicle, HIV-1(MN)/H9, and HIV-1(MN)/H9 Clone 4 preparations. These were purified by sucrose density gradient centrifugation. The microvesicle preparation (lane A) contains a single major ~42-kDa band (actin) and numerous minor protein bands. The HIV-1(MN)/H9 (lane B) and HIV-1(MN)/H9 Clone 4 (lane C) preparations contain some major bands of viral proteins (e.g., $p24^{CA}$, $p17^{MA}$, and $p6/p7^{NC}$). They also contain a major actin and numerous minor protein bands, similar to the ones seen from H9 microvesicles. The HIV-1(MN)/H9 Clone 4 preparation contains less material in the 30- to 40-kDa (HLA DR) region than the HIV-1(MN)/H9 preparation.

Both RNA and DNA were isolated as described under Materials and Methods from 20 mg of purified H9 microvesicles. Isolation of purified RNA and DNA from H9 microvesicles resulted in easily visualized nucleic acid pellets at the bottom of the tubes containing the CsCl pads. The guanidine isothionate layers were removed after centrifugation, reapplied to fresh, respective, 96 or

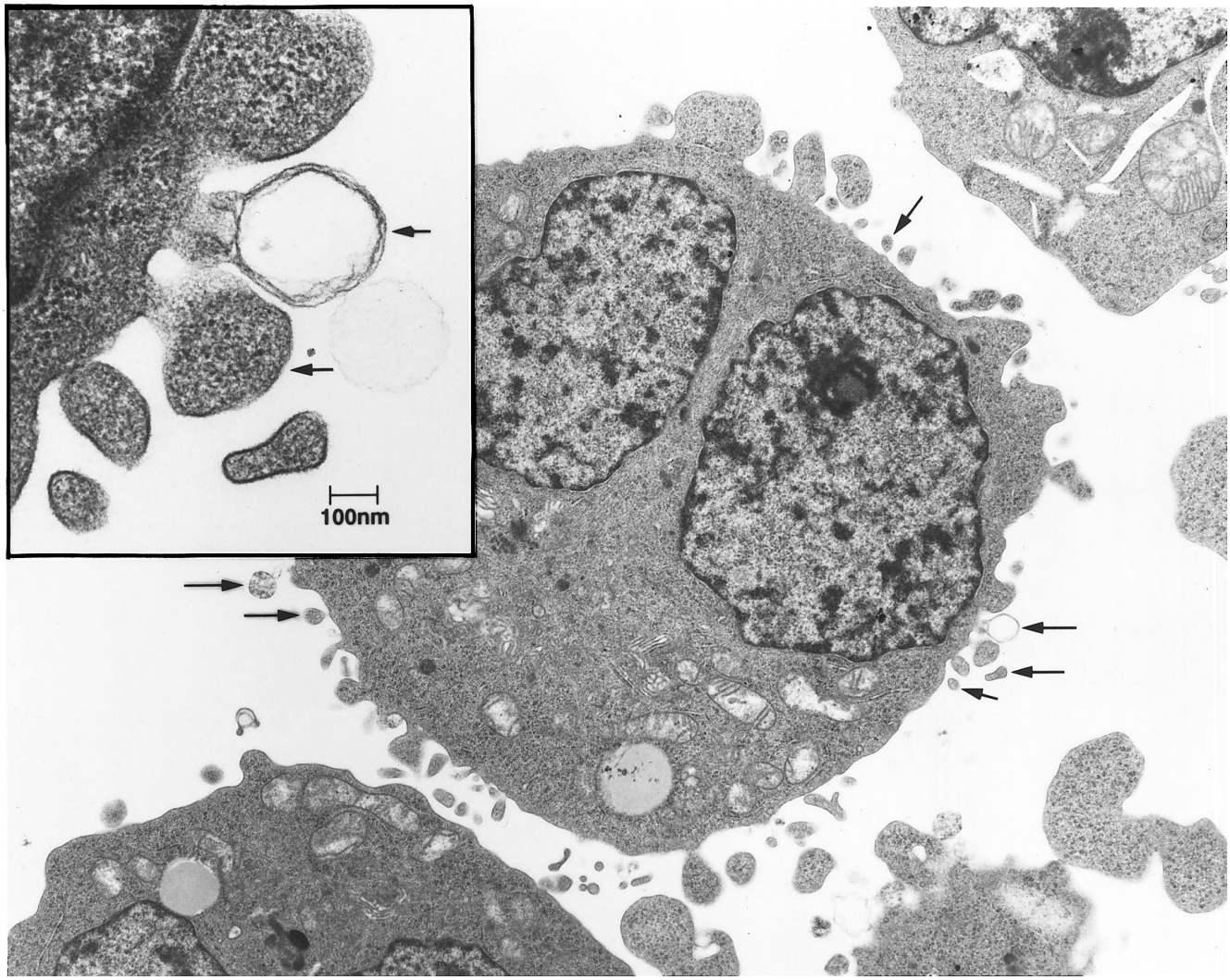

FIG. 2. Transmission electron micrograph of an uninfected H9 cell (12,000×). Note the presence of a number of microvesicles of various size, shape, and electron density near the cell's periphery. The inset shows some "budding" microvesicles at higher magnification (90,000×). Some of the microvesicles are indicated by arrows. The bar represents 100 nm.

67% (wt/vol) CsCl cushions, and centrifuged under conditions identical to the first nucleic acid isolation. This was performed to determine whether all of the polynucleotides were isolated from the disrupted H9 microvesicles. There was no detectable nucleic acid absorbance after the second centrifugation step. From one lot (P3552), 214 ± 4 μg of RNA and 41 ± 3 μg of DNA was obtained from a 20 mg protein-containing H9 microvesicle pellet. Another lot (P3548) yielded 200 ± 4 μg of RNA and 104 ± 3 μg of DNA. The nucleic acid isolated by this procedure was judged to be of high purity since the OD_{260}/OD_{280} ratio of all samples was ⩾2.0. RNA isolated by the CsCl cushion procedure was fractionated on a denaturing formaldehyde/agarose gel. Figure 4A shows the ethidium-stained gel after fractionating 2.5 to 40 μg of RNA. The major bands that are visible are the 28S and 18S ribosomal bands and a faint band at the bottom of the gel, probably tRNA and other low molecular weight RNA species. The RNA was transferred onto a nitrocellulose filter and probed with ^{32}P-labeled-cDNA probes. Figure 4B shows a Northern blot that was hybridized with a probe detect glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcripts. A significant amount of G3PDH mRNA was observed. No HIV-1 mRNA was detected (data not shown).

To determine if viral envelope proteins were associated with microvesicles, HIV-1(MN)/H9 was purified from six liters of cell culture supernatant by continuous flow ultracentrifugation in a sucrose density gradient. The gradient was monitored for UV absorbance (A_{280}) and fractions were collected. Fractions were analyzed for sucrose, $p24^{CA}$, HLA DR, and $gp120^{SU}$ concentrations (Fig. 5A). The 25 to 50% sucrose density gradient was found in fractions 11 through 40, respectively. The earlier fractions contained material that was not dense enough to penetrate 25% sucrose. Most of the sucrose density gradient fractions had similar A_{280} values, indicating that they had similar total protein concentrations. A discrete $p24^{CA}$ peak (i.e.,

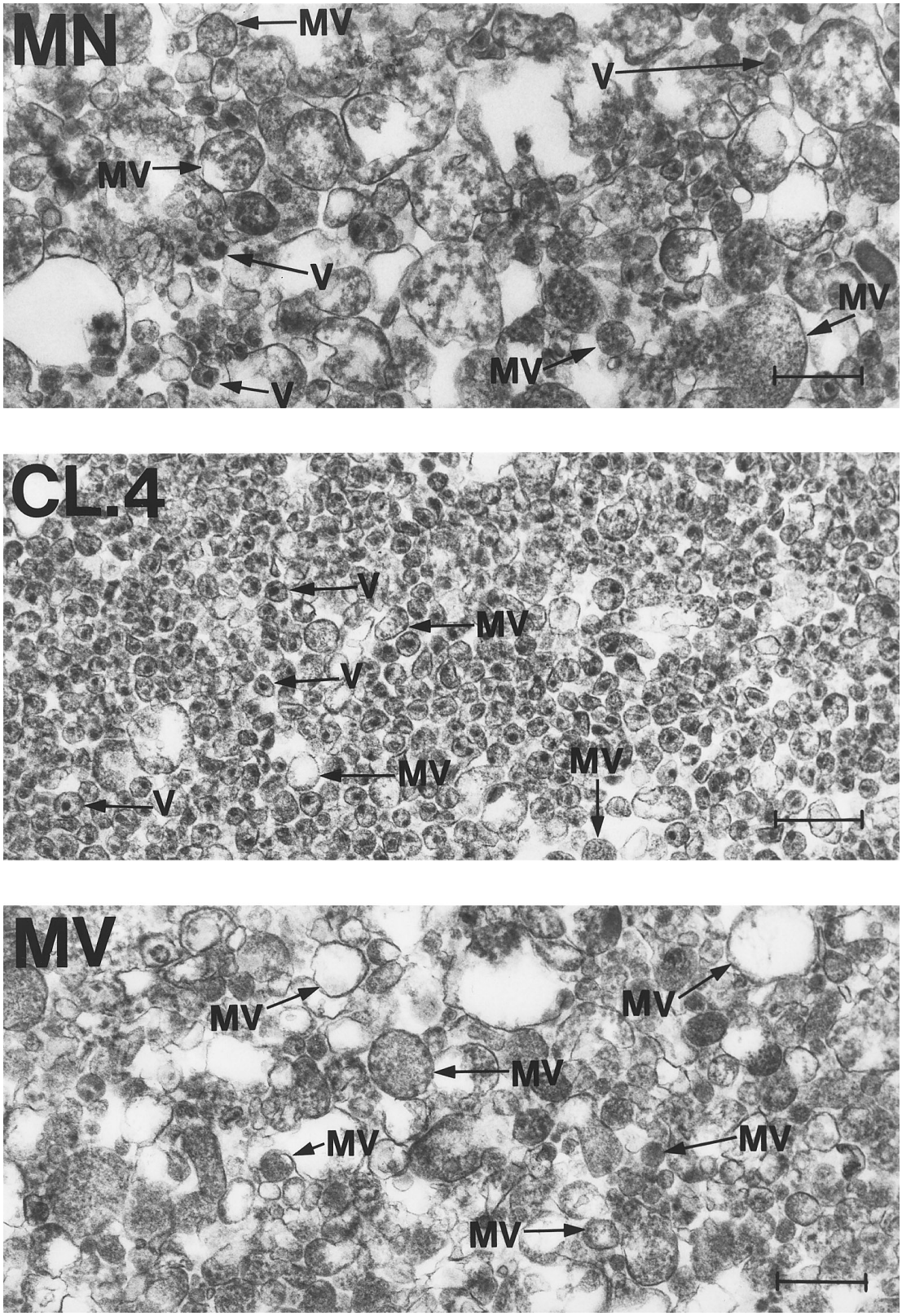

FIG. 3. Sucrose density gradient purified preparations were thin sectioned and examined by transmission electron microscopy (the bars represent 1 μm). (MN) Purified HIV-1(MN)/H9 containing some mature viruses and numerous nonviral particles (presumably microvesicles). (CL4) Purified HIV-1(MN)/H9 Clone 4 containing numerous mature virions and relatively few microvesicles. (MV) Purified H9 microvesicles. Some microvesicles (MV) and HIV-1 particles (V) are indicated by arrows.

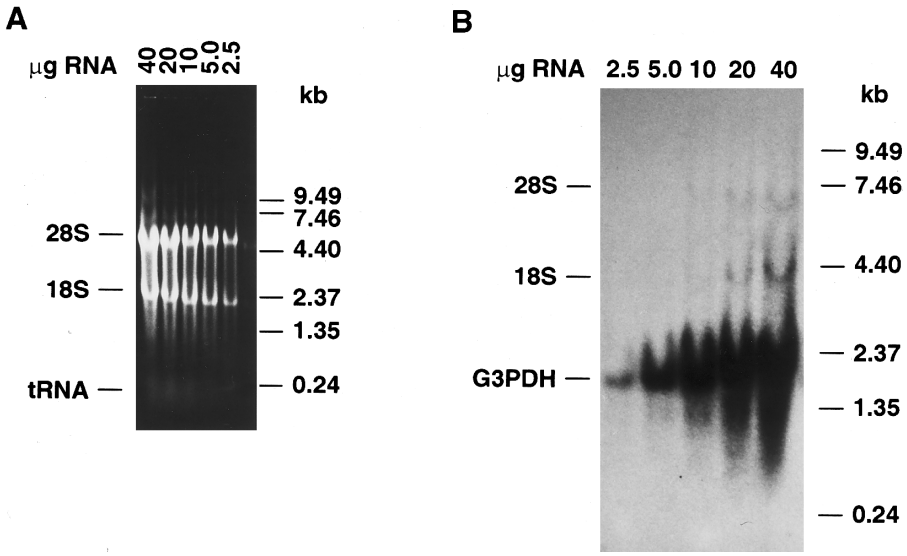

FIG. 4. Analysis of nucleic acids found in H9 microvesicles. (A) Various amounts of RNA from H9 microvesicles was fractionated on a denaturing formaldehyde/agarose gel and stained with ethidium bromide. Visible are 28S and 18S ribosomal bands and a minor band in the position where tRNA is usually found. (B) A Northern blot of H9 microvesicle RNA was prepared and hybridized with an 8088 BP AvaI HIV-1 fragment (from pNL4-3) that was ^{32}P-nick translated and a ^{32}P-nick translated human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA probe from Clontech Laboratories, Inc. (Palo Alto, CA). No HIV-1 RNA was detected (data not shown). As seen in the figure, G3PDH transcripts were readily detected. RNA size markers are shown.

the HIV-1 virions) was found between fractions 25 and 30. Particulate material containing HLA DR was found in all of the gradient fractions, demonstrating the presence of microvesicles in the entire sucrose density gradient. There appeared to be two peak concentrations of microvesicles in the gradient, one found between fractions 19 and 24 and the other between fractions 27 and 32. The distribution of gp120^{SU} closely paralleled that of p24^{CA} and not the bimodal HLA DR distribution, suggesting that little if any gp120^{SU} was found on the microvesicles. Approximately 5.8 times more p24^{CA} was found in the virus peak (fractions 25 through 30) than HLA DR. The same cultures, used as a source of material for this experiment, were passaged weekly for several weeks. Six liters of cell culture supernatant were processed and analyzed as before (Fig. 5B). The 25 to 50% sucrose density gradient was found in fractions 16 through 39, respectively. As in the previous experiment, a discrete HIV-1 peak (i.e., p24^{CA}) was observed among a broad distribution of HLA DR. However, the distribution of HLA DR was significantly altered. The amount of HLA DR in the first peak was greatly increased over that of the previous purification. Also, less virus was produced and now approximately 2.8 times as much HLA DR than p24^{CA} was found in the virus peak (fractions 22 through 30).

PHA-stimulated human peripheral blood lymphocytes (PBL) were analyzed to determine if activated cells also produced microvesicles. Approximately 7.5×10^8 PBLs were recovered from the Ficoll-Hypaque centrifugation of the PBL from a single lymphapheresis pack. PHA-activated PBL were cultured in roller bottles and harvested twice weekly until a total of 10 liters was obtained. The clarified culture supernatant was banded in a sucrose density gradient and the resulting fractions analyzed by HLA DR RIA and β2-M RIA. As can be seen in Fig. 6, the PBL microvesicles banded in a wide range of densities and contained both HLA DR and β2-M. An electron micrograph was prepared from the material in fraction 12 (data not shown). Numerous microvesicles were seen, demonstrating that these particles are produced not only by transformed T-cells such as H9, but also by human PBL.

DISCUSSION

There are a large number of publications supporting the concept that cellular proteins are physically associated with HIV-1 (Gelderbloom *et al.,* 1987; Kannagi *et al.,* 1987; Schols *et al.,* 1992; Arthur *et al.,* 1992; Meerloo *et al.,* 1993; Orentas *et al.,* 1993; Franke *et al.,* 1994; Ott *et al.,* 1995b, 1996; Gluschankof *et al.,* 1996). Since cellular proteins bound to nonviral particles (i.e., microvesicles) can copurify with virus, the finding of cellular proteins in purified virus preparations does not indicate that these proteins are necessarily physically associated with the virus particles. In this report, we characterize microvesicles which copurify with HIV-1 during sucrose density centrifugation. The amount and type of cellular proteins found in purified virus preparations were shown to be affected by the virus isolate and cell line. In fact, the amount of HLA DR exceeded the amount of p24^{CA} in purified preparations of two of the prototypical HIV-1 isolates, HIV-1(B)/H9 and HIV-1(MN)/H9. Preparation of single cell clones of HIV-1(MN)/H9 resulted in a biological clone, HIV-1(MN)/H9 Clone 4, which was found to yield

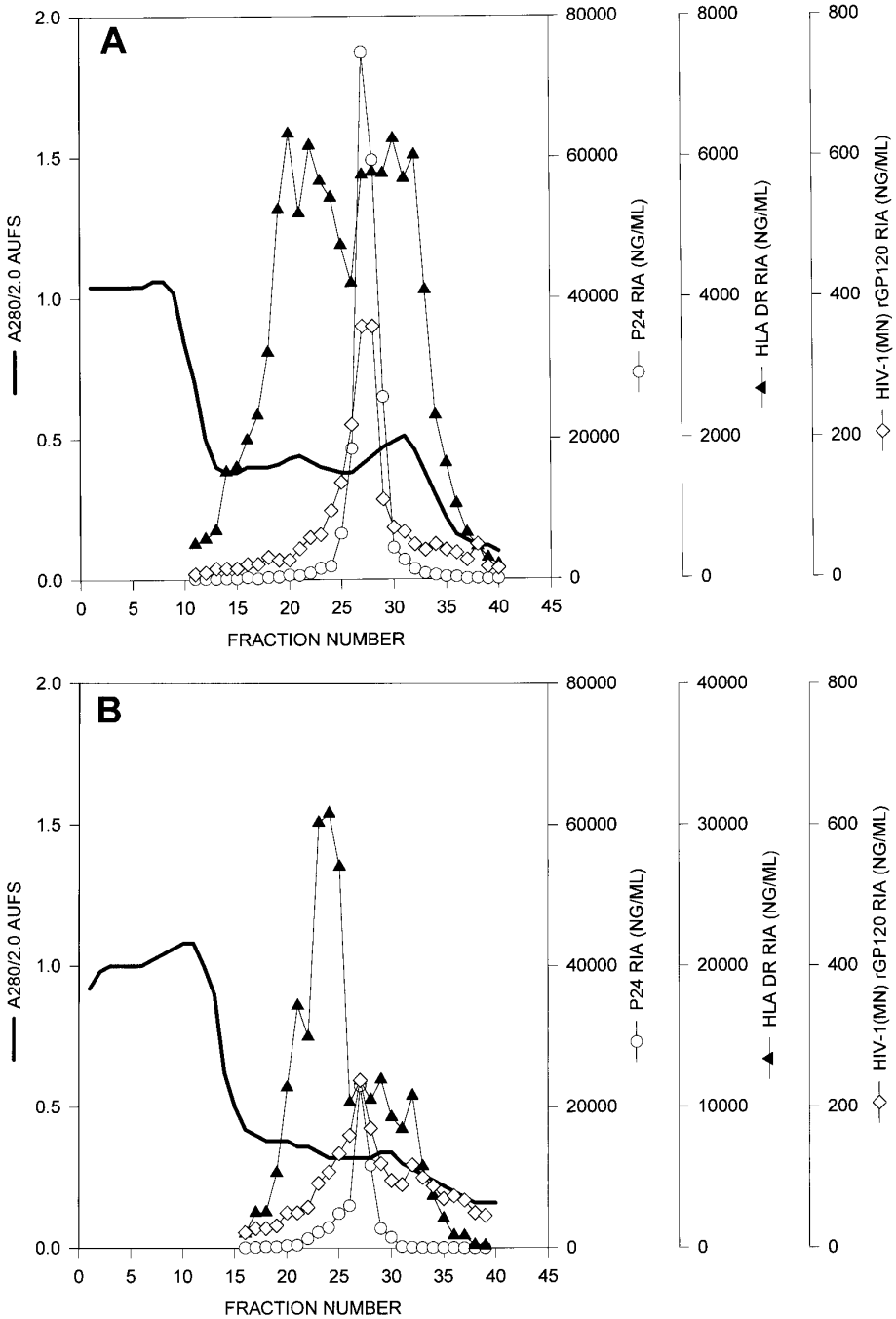

FIG. 5. Banding of HIV-1(MN)/H9. (A) Six liters of clarified cell culture fluid were banded in a sucrose density gradient. The material was monitored for UV absorbing material at 280 nm/2.0 absorbance units full scale (AUFS) during collection. The concentration of $p24^{CA}$, $gp120^{SU}$, and HLA DR were determined for each fraction by RIA. The 25 to 50% sucrose density gradient starts at fraction 11 and ends at fraction 40, respectively. The earlier fractions are material that was not dense enough to penetrate the gradient. HLA DR was detected in all of the fractions and in peak fractions at concentrations approximately one-tenth that of $p24^{CA}$. In general, viral envelope protein ($gp120^{SU}$) is found in parallel with $p24^{CA}$ and not in parallel with the HLA DR, suggesting that little or no $gp120^{SU}$ is associated with the microvesicles. (B) The same cultures used for the first experiment were passaged twice weekly for several weeks and six liters of cell culture fluid were processed as before. The sucrose density gradient starts at fraction 16 and ends at fraction 39. The amount of $p24^{CA}$ in peak fractions was approximately one-fourth of that found in the first experiment while the concentrations of HLA DR were approximately 5 times higher than before. Again, most of the $gp120^{SU}$ is found in parallel with $p24^{CA}$ and not in parallel with the HLA DR, suggesting that little or no $gp120^{SU}$ is associated with the microvesicles. However, some $gp120^{SU}$ may be associated with microvesicles that banded at a higher density than the virus particles. These two experiments demonstrated that over time the ratio of viral protein to HLA DR can be highly variable. Note that the HLA DR concentration scales are different in A and B.

not only much more virus per liter than the two prototypical cell lines, but also much more $p24^{CA}$ than HLA DR. These findings suggest that not only do the virus isolate and cell line affect the relative concentrations of viral to cellular proteins, but there may be variation between individual cells in a mixed population. At present, we have

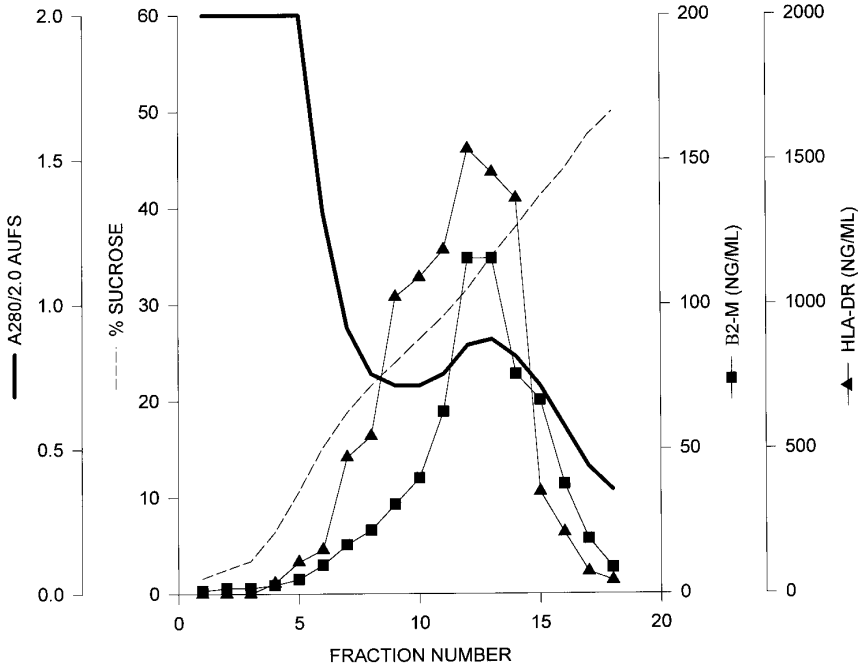

FIG. 6. Banding of human PBL microvesicles. Ten liters of clarified PBL cell culture fluid were banded in a sucrose density gradient. The concentration of HLA DR and β2-M were determined for each fraction by RIA. Like the microvesicles from H9 cells, these microvesicles banded in a wide range of sucrose densities and peak concentrations were observed between approximately 25 and 40% sucrose.

no data indicating whether this is a property of the cell clone, the two proviruses contained in this cell clone (Ott *et al.,* 1995a), or both. In addition to H9, all of the uninfected T-cell lines analyzed, CEM-SS, DAUDI, and MOLT-3, produced microvesicles that banded at the same density as HIV-1. Implicitly, microvesicles purified from cell lines such as CEM-SS and MOLT-3 that do not produce HLA DR did not contain any HLA DR but did contain β2-M. The reciprocal was found for the β2-M negative cell line DAUDI. Previous reports have indicated that infection of T-cell lines can up-regulate, down-regulate, or have no effect on the cell surface expression of various cellular proteins (Schols *et al.,* 1992; Wrightham *et al.,* 1991; Kerkau *et al.,* 1989; Stevenson *et al.,* 1987; Mann *et al.,* 1988; Noraz *et al.,* 1995). Here we observed that infection of CEM-SS, DAUDI, and MOLT-3 cells with HIV-1(MN)/H9 Clone 4 virus did not greatly effect the amount of HLA DR found in viral pellets relative to the analogous microvesicle preparations within the time period examined.

We have produced and purified a large number of HIV-1(MN)/H9 preparations and found them to have a wide range of HIV-1 $p24^{CA}$ to HLA DR ratios (data not shown). Two reasons for this variability are now evident. First, the amount of HIV-1 and microvesicles produced was shown to vary over time in HIV-1(MN)/H9 cultures. An early harvest yielded approximately 5.8 times as much HIV-1 $p24^{CA}$ than HLA DR in the virus peak while virus harvested from later passaged cells had approximately 2.8 times as much HLA DR than HIV-1 $p24^{CA}$. Second, the density of microvesicles is more heterogeneous than HIV-1, causing them to be distributed over a broader range in a sucrose density gradient. Consequently, the ratio of viral-associated protein to protein contributed by microvesicles is also affected by which fractions are selected for inclusion in the "virus pool." Typically, fractions are selected based on the A_{280} and sucrose density profiles. As shown in Fig. 5A, selection of fractions 25 through 30 would yield a HIV-1 $p24^{CA}$ to HLA DR ratio of 5.8:1. However, if fractions 22 through 35 had been pooled, the result would have been a HIV-1 $p24^{CA}$ to HLA DR ratio of 2.9:1.

Our finding that little if any $gp120^{SU}$ was found associated with microvesicles when fractionated by sucrose gradient centrifugation may indicate that $gp120^{SU}$ was not distributed in a random fashion on the surface of HIV-1-infected cells and that the viruses and microvesicles bud from different topological sites. However, there was some evidence that a little $gp120^{SU}$ may have been associated with microvesicles that banded at a slightly higher density than HIV-1 particles (see Fig. 5B). The finding that CD43 and CD63 appeared to be primarily virion-associated and HLA-DQ was associated only with microvesicles (Gluschankof *et al.,* 1997) is consistent with the concept of separate budding sites. The finding that HIV-1 buds almost exclusively from the basolateral surface of polarized VERO cells (Owens *et al.,* 1991) provides direct evidence that HIV-1 particles can bud from specific topological sites. Perhaps the budding site of microvesicles is from a different location. An alternative explanation for the lack of $gp120^{SU}$ on microvesicles is derived from the finding that envelope proteins require association with the viral Gag precursor under the

plasma membrane in order to be included in virions (Yu *et al.,* 1992). It is possible that without the Gag association with gp120^{SU}, either the viral envelope proteins are not included in microvesicles or they are unstably incorporated and consequently lost.

PHA activated human PBLs were also shown to produce microvesicles that incorporated cellular proteins (Fig. 6). It has recently been reported that B-lymphocytes secrete antigen presenting vesicles (Raposo *et al.,* 1996) that are similar to the microvesicles described here. We have previously shown that the HLA DR on HIV-1 is functional since it can serve in superantigen presentation to human T-cells (Rossio *et al.,* 1995). Thus, it is possible that cellular proteins on microvesicles and HIV-1 may have immune functions. Experiments are in progress to determine if class II molecules on HIV-1 and microvesicles can properly present antigens or if there is improper presentation resulting in anergy and/or apoptosis.

In addition to proteins, microvesicles were also shown to contain both RNA and DNA. Approximately 10 μg of RNA and 4 μg of DNA were found per mg of protein. The major RNA species in microvesicles were ribosomal 28S and 18S subunits and some low molecular species, perhaps tRNA. A significant amount of G3PDH mRNA was found, indicating microvesicles contained elements of the cell cytosol.

The presence of microvesicles in purified retroviruses has practical implications. Human cellular antigens have been found associated with HIV-1 preparations (Gelderbloom *et al.,* 1987; Henderson *et al.,* 1987; Hoxie *et al.,* 1987; Kannagi *et al.,* 1987; Schols *et al.,* 1992; Arthur *et al.,* 1992; Meerloo *et al.,* 1993; Orentas *et al.,* 1993; Franke *et al.,* 1994; Thali *et al.,* 1994; Ott *et al.,* 1995b, 1996; Gluschankof *et al.,* 1996) and were initially recognized as a source of some false positive results in immunoassays using purified HIV-1 preparations to detect anti-HIV-1 antibodies in human plasma samples (Hunter *et al.,* 1985; Kuhnl *et al.,* 1985; Sayers *et al.,* 1986; Blanton *et al.,* 1987; Drabick *et al.,* 1989; Schols *et al.,* 1992). Characterization of microvesicles may afford some insight on how to separate these particles from purified retroviruses. We have been unsuccessful in separating microvesicles from HIV-1 by centrifugation techniques (data not shown). However, lack of gp120^{SU} on microvesicles suggests that protocols employing immunoaffinity techniques may be useful in this separation. Clearly, future experiments utilizing purified viruses must be carefully controlled to account for the effects of cellular antigens present on microvesicles.

It does not appear that many of the predominant surface antigens (i.e., HLA DR, class I, and β2M) are required for infection *in vitro* because it is possible to produce infectious HIV-1 stocks devoid of these proteins by propagation in cells which do not contain these cellular proteins (Bess, unpublished data). However, it has been shown that cyclophilin associates with the p24^{CA} domain of the Gag polypeptide of HIV-1 (Luban *et al.,* 1993) and is required during the infection process by group M HIV-1 (Braaten *et al.,* 1996). It has also been shown that the presence of viral-associated cellular adhesion molecules will enhance viral infectivity of CD4-negative cells (Castilletti *et al.,* 1995). Cellular cytoskeletal proteins, actin, ezrin, moesin, and cofilin (Ott *et al.,* 1996) are located in the virion interior. It is unknown how these cellular proteins associate with the virus particles and if they participate in the virus replication cycle. Numerous other cellular proteins have been identified in purified preparations of HIV-1. It is not known if these are physically associated with HIV-1 particles and, if so, whether or not they have a role in the virus replication cycle. Identification of which cellular proteins are associated with the virus is a prerequisite to studying the potential functions of cellular proteins in the virus replication cycle. The development of various purification strategies to separate microvesicles from HIV-1 particles and the use of cell lines that produce fewer microvesicles will greatly enhance our ability to identify virion-associated cellular proteins.

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