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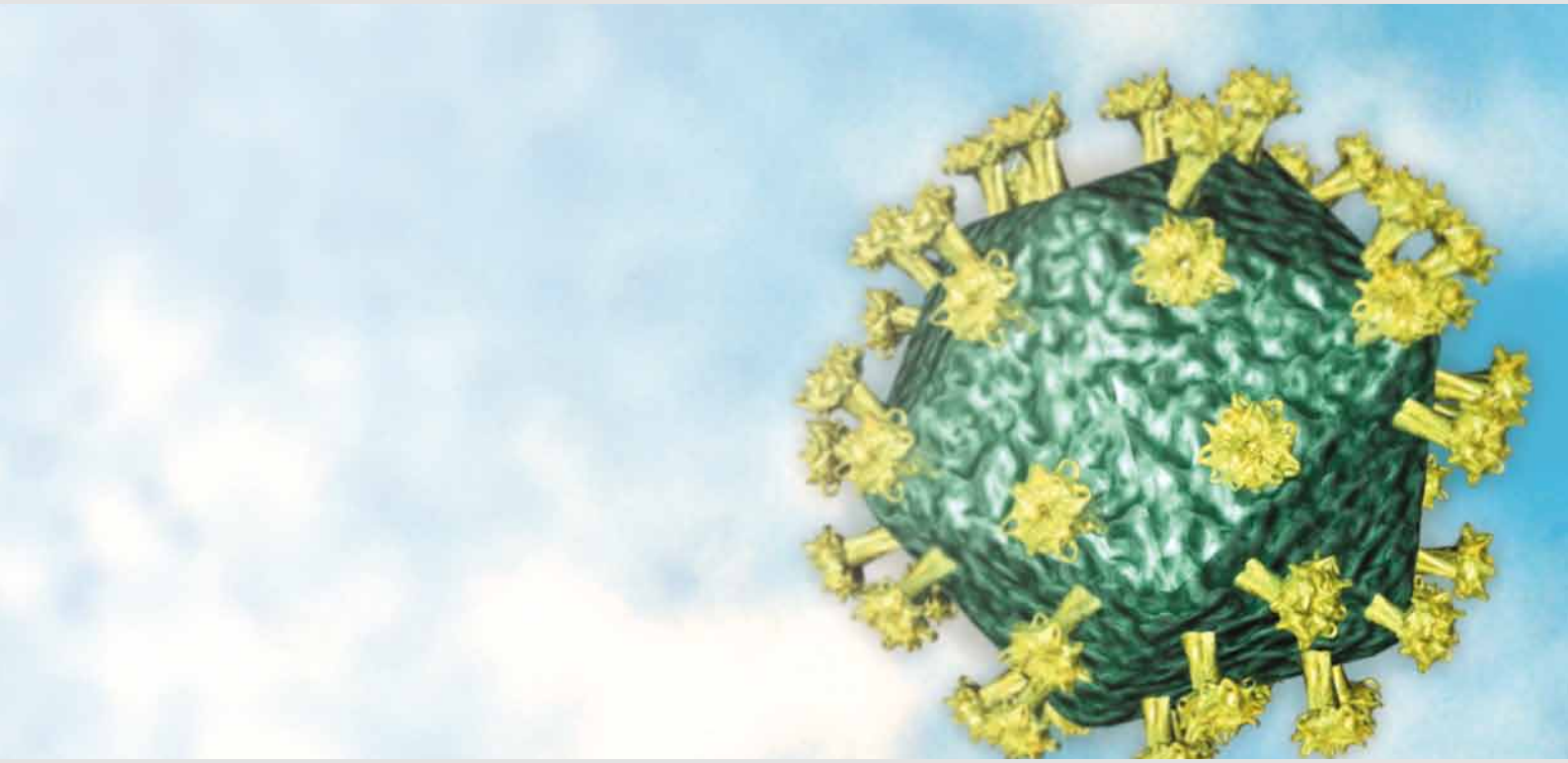

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When cells are infected by viruses and are forced to synthesize viral progeny, the budding particles take with them components of the host cell membrane. It is therefore possible to identify susceptible cell types by purification using appropriate markers. With MACS Technology, this can be achieved directly – without prior blood plasma processing.

Application of µMACS™ Streptavidin MicroBeads for the analysis of HIV-1 directly from patient plasma


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Introduction

It is established that host membrane proteins are incorporated into the retroviral envelope as HIV-1 virions bud from human cells (for a review see Tremblay *et al.*[1]). Direct or indirect mechanisms of host protein inclusion or exclusion from the retroviral envelope may be involved. This fact has permitted the ability to discriminate host cell types supporting viral replication by a targeted capture of virions directly from HIV-infected patient plasma via a previously described method[2–5]. However, this method required extensive processing of patient plasma to permit immunomagnetic capture of HIV-1 with high efficiency. In this report, we now describe marked improvements in the immunomagnetic capture protocol that permits analysis of virions directly from patient plasma without prior processing. These improvements not only allow direct analysis of patient plasma but they also open new approaches to the characterization of virions from HIV-infected individuals.

Materials and Methods

Preparation of samples.
Laboratory stocks of HIV-1 (strain Ba-L, subtype B), grown in either purified macrophages or $CD4^+$ T lymphocytes, were spiked into tissue culture medium or plasma at known viral particle numbers[2]. Either a commercial source of normal human plasma or a subtype B HIV-1-infected patient plasma with high anti-HIV antibody titers but undetectable viral load was used. Where indicated, HIV-1 spiked into specific antibody-containing plasma was processed by ultracentrifugation, salt dissociation, and spin column filtration, as described[2–5]. HIV-1-infected plasma samples with detectable viral loads were obtained from patients enrolled in a longitudinal TB therapy cohort under study in Kampala, Uganda, via collaboration with Dr. Zahra Toossi at Case Western Reserve University (Cleveland, Ohio). Appropriate informed consent was obtained by the Case Western Reserve Makerere Research Collaboration.

Immunomagnetic capture of HIV-1.
HIV-1 spiked into medium or plasma (processed or unprocessed) was first incubated with 0.5 µg of various antibodies (all obtained biotinylated from commercial sources) for 30 min at room temperature. To each sample, 20 µl µMACS™ Streptavidin MicroBeads (Miltenyi Biotec, Auburn, CA) were added and the binding reaction was incubated an additional 10 min at room temperature. Antibody-bound virus was then captured by magnetic separation with slight modifications from the manufacturer's protocols. Briefly, µ columns were placed in the µMACS™ magnetic separator attached to a MultiStand under a biological safety cabinet. Each column was prepared by prewetting with 100 µl protein equilibration buffer and rinsing twice with 100 µl PBS containing 2% fetal bovine serum (PBS/FBS). The entire volume of the virus capture reaction mixture (approximately 200 µl) was then applied to the column and allowed to drain completely. The columns were washed four times with 200 µl volumes of PBS/FBS. Fifty microliters of an appropriate lysis buffer (see below) were then added to the column, allowed to stand for 5 minutes at room temperature, and then followed by an additional 150 µl of the same buffer. All lysate elution fractions were collected as a pool. When capture efficiencies using MACS™ Technology were compared to the previous protocol using Dynal magnetic beads (Great Neck, NY), 2×10^7 streptavidin-conjugated Dynabeads (M-280) were used and the capture reactions were performed as previously described[2–5].

Analysis of captured virus.
For quantitation of captured HIV-1, two commercially available kits were used and assays were performed as directed. For the HIV-1 p24 antigen enzyme immunoassay (Coulter/Immunotech Inc., Westbrook, Maine), the lysis buffer consisted of PBS containing 0.5% NP-40. Whereas, for the more sensitive HIV-1 RNA quantitation assay, the lysis buffer was obtained from the commercial source of the NucliSens QT viral load assay (Organon Teknika/

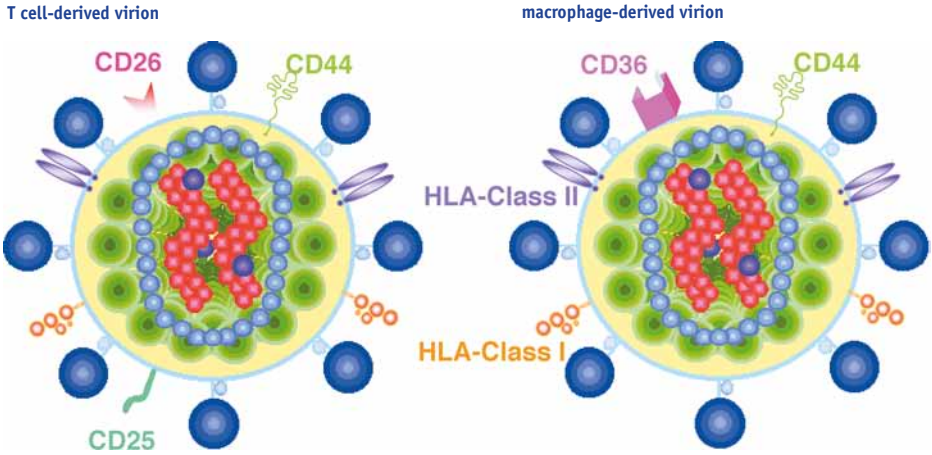

Figure 1 HIV-1 replication in different target cell populations results in selective incorporation of distinguishing host cell proteins. Virions derived from T cells (schematic on left) incorporate distinct T cell markers (i.e. CD26, CD25) while those derived from macrophages (schematic on right) acquire macrophage markers (i.e.. CD36). HIV-1 replication in either cell type allows for incorporation of dual markers, CD44 and HLA-DR. (modified from Lawn, S.D. (2004) J. Infect. 48: 1–12)

bioMérieux, Inc., Durham, NC). Viral RNA isolation and RT-PCR quantitation were performed using manufacturer's reagents and protocols. In some experiments, intact captured virus was recovered by removing the column from the magnetic separator and eluting with complete tissue culture medium. The infectivity of captured virus was then determined by direct inoculation onto purified $CD4^+$ T lymphocyte cultures that had been stimulated with PHA for 3 days. Cultures were fed with fresh media on day 7 and HIV-1 replication was monitored in cell-free culture supernatants by virion-associated, magnesium-dependent reverse transcriptase (RT) activity, as described[6].

Results and Discussion

Comparison of HIV-1 capture efficiency from processed and unprocessed plasma.

The ability to capture HIV-1 from plasma of infected persons has novel applications for understanding cellular compartments of viral replication (figure 1) and the impact of opportunistic infections. However, as we developed this technique[2–5], the need for extensive processing of plasma to overcome inhibition of capture by anti-HIV antibodies and serum reactive proteins was laborious, time-consuming, and damaging to the virus. Furthermore, plasma processing reduced the overall sensitivity of the technique due to aggregation or loss of virus. Because of their small size and behavior in suspension, we hoped that µMACS™ MicroBeads would show improved efficiency of virus capture directly from unprocessed plasma as compared to the much larger magnetic beads (Dynabeads) on which the original technique was developed.

Indeed, when the efficiency of capture with a positive control antibody (CD44) was determined for 10,000 HIV-1 particles spiked into plasma containing anti-HIV antibodies, the µMACS™ MicroBeads captured 100% of the input virions from unprocessed plasma while, as previously observed[2], capture by Dynabeads was markedly inhibited (figure 2). When plasma spiked with 10,000 HIV-1 particles was first processed, the input level of virions per capture fell (to approx. 4,000 copies) due to viral loss. However, the efficiency of capture by either µMACS™ MicroBeads or Dynabeads was similar, reaching about 50–60% of input (figure 2). The lack of complete capture by either system after plasma processing probably reflects virion aggregation, incomplete resuspension, or physical damage as a consequence of the ultracentrifugation. However, the use of µMACS™ Streptavidin MicroBeads permits the direct viral capture from plasma without the need for previous processing and therefore avoids this loss of input copy number. This also allows for the application to be transferred to patient material having lower viral loads, while saving the time and expense of plasma processing.

The efficiency of virus capture directly from the plasma was determined using the positive control marker (CD44) on samples from a HIV-1-infected cohort. As expected, the efficiency of virus capture varied among the 28 samples tested, with an average efficiency of 60% of input virus (figure 3). However, this variation was not dependent upon the patient viral load (ranging between 8×10^3 and 2×10^6 virion copies per ml in this sample set, figure 3) or the input amount of patient plasma (ranging between 100 and 150 µl in this study, data not shown).

Recognition of distinguishing host cell proteins incorporated in HIV-1.

To confirm that the use of µMACS™ MicroBeads with this technique can provide comparable results in regard to identifying host cell origins of viral replication (figure 1), HIV-1 stocks from the same strain (Ba-L) that were grown in either purified macrophage or $CD4^+$ T lymphocyte cultures were captured using a panel of antibodies against distinguishing host cell surface proteins. In accordance with the cellular

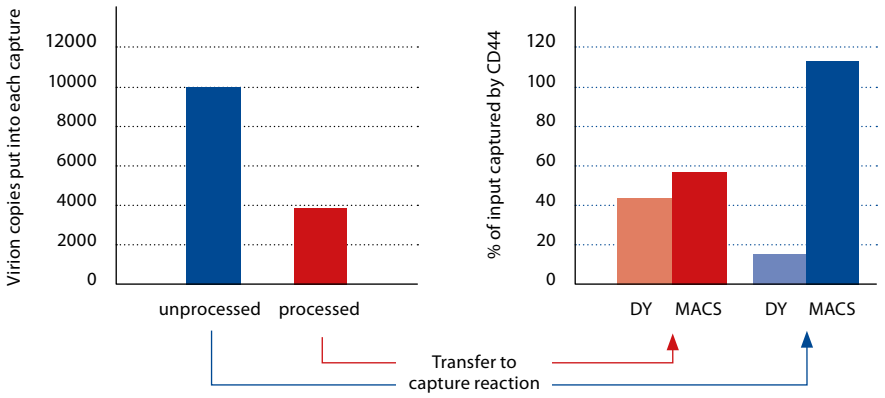

Figure 2 Efficiency of HIV-1 immunomagnetic capture when spiked into HIV antibody-positive plasma; with and without plasma processing. Ten thousand HIV-1 particles were spiked into human plasma with a high titer of anti-HIV antibodies and left unprocessed or taken through a plasma purification algorithm, as previously described[2]. These preparations were then subjected to the virus capture procedure using the universal marker CD44 and comparing efficiency of capture with Streptavidin-conjugated Dynal Dynabeads (Dy) and Miltenyi Biotec µMACS™ MicroBeads (MACS). Capture efficiency reflecting greater than 100% of input is due to the minor degree of quantitative variability inherent to the HIV-1 viral load (RT-PCR) assay.

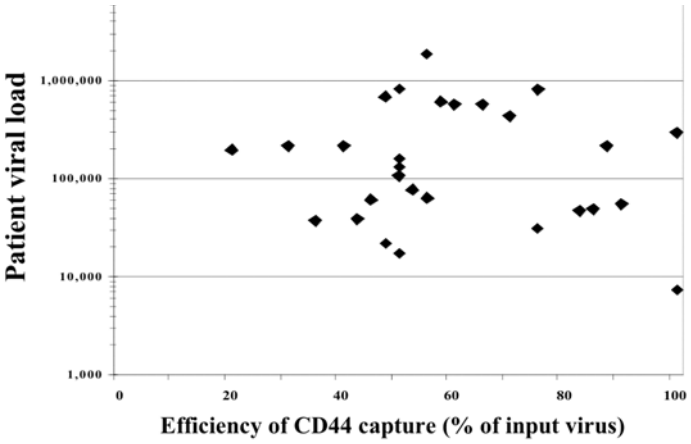

Figure 3 Efficiency of viral capture directly from HIV/TB-patient plasma. HIV-1-infected patient plasma (28 samples from 7 individuals over several time points) was used directly in the viral capture procedure. The efficiency of capture (percentage of virus input) by the universal marker CD44 was compared over the range of patient viral load determinations.

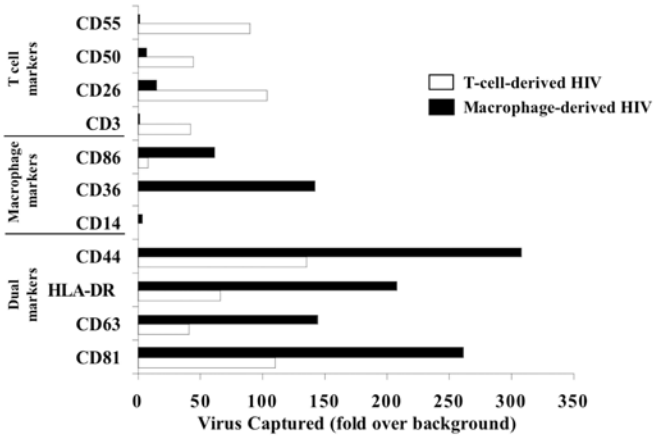

Figure 4 HIV-1 capture by antibodies targeted against cell surface markers selective for macrophages or CD4⁺ T lymphocytes. Antibodies against cell surface markers that had previously been shown to distinguish macrophage-derived and T cell-derived HIV-1, or present on both viral stocks (dual markers), were tested using µMACS beads in the capture procedure. Levels of captured virus were determined by HIV-1 p24 antigen immunoassay and compared to capture by a negative control antibody (CD19) to derive fold-over background values, as described[2].

origin of viral replication, HIV-1 stocks were selectively captured with host markers specific for the cell lineage (figure 4). These results are similar to those previously obtained using Dynabeads[2] except that the fold capture over background was substantially increased with µMACS™ MicroBeads due to low levels of nonspecific capture.

Analysis of captured virions for infectivity.
The previous method for HIV-1 capture directly from plasma included high salt treatment during processing to dissociate the outer viral envelope protein and any bound antibodies or other potential inhibitors of magnetic capture[2]. This treatment negated infectivity of captured virions. Furthermore, it seems unlikely that HIV-1 attached to a significantly larger magnetic bead could efficiently bind its co-receptors and fuse with a target cell. Since the processing steps were circumvented with the use of the much smaller and biodegradable µMACS™ Streptavidin MicroBeads, we directly examined the issue of infectivity of MicroBead-labeled virions. Known copy numbers of HIV-1 were spiked into and subsequently captured from normal human plasma and plasma containing anti-HIV antibodies. As previously observed (figure 2), using the positive control host cell marker (CD44) the efficiency of viral capture by µMACS™ MicroBeads from both plasma preparations approached 100% of input (data not shown).

Captured virions were eluted from the separation column and placed directly onto activated CD4⁺ T lymphocyte cultures. Over the course of the following two weeks, HIV-1 replication was observed in these cultures with as few as 2.4×10^5 virions spiked into plasma prior to capture (figure 5). Therefore, this application could be used to derive viral isolates from plasma or other biologic fluids, especially when cryopreserved patient peripheral blood cells are not available. Lower numbers of spiked virions also gave rise to positive infection cultures, but the successful outcome was variable (data not shown). Virions captured from anti-HIV antibody-containing plasma showed a delayed rise of viral replication but were not completely neutralized during the acute infection (figure 5).

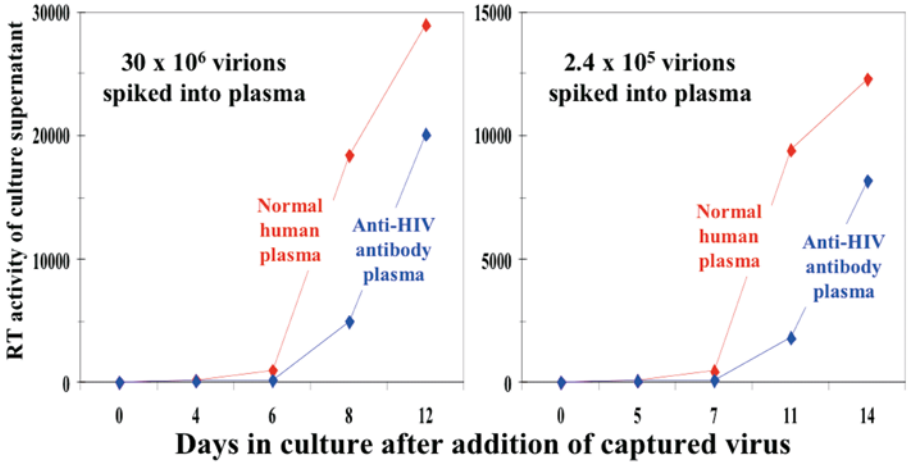

Figure 5 **Acute infection of CD4⁺ T lymphocytes with HIV-1_{Ba-L} captured (with anti-CD44) from spiked plasma.** Virions were spiked into plasma at the numbers indicated, captured by CD44 antibody recognition, eluted without lysis, and cultured overnight with activated CD4⁺ T lymphocytes. Cultures were continued for two weeks to determine extent of HIV-1 replication.

Conclusions and future applications

Study of HIV-1 directly from patient plasma or other biologic fluids can provide novel insight into cellular compartments supporting viral replication and aspects of viral pathogenesis. The improved ability to apply viral capture technology without extensive sample processing, due to the unique properties of µMACS™ MicroBeads, can advance this technique into new applications. By eliminating the need to remove inhibitors (antibodies, acute serum proteins etc.) from biologic fluids by processing, this will allow the approach described here to be utilized for bodily fluids for which extensive processing would not be feasible (breast milk, semen, etc.). A dual labeling technique could also be feasible using magnetic beads from different commercial sources. Furthermore, to study antibody neutralization, this technique could be modified to culture captured HIV-1 in the presence of increasing concentrations of autologous or heterologous plasma.

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