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Advances in methods for the production, purification, and characterization of HIV-1 Gag–Env pseudovirion vaccines

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Abstract

HIV pseudovirion or virus-like particle vaccines represent a promising approach for eliciting humoral and cellular immune responses. Pseudovirions present the envelope glycoprotein complex in its authentic trimeric form, and thus have the potential to generate neutralizing antibodies against relevant virion-associated epitopes that may be lacking in protein subunit vaccines. The development of pseudovirion particles as a viable vaccine approach for progression to clinical testing has been limited by a number of factors, including shedding of particle-associated gp120, practical limitations to large-scale production and purification, and the generation of antibodies against cellular proteins incorporated on the particle surface that confound the analysis of HIV-specific neutralizing antibody responses. Here, we review methods that address each of these challenges, with a focus on production methods for generating non-infectious Gag–Env pseudovirions. Mammalian cell lines that inducibly express HIV Gag and Env can overcome production limitations, and produce pseudovirions that retain gp120 following purification. Baculovirus production systems have the potential to provide higher quantities of particles, but cleavage of gp160 remains a current limitation. Anti-cellular antibody responses can be diminished by adsorption with cell lysates or whole cells. These technical advances should facilitate the further development of pseudovirion vaccine approaches in preclinical testing and future clinical trials.

Keywords: Pseudovirion; VLP; HIV vaccine

1. Introduction

The HIV/AIDS pandemic continues to expand, despite more than twenty-five years of research, education, and prevention efforts. The best hope for slowing or halting the spread of AIDS is through an effective preventive vaccine. A number of promising vaccine approaches have advanced to clinical trials, led at present by live vector-based regimens designed to generate cellular immune responses against the virus. Other vaccine attempts have failed, most prominently the use of subunit gp120 protein-based vaccines. Gp120 subunit vaccines were tested extensively in phase I and II trials and eventually in two phase III efficacy studies, where they were definitively proven ineffective in protecting against HIV infection [1]. New approaches are needed, in particular approaches that have the potential to generate meaningful neutralizing antibody responses against primary HIV-1 isolates.

Pseudovirions or virus-like particle (VLP) vaccines have a number of theoretical advantages for generating HIV-specific immune responses. Particulate antigens can be taken up by antigen presenting cells and processed by the class II presentation pathway. Particulate antigens can also be taken up into the alternative class I presentation pathway, where they are processed by the proteasome and presented by MHC class I molecules [2]. The envelope glycoprotein trimer is presented in its native state on the pseudovirion membrane, allowing B-cell recognition of epitopes that are normally

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presented on the surface of infectious virions. Presentation in this manner should minimize the generation of antibody responses directed against regions of the glycoprotein that are irrelevant to neutralization of virion particles. The concept that a VLP vaccine can be successfully engineered, produced, and advanced through human efficacy trial testing has been proven with the recent licensure of the papillomavirus VLP vaccine (Gardasil, Merck Pharmaceuticals) [3]. Enveloped HIV particles are likely to raise unique challenges, but the papillomavirus VLP success nevertheless reinforces the promise of particle-based vaccine approaches.

HIV pseudovirion immunization has been shown to generate promising cell-mediated immune responses in animal models. $Pr55^{Gag}$-based VLPs have elicited strong cytotoxic T lymphocyte (CTL) responses in BALB/c mice [4]. Intramuscular administration of unadjuvanted $Pr55^{Gag}$ pseudovirions in macaques resulted in long-lived cytotoxic T lymphocyte (CTL) responses directed against multiple epitopes [5–7]. Various strategies to include envelope glycoproteins in pseudovirions have revealed that Env-specific antibodies are raised by Gag–Env pseudovirions [5]. Some of these strategies have elicited strain-specific neutralizing antibodies, and our group recently demonstrated that breadth of neutralization is enhanced when using Gag–Env pseudovirions as compared with subunit protein of the same isolate [8]. Protection in macaque challenge models has not been clearly established. Accelerated clearance of SHIV in rhesus macaques immunized with SIV/HIV pseudovirions was reported, and correlated with an anamnestic response to gp120 [9]. However, poxvirus priming followed by pseudovirion boosting did not protect against pathogenic SIV challenge [10], and immune responses raised by VLPs alone failed to protect macaques from SHIV infection [11,12]. The potential of pseudovirions to raise desirable immune responses thus appears high, while definitive proof of protection in animal models is not yet established.

Given the promising potential of HIV pseudovirions, it seems remarkable that no HIV pseudovirion approaches have advanced beyond early preclinical testing. It is likely that the complexities of pseudovirion production, envelope protein incorporation and stability, and purification have limited enthusiasm from vaccine manufacturers. A Vero cell-produced pseudovirion preparation designed for human testing was pursued for a time by Aventis Pasteur [13]. This product was produced but never administered to humans, and the program was subsequently abandoned, at least in part due to complexities involved in the manufacturing process. Therefore, advances are needed in HIV pseudovirion design and in the methods used to produce and purify the product. Here we evaluate and compare production methods for pseudovirions, and describe efforts to enhance the efficiency of production and purification from mammalian cell lines. We also describe the generation of humoral responses against cellular proteins incorporated into the envelope of the particle that can complicate quantitation of HIV-specific neutralizing antibodies, and a method to adsorb away these anti-cellular antibodies. The discussion here will be limited to the use of purified Gag–Env particles as an exogenously administered immunogen, and does not deal with live vectors or other strategies that may result in particle production in host cells.

2. Materials and methods

2.1. Plasmids and transient transfection methods

Gag–Env pseudovirions were produced using a codon-optimized *gag* gene representing the HXB2 isolate in plasmid pVRC3900 [14] and a codon-optimized env gene encoding gp160 of the BaL isolate cloned into pcDNA3.1 as previously described [5]. The same *gag* and *env* genes were used to generate stable, inducible cell lines and recombinant baculoviruses. The tetracycline-responsive HIV-1 pseudovirion expression plasmid pRevTREGagEnv was produced by subcloning the Gag sequence from pVRC3900 and the BaL gp160 sequence from pcDNA3.1 (Zeo)-BaLgp160opt into pRevTRE (BD Clontech, Palo Alto, CA). The use of this vector to create one of these cell lines, XC-18, has previously been described [5]. Additional cell lines were created using the TRex expression system (Invitrogen, Carlsbad, CA). pcDNA4/TO-Gag was created by inserting the HindIII-BamH1 fragment encoding the gag gene from pVRC3900 into pcDNA4/TO. pcDNA5/TO-EnvBaL was created by inserting the env gene from pcDNA3.1 (Zeo)-BaLgp160opt as a HindIII-Xba1 fragment into pcDNA5/TO. The *Spodoptera frugiperda* insect cell line Sf9 was used to generate recombinant baculoviruses. SF9 cells were maintained in TNM-FH media (Modified Grace's Medium; Sigma–Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum and cultured in a 27 °C incubator under room air. AcNPV-derived baculoviruses were generated using the BaculoGold™ Starter Package (BD Pharmingen, San Diego, CA) according to manufacturer's instructions. pAcGag was created by non-directional PCR cloning of the codon-optimized *gag* gene from pVRC3900 into the BamH1 site of pAcUW51 (BD Pharmingen, San Diego, CA). pAcEnvBaL was created by inserting the BamH1–EcoR1 fragment encoding the codon-optimized *env* gene from pBABE/Env into the BglII–EcoR1 sites of pAcUW51. The pAcGag and pAcEnvBaL baculovirus transfer vectors were cotransfected into Sf9 cells together with linearized BaculoGold DNA to generate recombinant baculoviruses expressing the structural HIV-1 Gag and Env proteins. Recombinant baculoviruses were plaque-purified using standard methods and large-scale virus preparations were generated by infecting 4×10^6 Sf9 cells per 10 cm^2 culture dish at an MOI of 0.1. Baculovirus-containing supernatants were harvested 96 h post-infection, clarified by low-speed centrifugation, filtered through a 0.45 μm filter, and then purified by ultracentrifugation through a 5% sucrose cushion (40,000 × *g* for 1 h, 4 °C). Baculovirus par-

ticles were subsequently resuspended in PBS and stored at −80 °C.

2.2. Stable mammalian cell line production of pseudovirions

Generation of the XC-18 cell line has been previously described [15]. Briefly, the XC-18 cell line was generated by retroviral transduction of a 293 Tet-On cell line with the tetracycline-inducible pRevTREGagEnv pseudovirion expression construct previously described. Transduced 293 Tet-On cells were maintained in DMEM (Dulbecco's modified Eagle medium, high glucose) supplemented with 10% tetracycline-free, heat-inactivated fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin/streptomycin, 100 μg/ml G418, 100 μg/ml hygromycin, and 0.5 μg/ml puromycin. The XC-18 clone was selected for high levels of p24 and gp120 production in the cellular supernatant. Complete proteolytic processing of BaL gp160 glycoprotein into gp120 (SU) and gp41 (TM) subunits was another selection criteria. Pseudovirion production was induced by the addition of doxycycline at a concentration of 2 μg/ml to the XC-18 cell culture followed by supernatant harvests on day 4 and day 8 post induction. XC-18 supernatants were clarified by low-speed centrifugation, filtered through a 0.45 μm filter, and purified using several methods described below.

2.3. Baculovirus production methods

The recombinant baculoviruses, pAcGag and pAcEnvBaL were used to generate HIV-1Pr55^{Gag}/BaL^{Env} pseudovirions. Sf9 cells were plated into 10 cm^2 culture dishes at a density of 6.0×10^6 cells per dish. After a 1 h attachment phase, the cells were infected with the pAcGag and pAcEnvBaL viruses at an MOI of 1 for each virus in a 3 ml volume of TNM-FH (Invitrogen) media. After 4 h the virus containing media was removed and the cells were washed with fresh room temperature TNM-FH media. Following the wash procedures, 10 ml of fresh TNM-FH media was added to the infected cell population. Sf9 supernatants were harvested at 24 h periods following infection through day 7 and clarified by low-speed centrifugation and filtered through a 0.45 μm filter. The p24 and BaL gp120 content of the cellular supernatant was determined using a p24 and gp120 capture ELISA described below.

2.4. Pseudovirion analysis on sucrose gradients

HIV-1 pseudovirions were analyzed by centrifugation on linear 20–60% sucrose gradients. Immature Gag–Env pseudovirions were produced by induction of the XC-18 cell line with 2 μg/ml of doxycycline in 10 cm^2 dishes. Typically, three 10 cm^2 dishes of XC-18 cells were induced at 50–60% confluence and pseudovirion preparations were metabolically labeled overnight using 75 μCi ml^{-1} [^{35}S]cysteine/methionine applied in cysteine and methionine-deficient DMEM. Labeled pseudovirions were harvested after 24 h, clarified by low-speed centrifugation, filtered through a 0.45 μm filter, and then purified by ultracentrifugation through a 20% sucrose cushion (100,000 × *g* for 3 h, 4 °C). Pseudovirion pellets were then resuspended in 1 ml of PBS, and overlaid on linear 20–60% sucrose gradients. Ultracentrifugation was performed at 100,000 × *g* overnight at 4 °C in a Beckman SW41 rotor. Equal fractions were collected and the density of each fraction determined using a refractometer. Samples were subsequently diluted in PBS and disrupted using RIPA buffer (1% NP-40, 0.1% SDS in PBS) and immunoprecipitated using HIV positive patients' sera. Analysis was performed by SDS-PAGE and autoradiography.

2.5. Determination of Env concentration using a gp120 capture ELISA

The gp120 concentration of HIV-1 pseudovirions was measured by a capture ELISA. The anti-gp120 capture antibody, D7324 (Cliniqa, Fallbrook, CA), was coated onto 96-well plates at a concentration of 2 μg ml^{-1} in PBS overnight at 37 °C. The plates were blocked with 3% milk protein in PBS for 1 h at 37 °C. Pseudovirions were diluted in Env sample diluent (5% milk protein, 0.5% Triton X-100 in PBS) and incubated on D7324-coated plates for 2 h at 37 °C. The amount of captured gp120 was determined using pooled HIV positive patients' sera at a dilution of 1:6000 in Env sample diluent for 1 h at 37 °C. Detection was performed using a horseradish peroxidase-conjugated goat anti-human IgG (H + L) (Pierce, Rockford, IL) at a dilution of 1:6000 in Env sample diluent for 1 h at 37 °C. Colorimetric analysis utilized the Immunopure TMB Substrate Kit (Pierce, Rockford, IL) and absorbance was read at 450 nm with a reference at 650 nm. Recombinant BaL gp120, obtained from the μQUANT Core Facility (Institute of Human Virology, Baltimore, MD) was used for the standard curve. The linear detection range of this assay is less than 30 pg of gp120.

2.6. Determination of p24 concentration by ELISA

The p24 content of HIV-1 pseudovirion preparations was performed using a p24 antigen capture ELISA. The murine anti-p24 capture antibody 183-H12-5C (CA183) was obtained from Bruce Chesebro and Kathy Wehrly through the NIH AIDS Research and Reference Reagent Program. The capture antibody was coated onto 96-well plates at a dilution of 1:2000 in PBS and incubated overnight at 37 °C. Plates were blocked for 1 h at 37 °C with 5% fetal calf serum in PBS. Immature pseudovirions were incubated in 0.1% SDS solution for 5 min at 100 °C and subsequently diluted in p24 sample diluent (10% fetal calf serum, 0.5% Triton X-100 in PBS) and incubated on CA183-coated plates for 2 h at 37 °C. The detection of bound p24 was determined using HIV-Ig, obtained from NABI through the NIH AIDS Research and Reference Reagent Program, at a dilution of 1:20,000 for 1 h

at 37 °C. Colorimetric analysis was performed using TMB substrate and absorbance was read at 450 nm. Recombinant p24 was used for the standard curve and sensitive to less than 20 pg of p24.

2.7. Transmission electron microscopy

Immature Gag–Env pseudovirions were produced and purified as described above. Sucrose-purified pseudovirion pellets were fixed in 2% gluteraldehyde in phosphate buffer, post-fixed with 1% osmium tetroxide, stained with 1% uranyl acetate, dehydrated in ethanol, and embedded in Spurr resin. Thin sections were cut with an ultramicrotome and analyzed with a Philips model 3000 electron microscope.

2.8. Production of Gag–Env pseudovirion particles using a BelloCell Bioreactor System

BelloCell -500 disposable bioreactor bottles (Cesco Bioengineering, Hsin-Chu, Taiwan) were used to cultivate the XC-18 cell line and produce Gag–Env pseudovirions. A BelloStage 3000 (Cesco Bioengineering, Hsin-Chu, Taiwan) control console was used to regulate the up/down rate of compression and expansion of the bioreactor bottles. The control console was housed in a humidified 5% CO_2 incubator and regulates both the time at the bottom position and top position, referred to as bottom hold (BH) and top hold (TH) respectively. For operational use, a BelloCell -500 bottle was filled with 470 ml of DMEM supplemented with 10% tetracycline-free, heat-inactivated fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin-streptomycin (Tet- DMEM-GM). The bottle was then seeded with 4.0×10^8 XC-18 cells in 30 ml of Tet-DMEM-GM. The inoculation phase was maintained for 3–5 h to allow for cellular attachment to the BioNOC II matrix. For the inoculation period, the BelloStage was set at an up/down speed of 1.5 mm s^{-1} and a BH of 0 min and a TH of 1 min. During the culture phase, the up/down speed was set to 1.0 mm/s with a bottom hold (BH) of 1 min and a top hold (TH) of 5 min. Both the cell number and media glucose levels were monitored and when the glucose concentration fell below 1.0 g/L the culture was replenished with fresh Tet-DMEM-GM. The cell culture media was replaced every two days starting on the third day after inoculation. At day 5 post-inoculation the cell culture was supplemented with 2 μg/ml of doxycycline to induce pseudovirion production. Starting with day 7 post-inoculation the harvested culture supernatants were clarified by low-speed centrifugation, filtered through a 0.45 μm filter, and stored at −80 °C. An aliquot of culture supernatant from each harvest was used to measure p24 and gp120 antigen production by the capture ELISA methods described above.

2.9. Purification of pseudovirions by cross-flow filtration

Pseudovirion-enriched cell culture supernatants were clarified by low-speed centrifugation, filtered through a 0.45 μm filter, and then purified using a QuixStand (GE Healthcare, Piscataway, NJ) benchtop cross-flow filtration system according to the manufacturer's recommended operating procedures. The QuixStand system utilizes hollow fiber cartridges to both concentrate material as well as to remove contaminants from the solution. In order to efficiently concentrate HIV-1 pseudovirions a UFP-300-C-4X2MA hollow fiber cartridge with a 300 kDa pore size was selected. Briefly, phosphate-buffered saline (PBS) chilled to 4 °C was loaded into the feed reservoir to equilibrate the system to physiological conditions. Cell culture supernatant containing pseudovirion particles were subsequently loaded into the feed reservoir. The recirculation rate was set to 140 ml/s in order to preserve the integrity of shear sensitive pseudovirion particles. The solution was concentrated by a factor of 40 over 2–3 h of operation. The concentrated retentate was harvested from the feed reservoir and 75 ml of pre-chilled PBS was added to remove residual pseudovirion particles. The retentate was then purified by ultracentrifugation through a 20% sucrose cushion (100,000 × *g* for 3 h, 4 °C). Pseudovirion pellets were resuspended in 1 ml of PBS and stored at −80 °C.

2.10. Evaluation and adsorption of anti-cellular antibodies

HeLa and 293 cells were maintained in DMEM-GM in a humidified, 5% CO_2 incubator. HeLa cell monolayers were grown in T150 cm^2 culture flasks until confluent. After washing twice with PBS, the cells were detached from the flask by rolling 5 mm sterile glass beads over the monolayer. Pooled cell suspensions were centrifuged at 400 × *g* for 10 min and resuspended in PBS. The lysates were divided into 1 ml aliquots containing 1×10^8 cells and subjected to three cycles of freeze-thaw using an ethanol-dry ice bath. The cells were centrifuged for 10 min at 1500 × *g* and pellets were washed twice. The packed cell membranes were stored at −85 °C. Two hundred and ninety-three cells were harvested by scraping off the cells from confluent T150 cm^2 tissue culture flasks. Two hundred and ninety-three pellets containing 2×10^8 cells were washed twice with PBS and resuspended in 0.5 ml of ice-cold PBS containing protease inhibitors (Complete Mini EDTA free; Roche Diagnostics, Indianapolis, IN). After brief sonication in an ice bath (15 s at 50% cycle, the cell lysates were clarified by centrifugation (10,000 × *g*, 5 min) and the supernatant stored at −85 °C.

For optimal removal of anti-cell specific antibodies from pseudovirion immunized antisera, a procedure combining adsorption with packed cells and blocking with cell lysate was employed. Packed HeLa cells (1×10^8) were resuspended in 100 μl of PBS containing protease inhibitors and added to 100 μl of heat-inactivated hyper-immunized guinea pig serum. Following incubation with rotation at 37 °C for 1 h, the serum was recovered by low speed centrifugation. The cell pellet was washed once with 100 μl of PBS and the recovered fluid was added to the serum. For the second round, 1×10^8 packed HeLa cells were thawed, mixed gently with the serum

and 100 μl of 293 cell lysate was then added to the serum-cell suspension. The second incubation step was performed at 4 °C overnight with rotation. The serum was recovered by low speed centrifugation and was washed once with 100 μl of PBS. The PBS was added to the recovered serum and the process resulted in a six fold dilution factor of the original serum sample.

An accurate and sensitive assay for the measurement of cell-reactive antibodies in live cells was developed based on absence of auto-fluorescence in the infrared spectrum. Serum samples were diluted in 2-fold series, starting at 1:50 with DMEM-GM. TZM-bl cells were grown to confluence in 96-well tissue culture plates. After gentle aspiration of the media, 50 μl of the diluted serum samples were transferred to the live cell monolayer in duplicate wells. The plate was incubated at 37 °C for 1 h in a 5% CO_2 incubator. The samples were aspirated and the cell monolayers were washed three times with 150 μl of PBS supplemented with 1% FCS. The cell-reactive antibodies were probed with 50 μl of anti-guinea pig IgG labeled with infrared dye 800W (Rockland Immunochemicals, Gilbertsvile, PA). After a 30 min incubation in humidified CO_2, the wells were washed three times as previously described. The plate was scanned using the Odyssey Infra-Red Imaging System (Li-Cor Biosciences, Lincoln, NE) and analyzed with the In-Cell Western software module.

For adsorption of sera with whole cells, 293 cells grown to dense monolayers were dispersed with non-enzymatic cell dissociation solution (Mediatech, Herndon, VA), washed and packed by low speed centrifugation (180 × *g*, 10 min). An equal volume of serum sample was added and mixed with the cells by end-over-end rotation at room temperature in 1.5 ml Eppendorf tubes. The serum was then recovered by pelleting the cells. Three cycles of adsorption were performed, the first for 4 h, the second overnight, and the third cycle for 4 h. The treated serum sample was finally diluted to twice the starting volume and kept at 4 °C until used for testing.

2.11. Measurement of neutralizing antibody titers

Guinea pig sera from a previous study was utilized in the adsorption studies. Methods for the luciferase-based HIV neutralization assay were performed as in the previous study [5].

3. Results

3.1. Pseudovirion production by transient transfection of mammalian cells

The focus of this investigation was to determine the relative efficiency of several HIV-1 pseudovirion production methods. We sought to develop methods that could produce pseudovirions of uniform consistency and develop systems that could be adapted to large-scale production. In our laboratory, we have used HIV-1 Gag–Env pseudovirions for immunogenicity studies and are currently evaluating their ability to elicit antiserum with broad neutralization activity against primary HIV-1 isolates.

Table 1
Gag:Env ratio of HIV-1 pseudovirions

Production method	Gag:Env ratio[a]
293T cells	25–50:1
XC-18, XC-34	28–32:1
Baculovirus	10:1
C.F. retentate	28:1

[a] Gag:Env molar ratio of sucrose purified pseudovirions was determined using a p24 and gp120 capture ELISA. Shown are values following purification through a 20% sucrose cushion.

One common method used to generate HIV-1 pseudovirions is transient transfection of 293T cells. We evaluated this method directly in comparison with production from stable cell lines or recombinant baculoviruses. 293T cells were grown to 60–70% confluency in 10 cm^2 culture dishes and transfected with expression plasmids encoding codon-optimized versions of *gag* and *env* using Lipofectamine 2000 (Invitrogen). The release of Gag and Env into the cellular supernatant following transient transfection of 293T cells is shown in Fig. 1A. Gag and Env were efficiently released into the supernatant at 24 h post-transfection, achieving a level greater than 1 μg/ml of capsid (p24) protein. The secretion of Env glycoprotein into the supernatant was delayed compared with Gag polyprotein, peaked at 36 h post-transfection at a level of 340 ng/ml of gp120, and was reduced thereafter due to cytotoxicity. Variation in the Gag:Env ratio (Table 1) of sucrose purified pseudovirions generated by transient transfection produced an inconsistent product. A further limitation in applying the transient transfection method to generate pseudovirions was the inability to efficiently scale up the production process. We therefore generated stable, inducible mammalian cell lines to overcome problems with lot-to-lot variation and to facilitate product scale-up.

3.2. Pseudovirion production from stable, inducible mammalian cell lines

The need to produce pseudovirions of consistent quality and in large amounts for immunization studies led to the development of stable Gag–Env cell lines. Overexpression of the HIV-1 structural genes produced high levels of cytotoxicity in human cell lines when production was constitutive (data not shown). Therefore, we generated cell lines transduced with codon-optimized *gag* and *env* cassettes controlled by a tetracycline-regulated promoter in 293 cells (293 Tet-On; Clontech). The XC-18 cell line was developed in order to generate Gag–Env pseudovirions. The inducible production of Gag and Env antigens by XC-18 is shown in Fig. 1B. This figure demonstrates that while Gag production was strictly regulated, Env was not. Upon induction, Gag release into the cellular supernatant reached levels greater

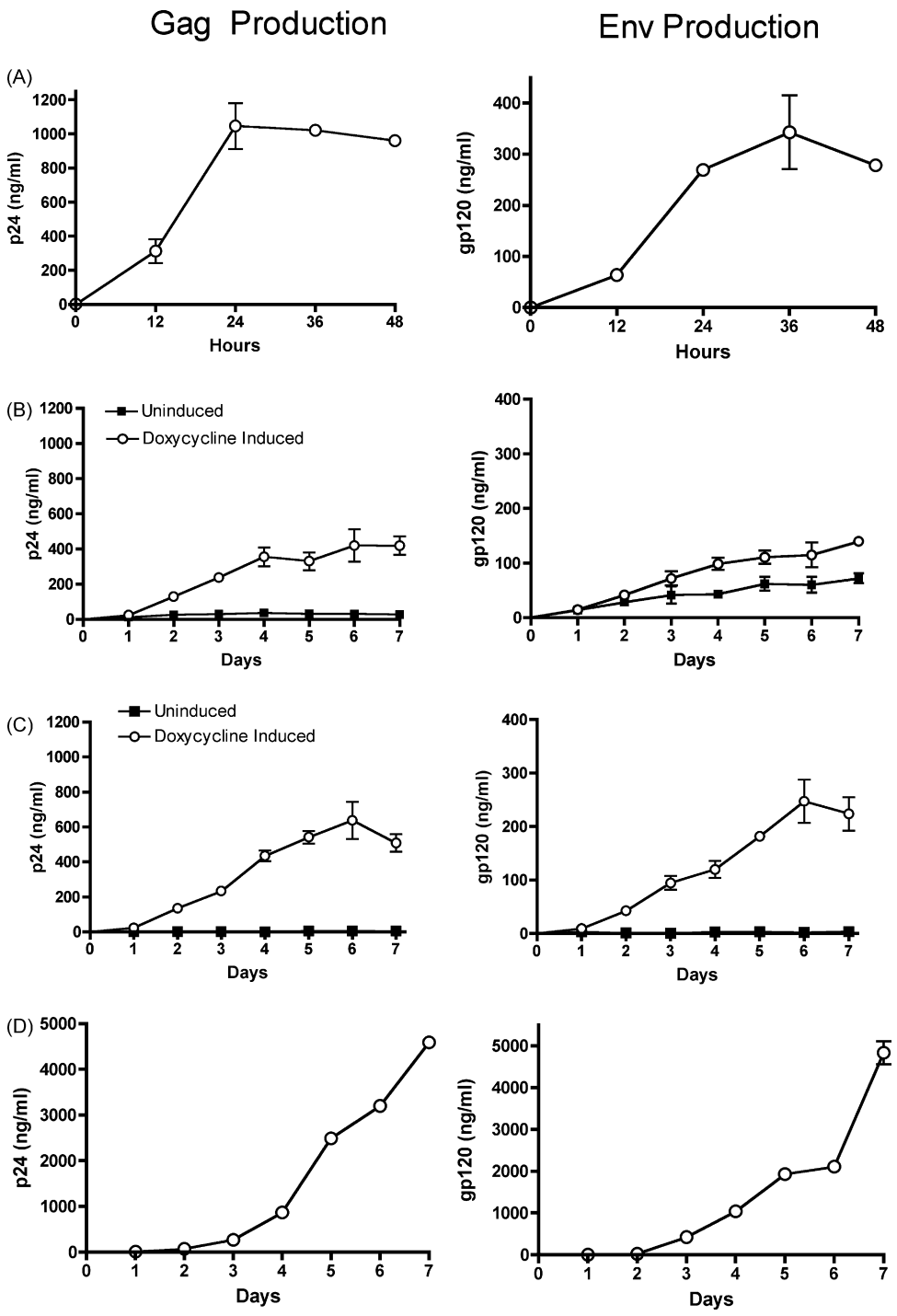

Fig. 1. Quantitative comparison of pseudovirion production methods. (A) Transient transfection of 293T cells. (B) Stable, inducible Gag–Env particle production from XC-18 mammalian cell line. (C) Stable, inducible Gag–Env particle production from XC-34 cell line. (D) Production from SF9 cells infected with recombinant Gag and Env baculoviruses.

than 450 ng/ml of p24. Similarly, the secretion of Env into the supernatant was efficient and obtained levels exceeding 100 ng/ml of gp120. Gag–Env pseudovirions produced by XC-18 exhibited a stable Gag:Env ratio (Table 1), allowing the generation of a pseudovirion immunogen of uniform consistency. Another tetracycline-inducible 293 Tet-On cell line, XC-34, was produced using the TRex retroviral transduction system (Invitrogen). The release of Gag and Env proteins by XC-34 is displayed in Fig. 1C. Two characteristics of the XC-34 cell line made it superior as compared with XC-18. Induction of XC-34 resulted in a 50% increase in Gag release and a 100% increase in Env secretion in comparison to XC-18. Additionally both Gag and Env antigen production were tightly controlled in XC-34 (Fig. 1C), resulting in an increase

in cell viability over time due to the absence of Env-dependent cytotoxicity. We note that pseudovirons from XC-18 have been utilized in our previous immunization studies, while the XC-34 cell line has thus far been less well characterized, so that many of the studies below will utilize particles from XC-18. Together, these results suggested that the development of inducible mammalian cell lines optimized for Gag–Env pseudovirion production was feasible, and thus these methods were moved to larger-scale production and purification as described later in this report.

3.3. Baculovirus production of Gag–Env pseudovirions

Baculovirus production methods have been shown to efficiently generate virus-like particles [16]. We compared baculovirus production methods to our mammalian cell production system, using the identical *gag* and *env* gene constructs. Recombinant baculoviruses expressing Gag (pAcGag) and Env (pAcEnvBaL) were used to produce HIV-1 Gag–Env pseudovirions. Sf9 cells were infected with Gag and Env expressing recombinant baculoviruses at an MOI of 1.0. The release of Gag and Env antigens into the Sf9 supernatant following infection is shown in Fig. 1D. The amount of Gag and Env release into the supernatant from infected Sf9 cells was on average one log greater than that generated from either transient transfection (Fig. 1A) or stably transduced cell line production systems (Fig. 1B and C). The release of Gag and Env from Sf9 cells in to the supernatant on day 7 post-infection reached levels greater than 4.5 mg/ml. Thus, if the quality of these particles is equal to that of mammalian cell production, the enhanced yield would make this a very desirable means of producing HIV pseudovirions (further discussion below).

3.4. Assessment of pseudovirion quality by density gradient analysis and electron microscopy

HIV-1 pseudovirions were analyzed by equilibrium density ultracentrifugation in order to qualitatively assess their structural characteristics and integrity. Immature pseudovirions generated by transient transfection of 293T cells generated particles with a peak density of 1.15–1.17 g/ml corresponding to the accepted HIV-1 retroviral particle density (Fig. 2A). Incomplete processing of the envelope glycoprotein was evident by the presence of gp160 in the peak particle fractions generated using transient transfection methods (Fig. 2A, top band). We were unable to produce Gag–Env pseudovirions by 293T transfection that generated completely cleaved Env, raising a potential additional limitation to this technique. In contrast, pseudovirions generated using XC-18 demonstrate complete processing of their envelope glycoprotein at the peak particle fractions of 1.15–1.17 g/ml (Fig. 2B). XC-18 particles separated on linear 20–60% sucrose gradients display a significant amount of shed gp120 in the low density fractions (Fig. 2B). Note that these particles were first sedimented through a 20% sucrose cushion,

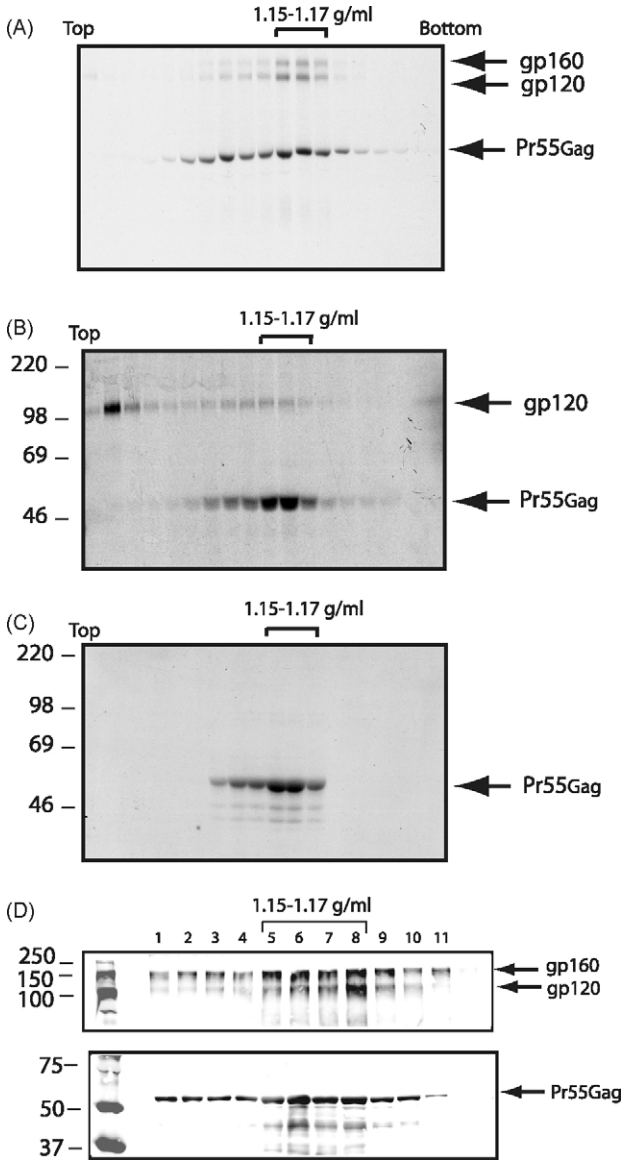

Fig. 2. Analysis of particle density and content by equilibrium centrifugation on sucrose gradients. (A) Analysis of particles from transient transfection of 293T cells. (B) Analysis of particles produced by stable, inducible cell line XC-18. (C) Particle gradient from stable, inducible Gag cell line XC-46. (D) Western blot showing $Pr55^{Gag}$ and gp160 bands for baculovirus-produced pseudovirions analyzed by sucrose density gradient centrifugation. The top blot was probed with a murine monoclonal anti-gp120 antibody; the bottom blot was probed with murine monoclonal antibody CA-183.

suggesting that the gp120 was initially associated with the particles but that some had shed during the gradient centrifugation process. An additional cell line was developed to generate immature Gag pseudovirions by stable transduction of codon-optimized Gag into 293 cells under a tetracycline-inducible promoter (XC-46). This cell line produces Gag pseudovirions for use as controls in immunization regimens, and similarly produced particles of the expected density that lack gp120 (Fig. 2C).

Baculovirus production of Gag–Env pseudovirions led to the incorporation of large amounts of envelope protein on

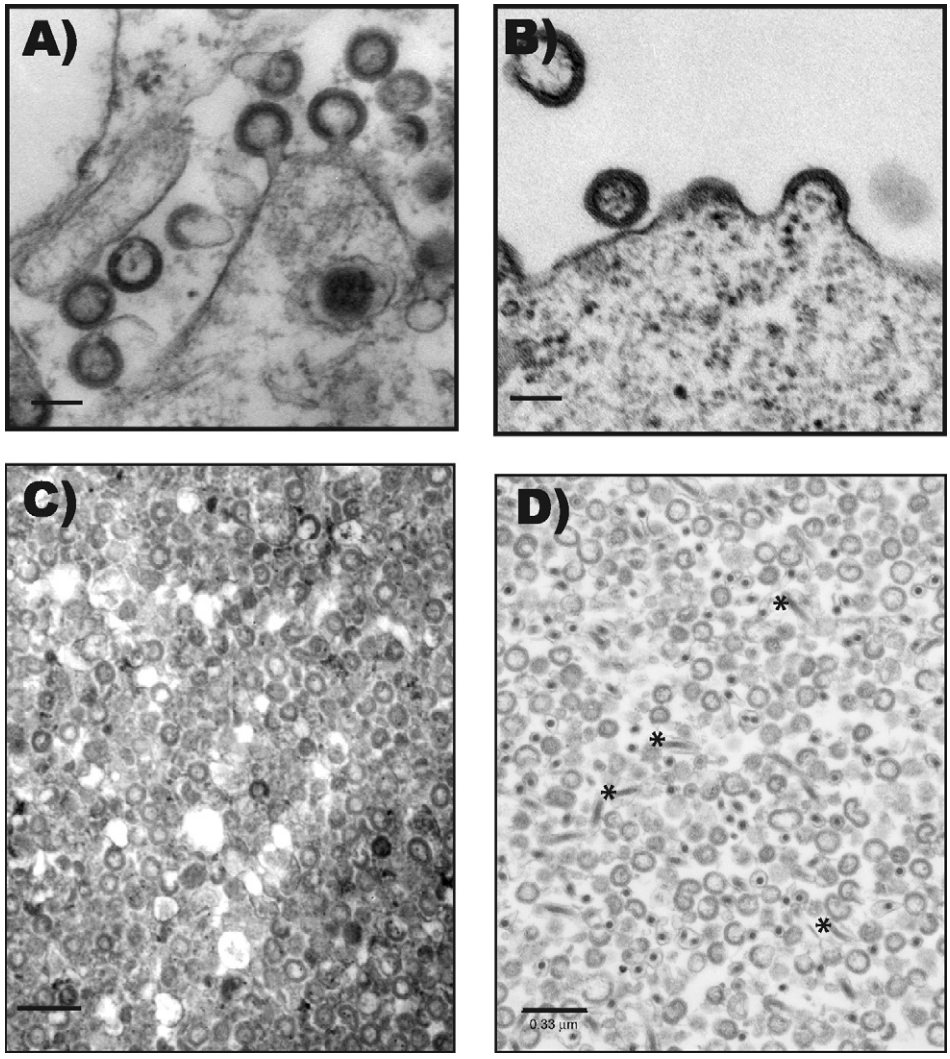

Fig. 3. Electron microscopy of Gag–Env particles produced by mammalian vs. insect cell production systems. (A) Gag–Env particles budding from XC-18 cell line. (B) Gag–Env particles produced from SF9 cells. (C) Particle pellet from XC-18 cells. (D) Particle pellet from SF9 cells. Asterisks indicate some of many baculovirus particles present in Gag–Env cell pellet.

the particles (Figs. 1 and 2D). However, a significant portion of the incorporated Env was in the form of uncleaved gp160 (Fig. 2D,). We were unable to significantly enhance the cleavage of gp160 using overexpression of a recombinant baculovirus expressing furin, or by introducing targeted modifications of the gp120/gp41 cleavage site (data not shown). For the purpose of presenting native Env trimers on pseudovirion particles, we considered this a limiting factor. Thus while the quantity of particles produced and the quantity of gp160 incorporated were quite encouraging, further work is needed to achieve Env cleavage and present native trimers using baculovirus production systems.

The molar ratio of Gag:Env on pseudovirion particles was consistently 30:1 (Table 1). Note that this is approximately twice the amount on infectious particles as determined by Chertova et al. [17], and using an estimate of 2500 Gag molecules per virion would represent approximately 83 Env molecules or 28 trimers per particle. Gag–Env ratios from 293T transfection were sometimes higher but were quite variable (Table 1).

Immature pseudovirion release from XC-18 and baculovirus-infected Sf9 cells and sucrose particle enrichment were next evaluated by transmission electron microscopy. XC-18 and infected Sf9 cells demonstrate immature Gag–Env pseudovirion budding and particle release as expected (Fig. 3A and B). The morphology of the released particles was essentially identical for these two methods. We then evaluated the quality of the purified immunogens by electron microscopy. XC-18 and baculovirus-infected Sf9 cells generated pseudovirions were enriched through a 20% sucrose cushion and then fixed and processed for transmission electron microscopy. Pseudovirions generated from XC-18 displayed enrichment of immature pseudovirions, although some degree of microvesicular contamination was evident (Fig. 3C). Enriched baculovirus-derived pseudovirions from infected

Sf9 cells also demonstrated efficient purification of immature particles (Fig. 3D). One notable difference is the presence of numerous baculoviruses in the immunogen prep (asterisks, Fig. 3D). The sucrose purification of baculovirus-derived pseudovirions therefore includes the co-enrichment of baculovirus particles. Baculovirus particles were quite distinct from that of immature HIV-1 pseudovirions, with an elliptical enveloped virion of approximately 300 nm in length. It remains uncertain what effects the co-purification of baculoviruses with Gag–Env particle preparations intended for immunization studies may have on product tolerability and immune responses.

3.5. Enhanced production of pseudovirions using a BelloCell bioreactor system

The BelloCell bioreactor system uses a non-woven polyester (PET) fabric surface-treated to make it hydrophilic and biocompatible. The PET matrix provides a large surface area for cellular adherence and growth at high cell density. The BelloStage apparatus provides aeration, gas exchange, limits the buildup of toxic cell metabolites, and provides a nutrient rich environment. The tetracycline-inducible, Gag–Env pseudovirion producing cell line, XC-18, was grown in the BelloCell system. The apparatus was seeded with 8.0×10^8 XC-18 cells and induced with 2 μg/ml Dox upon reaching 1.0×10^9 cells/bottle on day 5 (Fig. 4A). XC-18 cells continued to proliferate, reaching a maximum density on day 13 (3.25×10^9 cells/bottle) and maintained approximately 2.5×10^9 cells/bottle until day 23.

Pseudovirion production using the BelloCell bioreactor system is shown in Fig. 4B. Gag and Env production peaked on day 13 at 275 ng/ml and 425 ng/ml respectively. High levels of Gag and Env release were maintained over the course of the 3-week experiment, and there existed a direct correlation between the cell density and antigen production. XC-18 cells were maintained in the apparatus for 23 days over which time there were nine antigen harvests resulting in the production of approximately 1 mg of pseudovirion-associated p24. This level of antigen production from one bottle was equivalent to the production capacity of approximately one hundred and seventy, 150 cm^2 culture flasks. From a practical perspective, this production system saved a tremendous amount of incubator space, could be maintained for a long period of time, and allowed us to generate quantities of pseudovirions sufficient for immunization of small animals in a shorter period of time than had been possible with growth in individual flasks. This advance took us one step closer to making Gag–Env pseudovirion production efficient and practical for large scale experiments.

We note that very high levels of Env and Env/Gag ratios in the supernatant are shown in Fig. 4B. Most of the gp120 in the supernatant prior to pelleting represents free gp120 shed from producer cells. Interestingly, despite very high levels of Env production, the ratio of Gag/Env on purified pseudovirions was remarkably constant (as in Table 1).

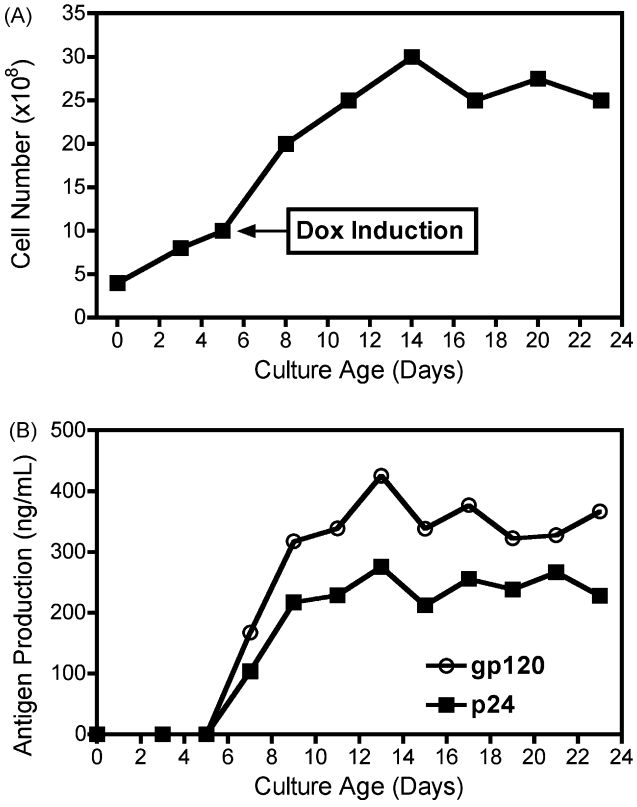

Fig. 4. Time-course profile of Bello Cell$^-$ produced immature $Pr55^{Gag}/BaL^{Env}$ pseudovirions. (A) XC-18 cell number over the course of the experiment. (B) Quantitative measurement of p24 and gp120 antigen production. Note that during times of peak production, 500 ml of supernatant was removed daily, allowing sustained particle production over the course of the experiment.

3.6. Cross-flow filtration for pseudovirion purification and concentration

The process of cross-flow (CF) filtration utilizes tangential movement of solution cross a hollow-fiber cartridge membrane resulting in the concentration of desired material. Concentrated solution remaining in the fluid stream is known as retentate, while the solution that moves across the membrane is called permeate. Concentration is achieved under low-stress conditions and makes this process ideal for the purification of shear sensitive components such as HIV-1 pseudovirions. CF filtration not only concentrates desired components within the retentate but allows for contaminants to be removed along with the permeate solution. This method has the potential to eliminate cumbersome concentration steps achieved by pelleting from large volumes of supernatant.

Two liters of clarified XC-18 supernatant were concentrated using CF filtration over 2.5 h resulting in a retentate volume of 100 ml. The concentration factor of the solution was 20-fold while the recovery of p24 and gp120 antigens was greater than 95% (data not shown). Upon concentration, the pseudovirion containing retentate was further purified through a 20% sucrose cushion, and the pseudovirion pellets

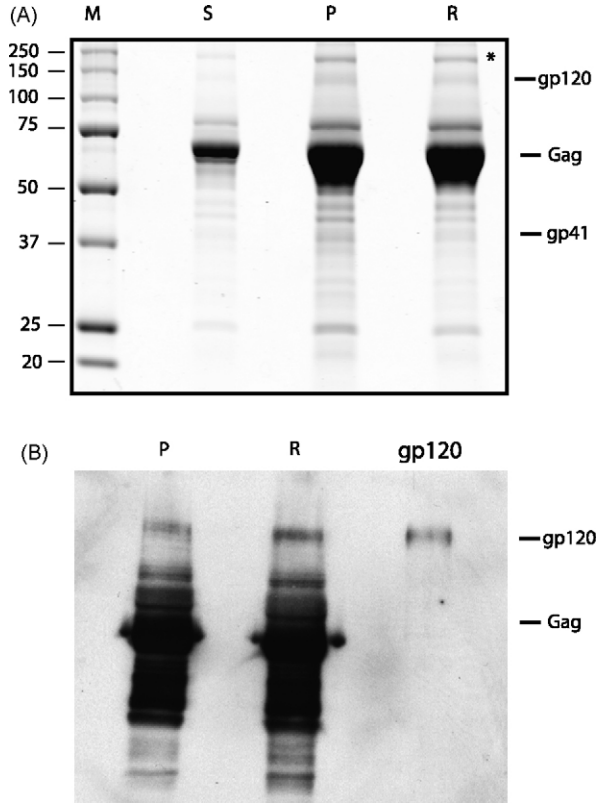

Fig. 5. Particle purity following cross-flow filtration. (A) Coomassie-stained protein from XC-18 supernatant (10 μl), S; pelleted material from 1 μl (P), and retentate (R) following cross-flow filtration. (B) Western blot showing that only fully-cleaved gp120 is present in purified samples from A. Blot was probed with pooled HIV+ patient sera. P; pelleted material from supernatant; R, retentate; gp120, purified gp120 protein as a marker control.

were resuspended in PBS. The Gag:Env ratio of CF concentrated pseudovirions was identical to that of pseudovirions generated using conventional purification methods (Table 1). The process of CF filtration did not qualitatively affect the integrity of pseudovirion particles. The concentration of Gag and Env by this method is illustrated by Coomassie staining in Fig. 5A. Note that 10 μl of supernatant is shown (S) in comparison to 1 μl of either pelleted supernatant (P) or CF retentate (R). The asterisk indicates a copurifying band that is not uncleaved gp160. The presence of completely cleaved Env was verified in CF retentates by Western blot analysis (Fig. 5B), and the particles did not lose gp120 during the CF process (Table 1). Thus, the concentrated pseudovirions remained structurally intact. This procedure, combined with the BelloCell factory production method described above, allows efficient production and purification of Gag–Env pseudovirions intended for vaccination.

3.7. Analysis and adsorption of anti-cellular antibodies

The analysis of the HIV-1 neutralizing potential of pseudovirion-immunized animal serum can be complicated by the presence of anti-cell antibody reactivity [5]. Pseudovirion immunogens generate antibodies reactive against membrane antigens incorporated onto particles released from producer cell lines. These antibodies result in non-envelope glycoprotein specific neutralization of primary HIV-1 isolates in neutralization assays involving human cell lines. In order to address this phenomenon we developed novel adsorption methodologies to reduce non-specific reactivity against particle surface components. Pseudovirion-immunized guinea pig serum was incubated with 293 or HeLa cell lysates, resulting in the specific adsorption of antibodies reactive against cell membrane antigens. Cell lysate adsorption procedures resulted in a 9-fold reduction in immunized serum cell reactivity against the producer 293 cell (Fig. 6A). The adsorption procedure only slightly reduced gp120-specific reactivity as determined by antibody titration in an ELISA format (Fig. 6B, filled triangles vs. open circles). We then utilized these cell lysates in neutralization assays, in an attempt to remove neutralization that was due to anti-cellular antibodies. Unfortunately, the lysates themselves created substantial background problems in the TZM-bl neutralization assay, so that the adsorption using this technique was not of practical utility in removing false neutralization activity from sera of pseudovirion-immunized guinea pigs (data not shown).

TZM-bl cells are HeLa-derived cells that were engineered to express CD4 and CCR5 and to contain integrated reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under the control of an HIV-1 long terminal repeat (LTR) [18]. We developed a cell-based Western to further evaluate the cellular reactivity of Gag–Env pseudovirion-immunized animal serum. Supernatants were adsorbed with HeLa cells, and the reactivity against TZM-bl measured in the in-cell Western format. This assay provided a visual confirmation of the existence of anti-cellular antibodies in guinea pig sera from pseudovirion-immunized groups, which could be detected even at a dilution of 1:4050 (Fig. 5C, pre-adsorption column). The adsorption procedure led to a substantial reduction in cellular antibody reactivity against TZM-bl cells as indicated by this technique (Fig. 6C, post-adsorption). We then attempted to apply the whole cell adsorption technique to remove anti-cellular antibodies and re-assess "true" neutralization. The titer of antibodies directed against 293 cells was reduced by this technique as shown by ELISA (Fig. 6D), although the reduction was not as substantial as that seen with the cell lysate (Fig. 6A). Whole cells also removed specific anti-gp120 reactivity more profoundly than the cell lysates (Fig. 6E vs. Fig. 6B). We were able to perform neutralization assays following adsorption with whole cells without difficulty. The post-adsorption sera had reduced levels of non-specific neutralization represented by neutralization of MLV, with mean 50% neutralization values falling from 970 to 328 (Fig. 6F, top graph). Post-adsorption sera also demonstrated a reduction in neutralization of BaL, the envelope strain utilized in the initial immunization protocol. Mean 50% neutralization titers for these sera fell from 1591 to 843 (Fig. 6F, bottom panel). Because of the simultaneous reduction of anti-gp120 reactivity with this technique, it was unclear what portion

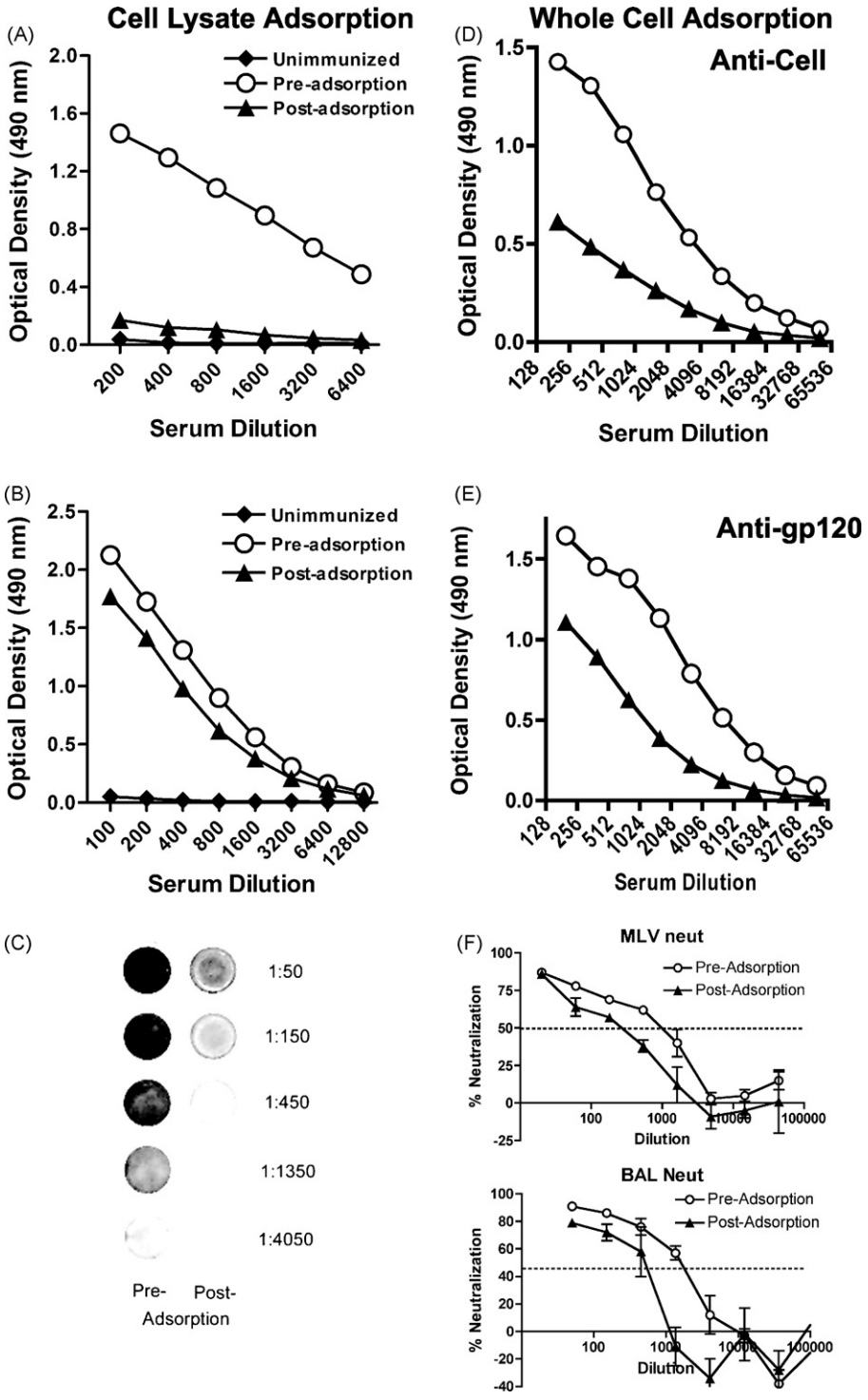

Fig. 6. Adsorption of anti-cellular antibodies. (A) Titration of pseudovirion immunized guinea pig serum reactivity against 293 cells before and after adsorption treatment to remove anti-cell specific antibodies. (B) HIV-1 BaL gp120 antibody titration of pseudovirion immunized guinea pig serum before and after adsorption treatment. Antibody titers were determined in an ELISA format using wells coated with purified gp120. (C) In-cell Western technique used to demonstrate anti-cell reactivity to membrane cell antigens before and after adsorption treatment. Cells in this experiment were TZM-bl. (D) pseudovirion-immunized guinea pig serum was adsorbed with whole 293 cells, then assessed for anti-cell activity as in (A). (E) anti-gp120 reactivity of pseudovirion-immunized guinea pig serum after adsorption with whole 293 cells. (F) Neutralization curves against MLV (top) or HIV-1 BaL (bottom), generated in TZM-bl luciferase-based assay.

of this drop in titer was due to removal of the anti-cellular activity vs. undesired adsorption of the specific anti-BaL reactivity.

4. Discussion

This study outlines practical strategies for the production and purification of Gag–Env pseudovirions for vaccine preparation. Production issues are rarely the focus of early preclinical studies of VLP approaches, yet they are critical to the success of any HIV pseudovirion immunization program. The concentration of particles derived from transient transfection of mammalian cells is reasonably high for the small-scale particle analysis required for cellular and molecular studies. However, transient transfection becomes impractical when one considers vaccine doses. Consider a vaccination protocol in which ten guinea pigs will be immunized on three occasions with pseudovirions, using a dose of 5 μg of particle-associated gp120 per dose (total requirement of 150 μg of particle-associated gp120). Supernatants from ten 10 cm^2 dishes may yield 100 μg of particle-associated p24 and only 10 μg of gp120. Estimating a loss of 25% of particle yield during purification and resuspension, this small trial would require production from two hundred 10 cm^2 dishes.

We contend that stable mammalian cell lines that can produce Gag–Env particles of consistent quality and quantity have an advantage as sources of vaccine product. Notable advantages include the avoidance of transfection reagents, predictable kinetics of particle production, the ability to select desired characteristics such as fully-cleaved gp120/gp41 trimers, and the capacity to adapt cells to bioreactors or cell factories for efficient large-scale production. The cell lines described here represent a means of achieving these goals.

Baculovirus production methods are clearly superior to mammalian cell production in terms of particle yield. Concerns remain, however, that baculovirus production may not result in authentic VLPs that present a fully cleaved gp120/gp41 complex. Despite the ability to enhance cleavage with co-expression of furin [19,20], the large-scale production of particles with fully-cleaved Env in insect cells is problematic. There is a growing body of evidence that presentation of uncleaved complexes is likely to be undesirable, and that neutralizing antibodies and not non-neutralizing antibodies bind to native trimers on particles [21]. Therefore the use of Gag–Env particles bearing significant amounts of uncleaved gp160 remains potentially limiting at present. A second potential hurdle for clinical development is the separation of baculoviruses from the HIV pseudovirion. As illustrated in Fig. 3D, this is not achieved simply by pelleting released particles through a sucrose cushion.

HIV virions and pseudovirions are known to incorporate a variety of host cell surface molecules on their lipid envelope. It should therefore not be surprising that pseudovirions used as immunogens will raise antibodies to these cell surface molecules. We report here that this type of response can be problematic, and that it needs to be considered carefully in the interpretation of neutralizing antibody results. We were able to remove anti-cellular activity almost completely using adsorption of sera with a cell lysate, but the lysate itself caused difficulties in the neutralization assay. A second adsorption technique employing whole cells partially removed the anti-cellular reactivity, but also removed some anti-gp120 reactivity. We conclude that adsorption techniques can be attempted to partially remove the anti-cellular activity and derive true HIV neutralization titers. However, the techniques described here have not yet been refined to a satisfactory level to allow the specific anti-HIV neutralization titer to be determined with confidence. Investigators utilizing pseudovirions should be aware of this potential problem, should include non-enveloped particles as a control arm in the immunization regimen, and should test carefully for neutralization of viruses such as MLV or SIV to define the extent of this activity in polyclonal sera. Future studies will be needed to demonstrate the true potential of Gag–Env pseudovirions to neutralize HIV without the complication of anti-cellular reactivity.

It is not yet clear how effective pseudovirion vaccines will be in generating neutralizing antibodies against HIV primary isolates. We believe that this approach is promising, but that further preclinical studies are warranted to clearly show that Gag–Env pseudovirions can achieve better results than recombinant gp120 protein, optimally through the generation of enhanced breadth of neutralization. In order for these experiments to succeed, tractable and reproducible production and purification techniques are essential. Our purpose in this report has been to summarize advances in this area that should allow enhanced quantities of pseudovirion particles of uniform character to be produced.

5. Conclusions

HIV pseudovirion or VLP vaccine approaches for eliciting neutralizing antibodies remain promising but have not yet lived up to their potential. Technical advances have been made for production and purification of particles, and should enhance the feasibility of using HIV pseudovirions as a vaccine platform in humans. Expression and purification of Gag–Env particles from stably-transduced cell lines as described in this report allows consistent, reproducible particle yields. Further development of baculovirus methods to achieve complete cleavage of gp160 during large-scale production would be advantageous.

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