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Cell Membrane Vesicles Are a Major Contaminant of Gradient-Enriched Human Immunodeficiency Virus Type-1 Preparations

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During preliminary experiments to establish the proportion of virus-coded p24 protein to virus membrane-associated HLA-DR in gradient-enriched HIV-1 preparations, we became aware of a large variability between experiments. In order to determine whether HLA-DR-containing cellular material was contaminating the virus preparations, we carried out enrichment by gradient centrifugation of clarified supernatants from noninfected cells and tested this material for HLA-DR content. We found that, independently of the cell type used, gradient enrichment resulted in the isolation of large quantities of HLA-DR-containing material which banded at a density overlapping that of infectious HIV. Electron microscopy of gradient-enriched preparations from supernatants of virus-infected cells revealed an excess of vesicles with a size range of about 50–500 nm, as opposed to a minor population of virus particles of about 100 nm. Electron micrographs of infected cells showed polarized vesiculation of the cell membrane, and virus budding was frequently colocalized with nonviral membrane vesiculation. Analysis of the cellular molecules present in the fractions containing virus or exclusively cellular material demonstrated that virus and cellular vesicles share several cellular antigens, with the exception of CD43 and CD63, found mainly at the virus surface, and HLA-DQ, which was found only in the cellular vesicles. © 1997 Academic Press

INTRODUCTION

The human immunodeficiency virus (HIV) infects predominantly cells of the immune system such as T lymphocytes and macrophages, which carry the CD4 molecule, the principal HIV receptor (reviewed in Sattentau and Weiss, 1988). The virus life cycle begins with virus binding and entry into receptor-bearing cells, proceeds with replication of the viral genome by reverse transcription and proviral integration into the host cell genome, and ends with the production and assembly of new viral proteins and RNA into progeny virions (reviewed in Levy, 1994). New particles are released from the cell by a polarized process of budding by which the virus acquires its envelope from the plasma membrane (Gelderblom, 1991).

The viral envelope contains glycoproteins of viral origin, gp120 and gp41, which reach the plasma membrane through the secretory pathway and which are essential for binding and entry of the virions into new target cells (reviewed in Moore *et al.,* 1993). Also present in the HIV envelope are molecules of cellular origin (Gelderblom *et al.,* 1987; Henderson *et al.,* 1987; Hoxie *et al.,* 1987; Arthur *et al.,* 1992; Meerloo *et al.,* 1993). Cellular molecules appear to be selectively incorporated into virions, since some are overrepresented in comparison to the relative amount in the cell membrane, whereas others appear to be absent (Hoxie *et al.,* 1987; Arthur *et al.,* 1992). This phenomenom is not HIV-specific, since the presence of cellular proteins in the membranes of other retroviruses has been observed (Bubbers and Lilly, 1977; Azocar and Essex, 1979; Lee *et al.,* 1982; Lando *et al.,* 1983). HIV-1 and simian immunodeficiency virus (SIV) envelopes are particularly enriched for HLA class-I and class-II DR molecules (Gelderblom *et al.,* 1987; Henderson *et al.,* 1987; Schols *et al.,* 1992; Arthur *et al.,* 1992; Cantin *et al.,* 1996). However, in none of the studies demonstrating the association of molecules of human origin with HIV or other retroviruses by biochemical or serological means has the purity of the virus preparation been verified. Virus to be used for biochemical and serological analyses or as an immunogen is frequently prepared by centrifugation through sucrose gradients. The fractions containing viral antigen and/or infectivity are considered to contain a population of relatively pure virus particles.

We have analyzed gradient-enriched virus preparations and found that there is contamination with an excess of nonviral membrane vesicles of cellular origin. This was the case when the virus was grown in an immortalized cell line or in activated peripheral blood mononuclear cells (PBMC). These vesicles, which are released from both noninfected and HIV-1-infected cells, contain a selection of cellular membrane proteins similar to, but not identical to, those in the virus particles. Contamination of gradient-en-

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riched virus by vesicles of cellular origin has implications for the preparation and use of vaccine formulations derived from whole-virus preparation.

MATERIALS AND METHODS

Cell culture and HIV infection

The immortalized T (H9) cell line was cultured in RPMI medium complemented with 10% fetal calf serum, penicillin, and streptomycin (growth medium, GM). PBMC were from the Marseille Blood Transfusion Center (CTS, Marseille, France) and were obtained as a preenriched fraction from individuals having undergone leukopheresis. The cells were activated prior to virus infection with 1 μg/ml phytohemagglutinin (PHA; Gibco BRL, Cergy Pontoise, France), then cultured in GM supplemented with 20 IU/ml IL-2 (Boehringer Mannheim, France), and infected at Day 5 after activation. Virus infection of H9 cells and activated PBMC was carried out as follows. Fifty million cells in 6 ml GM were infected with the HIV-1 molecular clone Hx10 (Fisher *et al.,* 1988) by agitation of the cells for 2 hr at 37° in the presence of virus at an m.o.i. of about 0.1. The cells were then kept at a density of 5×10^5 to 10×10^5/ml, and virus production was followed by ELISA detection of viral p24 in the culture supernatant as previously described (Moore *et al.,* 1990). Culture supernatants from different time points were centrifuged at 400 *g* for 10 min and filtered through 0.45-μm filters to remove gross cell debris, then frozen at −80°. Supernatants subsequently determined as containing the maximum amount of p24 were used for enrichment of viral material. Titration of virus infectivity was carried out by limiting dilution analysis on H9 cells. Briefly, serial fivefold dilutions of the virus preparation were incubated with 10^5 H9 cells/well in a U-bottomed 96-well plate. The tissue culture infectious dose (TCID) was determined as the last dilution giving a positive signal for soluble p24 antigen by ELISA on Day 5 after infection.

Enrichment of HIV and vesicular material

Virus and cellular vesicles were enriched from clarified cell culture supernatants at the time point giving maximal p24 production in the supernatant of infected cells or the corresponding time point in uninfected cells, by the following procedure. Precleared supernatants were centrifuged at 70,000 *g* for 45 min, and the pellet was resuspended in 500 μl of sterile PBS and centrifuged through 1 ml of a 20% sucrose/PBS cushion for 10 min at 70,000 *g.* The pellet containing virus/cellular material was resuspended in 250 μl of PBS and centrifuged through a discontinuous sucrose gradient (500 μl of 60% sucrose/PBS + 750 μl of 50% sucrose/PBS + 750 μl of 40% sucrose/PBS + 750 μl of 30% sucrose/PBS) for 15 min at 100,000 *g.* Twelve fractions of 250 μl each were taken from top to bottom, and stored at −80° until use. The sucrose concentration of each fraction was determined by refractometry.

ELISA assay for HLA-DR

HLA-DR protein in virus and vesicular material was quantified by twin-site ELISA. A 96-well ELISA plate (Nunc Life Technologies S.A.R.L., Cergy Pontoise, France) was coated overnight at 4° with 50 μl/well of 5 μg/ml L243 monoclonal antibody (mAb; obtained from the American Type Culture Collection) in 0.1 *M* bicarbonate buffer, pH 8.6. After washing, nonspecific sites were blocked with PBE (PBS, 5 m*M* EDTA, 2% BSA, 0.5% NP-40), and 50 μl/well of each of the test samples was incubated overnight at 4°. After nine washes with PBS/0.05% Tween 20, 50 μl of a 1/200 dilution of biotinylated anti-HLA-DR clone B8.12.2 (Immunotech, Marseille, France), a mAb reactive with an epitope that does not overlap with L243, was added for 1 hr at room temperature (RT). After further washing, streptavidin–alkaline phosphatase conjugate (Sigma-Aldrich, St. Quentin Fallavier, France) was added at a 1/1000 dilution for 1 hr at RT before washing. The rest of the reaction was carried out as proposed in the instructions for the AMPAK amplification kit (DAKO S.A., Trappes, France). The concentration of HLA-DR present in the sample was quantified by the inclusion of a standard curve generated with purified MHC HLA-DR4. This molecule, derived from insect cells coinfected with HLA-DRα and β DAF recombinant baculoviruses (Scheirle *et al.,* 1992) and purified as described elsewhere (Cammarota *et al.,* 1992) was kindly provided by D. Piatier-Tonneau (UPR420 CNRS, Villejuif, France).

Solid-phase antibody capture of virus and cellular material and measurement of captured virus infectivity

This assay is a modified version of the one described elsewhere (Abbate *et al.,* 1995). Briefly, 96-well ELISA plates were coated with a panel of mAb directed to different lymphocyte surface antigens. The mAbs ICO-80, By88, 10G7, 212.3, and RUU-SP2.28, directed to CD5, CD30, CD43, CD44, and CD63 were obtained from the fifth International Leucocyte Typing Workshop antibody panel, and mAb SPVL3 directed to HLA-DQ was purchased from Immunotech, France. HLA-DR-positive material from infected cell supernatants was captured onto the solid phase by coating plates with mAb L243 as described above. Nonspecific sites were blocked with PBS/1% FCS (wash buffer, WB) for 1 hr at RT, then 25 μl of each fraction from the sucrose gradient was added in WB to yield a final volume of 100 μl. After 4 hr of incubation at RT, the unbound material was removed. To determine the relative proportion of the different cellular antigens captured onto the solid phase from uninfected cell supernatants, the bound fractions were analyzed for the presence of HLA-DR on the surface of cellular vesicles

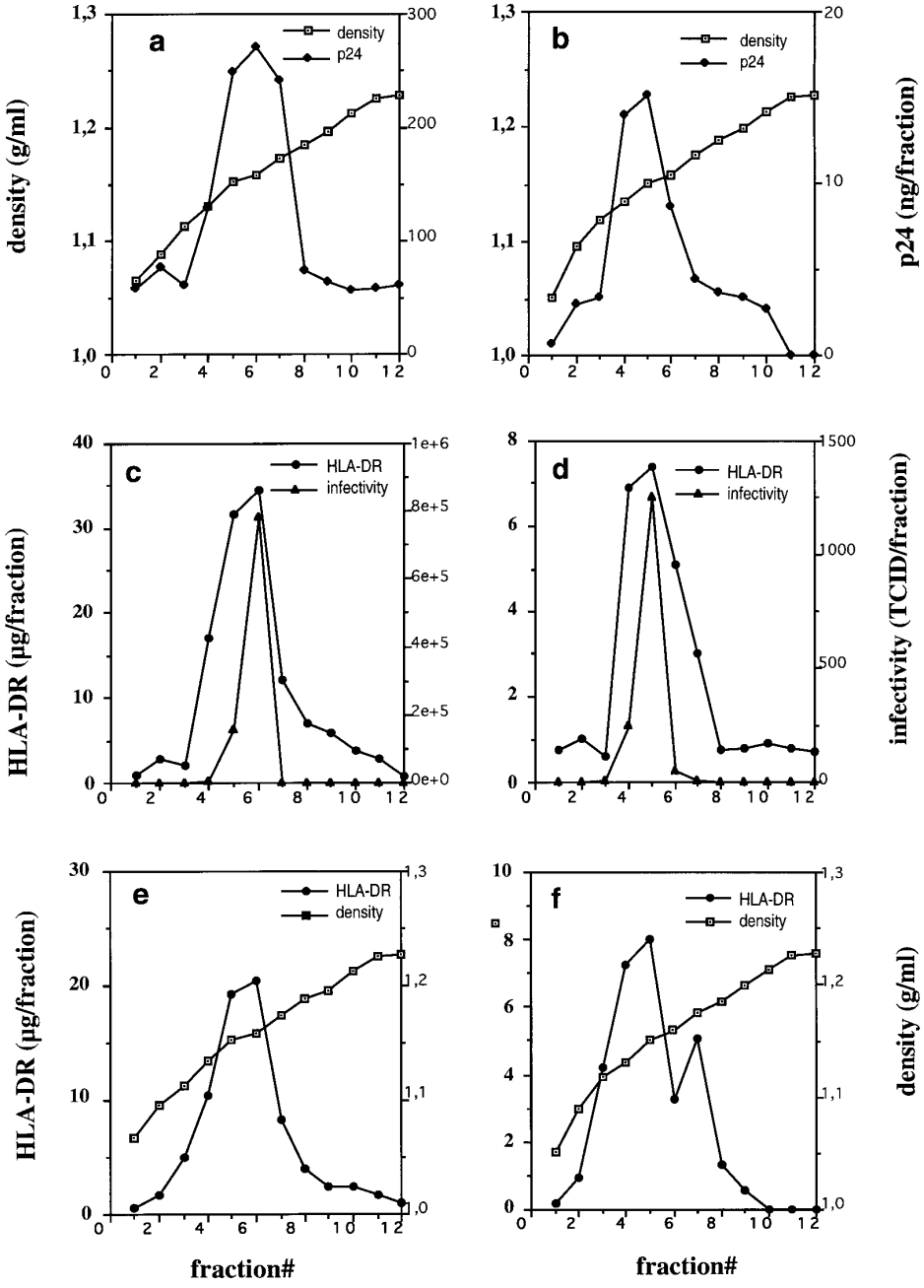

FIG. 1. Density gradient purification of HIV-1 and cellular vesicles from supernatants of infected and noninfected cells. Material pelleted at 70,000 *g* from supernatants of HIV-1-infected H9 cells (a and c) or activated PBMC (b and d) or from supernatants of noninfected H9 cells (e) or activated PBMC (f) was loaded onto a discontinuous sucrose gradient as described under Materials and Methods. Twelve 250-μl fractions were taken from top to bottom. The density, amount of p24 capsid (a and b), infectivity (c and d), and HLA-DR antigen (c, d, e and f) were determined for each fraction.

by ELISA as previously described (in this case, PBE was replaced by WB).

The unbound material from infected cell supernatants was titrated onto 4×10^5 H9 cells/well in a separate plate, and the TCID was determined by measurement of p24 antigen in the supernatant at Day 7 after infection as previously described. The plate containing the bound material was washed, and 4×10^5 H9 cells were added. At Day 7, the virus produced was titrated onto 4×10^5 H9 cells/well in a separate plate, and the TCID was determined as for the unbound material. All manipulations were carried out under sterile conditions using sterile buffers and media.

Electron microscopic analysis of virus and cellular vesicles

HIV-1 Hx10-infected cells and sucrose-gradient-enriched material from infected and noninfected cell culture

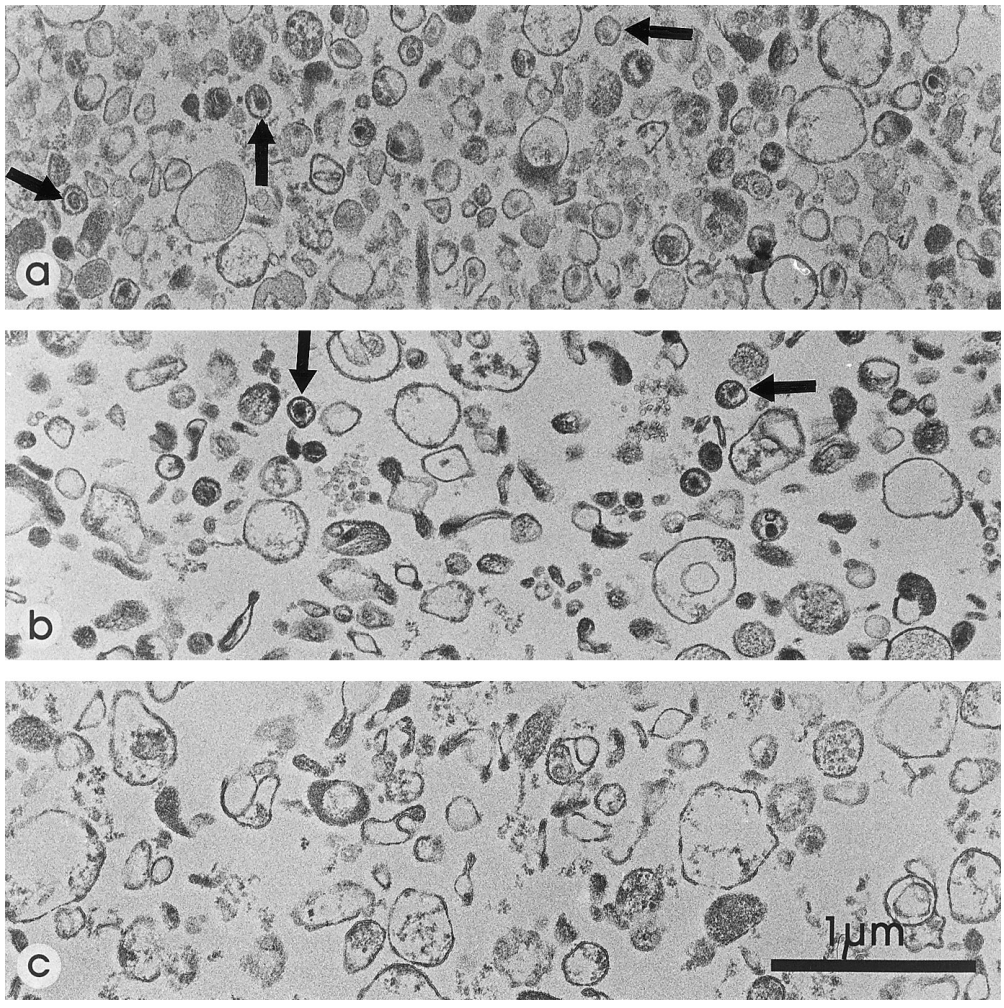

FIG. 2. Purified HIV-1 preparations are contaminated by cellular vesicles. Purified vesicles from infected H9 cells (a) and activated PBMC (b) supernatants (fraction 6 from Fig. 1a and fraction 5 from Fig. 1b, respectively) or from noninfected H9 cells (c) (fraction 6 from Fig. 1e) were treated for electron microscopic analysis as indicated under Materials and Methods. The cellular vesicles appear to be a heterogeneous population of both electron-lucent and electron-dense membrane delineated vesicles ranging in size from about 50 to 500 nm. (Original magnification ×36,000). Virions are indicated by arrows.

supernatants were fixed in 2.5% glutaraldehyde in PBS at room temperature. The specimens were enclosed into agarose blocks for ease of handling. After additional treatment with tannic acid and uranyl acetate, the specimens were embedded into Epon following routine techniques (Gelderblom *et al.,* 1987). Thin sections were poststained with lead citrate and evaluated using a Zeiss EM-10 A electron microscope.

RESULTS

In initial studies in which we attempted to establish the ratio of virion p24 capsid to cellular HLA-DR protein in HIV-1 virion-containing supernatants prepared from a variety of different cell lines, we found a very large variation between experiments (data not shown). We therefore decided to investigate the possibility that cellular material containing HLA-DR, and perhaps other molecules of cellular origin, might be a contaminant of sucrose gradient "purified" virus preparations. Indeed, electron microscopy of the sediments of culture supernatants revealed a high proportion of empty, membrane-bound structure (data not shown).

Clarified culture supernatant from noninfected cells and cells infected with a molecular clone of HIV-1 (Hx10) were fractionated on a discontinuous sucrose gradient. We quantified the amount of HLA-DR for each fraction and measured the concentration of p24 protein and infectivity for fractions derived from the infected cellular material. Figure 1 (a to c) shows the profile of HIV-1-containing fractions obtained from gradient centrifugation of supernatant from infected H9 cells and PHA-activated PBMC. As previously reported (Cheng-Mayer *et al.,* 1991), we also observed a marked difference in TCID values (about 2 to 3 log) between virus grown in activated PBMC or in a T cell

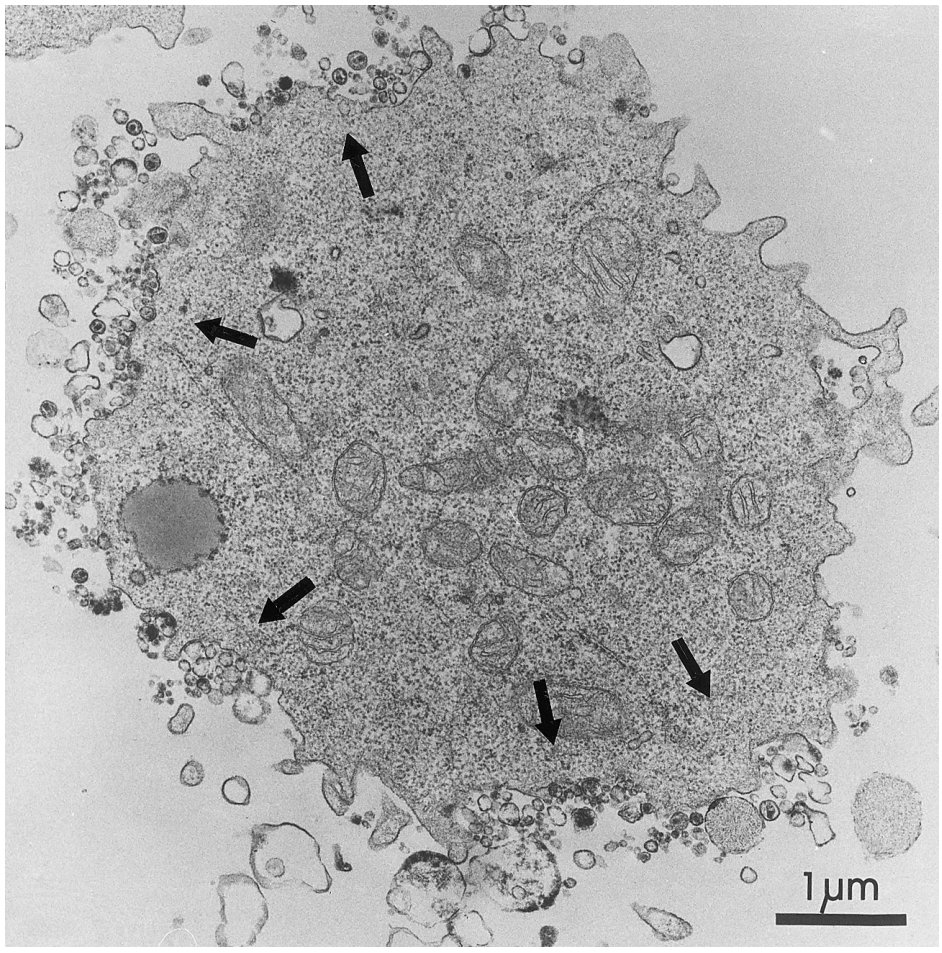

FIG. 3. Cellular vesicles and viral particles appear to bud from the same region of the plasma membrane. Infected activated PBMC were treated for electron microscopy analysis. Polarized HIV budding and cellular vesiculation can be seen at several distinct regions of the cell membrane. (Original magnification ×25,000). Arrows indicate major regions of virus budding and cell membrane vesiculation.

line. Infectious virus particles were found predominantly in fractions 5 and 6, which correspond to a sucrose density of approximately 1.15. Both p24 and HLA-DR were found in fractions 4–7 (sucrose density 1.14–1.17), implying that infectivity, p24, and HLA-DR are all associated with material of the same density, irrespective of the cell type used to grow the virus. Analysis of clarified supernatant from uninfected cells, however, revealed that HLA-DR was also present in the sucrose gradient (Figs. 1e and 1f). The distribution through the gradient was similar to the fractionated infected material, in that significant amounts of HLA-DR were detected between fractions 4 and 7. Thus HLA-DR-containing material present in noninfected cells has a density overlapping that of HIV-1 particles. In order to characterize this cellular material, we carried out electron microscopy of sample sediments derived from gradient-prepared supernatants from infected and uninfected cells. Figure 2 shows representative sections of the gradient-enriched preparations; pleomorphic vesicles, which range in size from about 50 to 500 nm and either are empty or stain slightly opaque, are present in the noninfected cellular material. The same vesicles can be seen in the preparations from the HIV-infected cells along with HIV-1 particles. The majority of the virus particles have the classical morphology, i.e., they can be identified by the relatively homogeneous diameter of about 110 nm, the dense cone-shaped core, and the "lateral bodies." In addition, a small fraction of immature HIV is also present. HIV particles are a minority population, comprising about 25 to 50% of the vesicles in both infected preparations. We conclude from these results that cellular vesicles appear, at least under the culture conditions used here, to be a major contaminant of HIV preparations enriched by sucrose gradient centrifugation. In an attempt to define the origin of these vesicles, we examined sections of HIV-infected cells for evidence of vesiculation. As shown in Fig. 3, infected cells showed polarized vesiculation, yielding membrane vesicles similar to those seen in the gradient-enriched material. From the images examined, it ap-

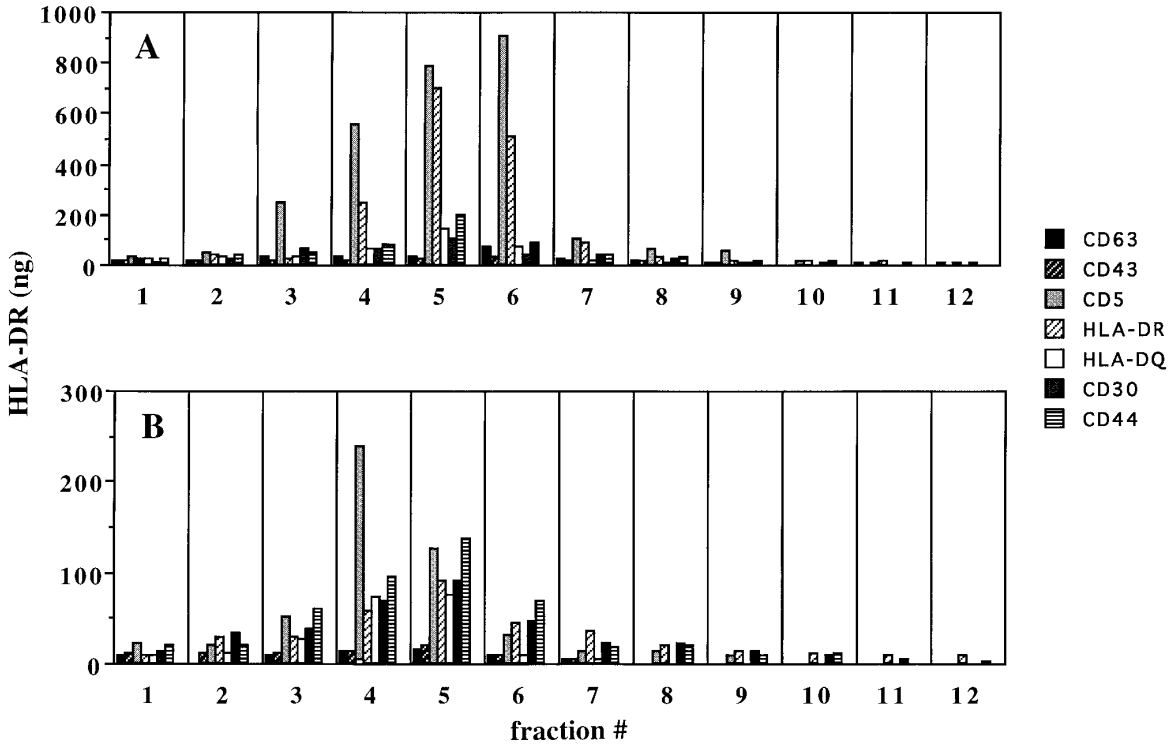

FIG. 4. Cellular vesicles carry cell membrane-associated antigens at their surface. Vesicles from density-gradient fractionated material obtained from H9 cells (A) or activated PBMC (B) supernatants were captured onto the solid phase by a panel of mAbs to various cellular antigens. The capture of bound vesicles was detected by titration of HLA-DR molecules present in the membrane of the vesicle using a twin-site ELISA. mAbs ICO-80, By88, 10G7, 212.3, RUU-SP2.28, L243, and SPVL3 directed to CD5, CD30, CD43, CD44, CD63, HLA-DR, and HLA-DQ, respectively, were used for capture, and biotinylated anti-HLA-DR clone B8.12.2 was used for detection.

pears that the cells undergoing vesiculation are healthy and not undergoing apoptosis, implying that such vesiculation may be part of a normal cellular function.

It has been reported that HIV-1 contains a number of cellular molecules in addition to HLA-DR. In order to better define the phenotype of the vesicles released by noninfected cells, we developed an assay to determine the presence or absence of different cellular antigens in both the virus particle and the cellular vesicle populations. Gradient-fractionated material was adsorbed to 96-well plates coated with mAbs specific for seven different lymphocyte antigens which have previously been determined to be present on the surface of the cell types used. Bound and unbound material was separated by washing, and the presence of vesicles in the two fractions was detected by a sandwich ELISA using anti-HLA-DR. The specificity of the capture system was tested by using supernatants from infected HLA-DR-negative A3.01 cells. Despite yielding large amounts of infectious virus, none of the A3.01-derived material was captured onto HLA-DR-coated plates, and all was present in the unbound fraction (data not shown). The presence of cellular antigens in the virus membrane was determined by measuring the infectivity present in the bound and unbound fractions derived from PBMC and H9 cells. Figure 4 shows the presence of the different antigens in the bound vesicle fractions from noninfected cells. Both H9- and PBMC-derived material contained CD5, HLA-DR, HLA-DQ, CD30, and CD44, which was broadly distributed between fractions 3 and 7, but little or no CD43 and CD63. The distribution of cellular molecules in the infectious, H9-derived HIV-1-associated fraction was somewhat different from that observed for the cellular vesicles (Fig. 5A). Not only were CD5, HLA-DR, and CD44 found to be associated with infectivity, but also CD43 and CD63, which were not detected in the cellular vesicles. Moreover, HLA-DQ which was associated with the cellular vesicles was absent in the infectious fraction. In the infected PBMC-derived material the situation was less clear; although CD63 and HLA-DR were strongly associated with HIV, CD30, CD44, CD5, and CD43 were less well represented. However, the fact that no CD5-, CD43-, CD30-, or CD44-positive infectious HIV was recovered in the unbound fraction suggests that the virus population carrying these antigens may be less infectious than the one containing HLA-DR and CD63 in their envelope. The only antigen which was obviously present in the unbound fraction was HLA-DQ, confirming that this molecule is excluded from infectious HIV-1 particles (Fig. 5B). Although we were unable to measure the quantity of cellular molecules incorporated into either the cellular vesicles or the

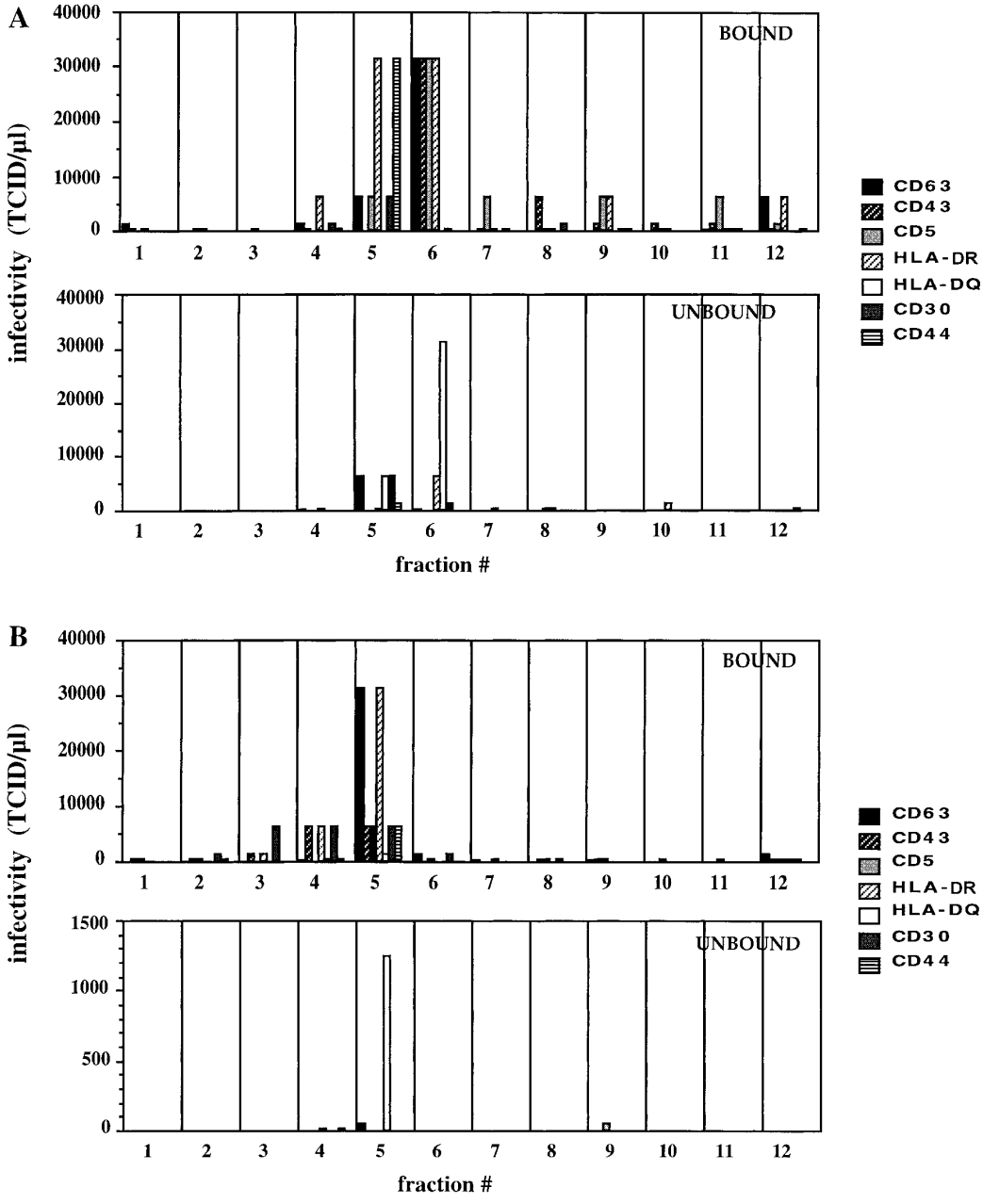

FIG. 5. The envelope of infectious viral particles shares some cellular antigens with the membrane of cellular vesicles. Infectious material present in fractions from density gradient-purified HIV from infected H9 cells (A) or activated PBMC (B) supernatants was captured by a panel of mAbs to various cellular antigens (as in Fig. 5) in a solid-phase capture system. The amount of infectious viral particles bound or unbound to the solid support was quantified (TCID/μl) by titration of the virus onto H9 cells and measurement of viral p24 protein in the infected cell supernatant.

infectious viral fractions, no obvious relationship between viral infectivity and the level of expression of these molecules at the cell surface (data not shown) could be found.

DISCUSSION

Here we show that in preparations of the HIV-1 molecular clone Hx10 enriched by sucrose gradient centrifugation from supernatants obtained from two different cell types, vesicles of cellular origin are a major contaminant. These vesicles contain a number of molecules of cellular origin, which are similar to, but not identical to, those found in the virus envelope. At present it is unclear why these cellular vesicles, many of which appear "empty" by electron microscopy, should have the same density as HIV-1 virus particles. One reason might be that the vesicles contain large amounts of protein and nucleic acid which are unstructured (Bess *et al.,* 1997) and thus are transparent by electron microscopy.

A large number of studies have used HIV antigen which has been "purified" by density gradient centrifugation. Early ELISA tests were based on lysed whole virus obtained from growth in tissue culture; false-positive results were obtained in a percentage of sera as a result of reactivity with HLA-DR antigen present in the viral lysate (Hunter and Menitove, 1985; Kuhnl *et al.,* 1985). It was assumed at the time that the contaminating HLA antigen was contained within the virus membrane (Henderson *et al.,* 1987), but it now seems likely that much of the contamination was from cellular vesicles cosedimenting with the virus (this study and (Bess *et al.,* 1997). Similarly, early vaccine trials were based on the use of inactivated material derived from gradient-enriched supernatants from SIV-infected human cells (Desrosiers *et al.,* 1989; Girard *et al.,* 1991; Murphey-Corb *et al.,* 1989). Other immunogens have taken the form of immunostimulating complexes (ISCOMS) (Morein *et al.,* 1978, 1984). When ISCOMS were prepared from material derived from HIV-1 grown in human cells, a major contaminant was found to be human HLA-DR antigen, and sera from animals immunized with these ISCOMS had high antibody titers against human HLA-DR (Henderson *et al.,* 1987). Although some of the antigenic material of cellular origin present in these vaccine preparations would have been contained within the virus envelope, much would probably have come from contaminating cellular vesicles.

Aside from leading to unwanted antigenicity and the prospect of false-positive results in early ELISA tests, the presence of anti-human HLA-DR and HLA class-I reactivity has been shown to correlate with protection of macaques against challenge with virus carrying these cellular molecules (Arthur *et al.,* 1995; Chan *et al.,* 1995). This provides an interesting possibility for investigating the mechanism of virus elimination in these animals and supposes the possibility of a vaccine based on immunization against alloantigens. However, in many situations, such as in studies of virus-specific immune responses and their correlation with protection from infection, cellular components present during immunization may be considered an undesirable contaminant and even potentially dangerous. Caution must therefore be exercised in terms of the presence of cellular vesicles when viral immunogens are density gradient enriched. Recently, immunogens for use in animal models have been prepared from virus grown in the cells of the animal to be immunized. This will avoid xenoreactivity, but alloreactivity against polymorphic antigens such as HLA molecules will remain a major immunogenic component.

It has been reported that human reticulocytes (Pan *et al.,* 1985) and B cell lines (Raposo *et al.,* 1996) secrete constitutively small vesicles called exosomes. Interestingly, the cellular vesicles produced by H9 cells and activated PBMC appear to be related to these B cell line-derived exosomes, as they share common features; a high concentration of HLA-DR molecules at their surface and an equilibrium density of about 1.13 g/ml (Raposo *et al.,* 1996). At present the role of this vesiculation in normal cell function is undefined, but may involve the elimination of excess membrane or membrane-associated proteins or, alternatively, a novel mechanism of antigen presentation (Raposo *et al.,* 1996).

It is unclear whether the production of cellular vesicles by the host cell has any significance for virus budding and release. The finding that many of the cellular molecules found in the virus are also present in the cellular vesicles may imply a common pathway. Indeed, the sites of the budding of virus and the apparent blebbing of nonviral vesicles appear to be colocalized at distinct polar regions of the cell surface (Fig. 3 and results not shown). Similar colocalization of cellular and viral antigens within regions of intense membrane vesiculation has been observed in cells infected with Sindbis virus and undergoing apoptosis (Rosen *et al.,* 1995). It is therefore interesting to speculate that HIV, and perhaps other viruses, may benefit from a normal cellular process in order to facilitate exit from the cell. Alternatively, it has been shown that membrane vesicles accumulate at sites of plasma membrane damage in a process thought to be involved in membrane repair (Miyake and McNeil, 1995). It may therefore be that HIV budding disrupts membrane integrity, resulting in the accumulation and polarized release of such vesicles. Studies concerning the role of plasma membrane vesiculation in HIV release from the cell are currently under way in this laboratory.

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