Efficient Human Immunodeficiency Virus (HIV)-1 Gag-Env Pseudovirion Formation Elicited from Mammalian Cells by a Canarypox HIV Vaccine Candidate

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Canarypox viruses undergo abortive replication in mammalian cells. Despite this restriction on replication in mammalian cells, significant immune responses have been shown in animals and in humans receiving recombinant canarypox vaccine vectors expressing heterologous immunogens. A recombinant canarypox vaccine candidate (vCP205), which expresses human immunodeficiency virus (HIV)-1 Gag, Env, and protease proteins, is presently under investigation in phase I and phase II human trials in the United States and elsewhere. In this study, the ability of vCP205 to elicit HIV Gag-Env pseudovirion formation in avian and mammalian cells was investigated. Gag-Env pseudovirions were produced from both avian and mammalian cell lines infected by this vaccine vector. A subset of mammalian cells was identified in which pseudovirion production and release was very efficient, surpassing the production from infected avian cells. The production of Gag-Env pseudovirions by canarypox HIV vaccine vectors may have important implications for future HIV vaccine design.

The design of a safe and effective human immunodeficiency virus (HIV) vaccine is a goal of intense national and global significance. An effective vaccine must be capable of eliciting broadly reactive immune responses to protect against the diverse HIV quasispecies that exist worldwide. Although the correlates of immune protection against HIV are not precisely defined, desirable characteristics of vaccine candidates include the potential to elicit both broad-based humoral and cellular immune responses [1]. Human trials employing recombinant envelope glycoprotein subunit vaccines, although successful in generating virus-neutralizing antibodies against laboratory strains of HIV, have thus far failed to show neutralization of primary isolates representing commonly transmitted viruses [2, 3]. Anti-HIV cytotoxic T lymphocyte (CTL) responses directed against epitopes present in more conserved regions of the virus may provide a more broad-based response. Recently, it was shown that a recombinant canarypox (ALVAC)-based HIV-1 immunogen, which includes clade B gag, env, and protease components, elicits broad CTL reactivity against a panel of primary isolate targets and against cells infected with diverse HIV-1 subtypes [4]. Human trials with this vaccine product have thus far produced measurable CTL responses in over half the recipient volunteers [5]. In addition, the duration of CTL response in the majority of the responders has been >15 months [1]. This vaccine is presently being tested in a phase II trial in the United States and will be soon be evaluated in further trials in the Caribbean and in Uganda.

Canarypox viruses are members of the avipox genus of the Orthopoxvirus family of DNA viruses. Productive replication of avipox viruses is restricted to avian cells [6, 7]. The block in replication in human and other mammalian cells is incompletely understood but is known to precede viral DNA replication. Despite undergoing abortive replication in mammalian cells, canarypox virus expressing heterologous immunogens have been used successfully as vaccines against veterinary pathogens in nonavian species. Mice inoculated with a recombinant canarypox virus expressing immunogens from Japanese encephalitis virus were protected against a lethal challenge [8]. Similarly, cats vaccinated with a recombinant canarypox virus expressing the feline leukemia virus (FeLV) A subtype env and gag proteins were protected against persistent viremia after exposure to a homologous FeLV challenge [9]. These results, together with the results of ongoing phase I and phase II trials in humans of canarypox vaccine candidates expressing HIV immunogens, suggest that despite an abortive replication cycle, canarypox recombinants produce sufficient antigen within host mammalian cells to produce demonstrable immune responses. For HIV, as described earlier, the most promising results elicited by canarypox recombinants thus far have been in inducing HIV-specific CTL responses. An understanding of the precise mechanism through which canarypox-HIV recombinants successfully induce a CTL response is desirable, to design future vaccine vectors capable of inducing even more potent CTL responses.
Detailed analysis of the production of HIV antigens by the current generation of live vector vaccines may also allow for the design of modifications that enhance the humoral response directed against the viral envelope protein.

HIV Gag proteins produced in the absence of all other viral gene products within a variety of cell types elicit the formation of virus-like particles, or pseudovirions [10–14]. The production of pseudovirions is an attractive vaccine strategy, which allows the presentation of viral antigens in native, particulate form. The present study was performed to test the hypothesis that recombinant canarypox viruses expressing Gag and Env are capable of eliciting HIV Gag-Env pseudovirion formation from mammalian cells. The production of Gag-Env pseudovirions by the vaccine vector currently under evaluation in phase I and phase II trials, designated vCP205 (Virogenetics, Troy, NY), was evaluated in both avian and mammalian cells. Despite the inability to replicate in nonavian cells, p24 antigen production by vCP205-infected mammalian cells was rapid and achieved levels within cells that were higher than that seen in avian cells. Gag-Env pseudovirions were produced and released from both avian and mammalian cells in tissue culture. The kinetics of release of pseudovirions from some mammalian cells was strikingly different from that of avian cells, showing a more rapid and efficient particle release. However, the rapid, efficient release of Gag-Env pseudovirions was not generalizable to all mammalian cells but was strictly cell-type dependent.

Materials and Methods

Construction of vCP205. vCP205 was provided by Virogenetics (Troy, NC). The construction of vCP205 has been described elsewhere [15]. Briefly, expression cassettes were generated to express Gag, Env, and protease from HIV-1 under the control of poxvirus promoters, and inserted into the C3 locus of the ALVAC genome by use of standard methods. The Env-expression cassette includes the region of the HIV-1 MN env gene corresponding to gp120 linked to the sequence encoding the HIV-1 gp41 transmembrane anchor sequence from HIV-1 IIIB (28 amino acids) and was placed 3′ to the vaccinia virus H6 promoter. The Gag cassette included the HIV-1 IIB gag open-reading frame and extending into the 5′ portion of pol corresponding to the protease gene. The gag-pro tease cassette was placed under control of the vaccinia virus I3L promoter region.

Infection of avian and mammalian cells and measurement of p24 antigen production. Primary chick embryo fibroblasts (CEFs) were prepared from 10–11 day chicken embryos and were maintained in Media 199 with 9% fetal calf serum (FCS), 2% Hi-Chick serum, 10% tryptose-phosphate broth, 0.056% sodium bicarbonate, 100 U/mL penicillin, and 100 μg/mL streptomycin. To generate canarypox stocks, 150-cm² flasks were seeded from a plaque-purified master stock of vCP205 at an MOI of 0.1 pfu/cell. The infected flasks were incubated at 37°C for 3–5 days until maximum cytopathic effect was apparent. Working stocks were generated by freeze-thawing and sonication of the infected cell lysates, followed by removal of cell debris by centrifugation at 1000 g for 10 minutes at 4°C. Canarypox stocks were then plaque-titered by preparing serial 10-fold dilutions of the stock virus in Dulbecco’s MEM (DMEM)-2% FCS. Diluted virus was placed on CEFs in 60 mm² dishes for 1 h with rocking. The inoculum was then removed and the dishes overlaid with DMEM containing 0.75% agar. Plaques were counted after staining with neutral red and the plaque titer of stocks recorded. Resulting virus stocks were stored at −70°C until needed. BSC-40 cells (an African green monkey kidney cell line) were maintained in DMEM with 10% heat-inactivated FCS supplemented with penicillin and streptomycin. Experiments to determine p24 antigen production and release from vCP205-infected cells were performed in 100-mm² tissue culture dishes. Cells were plated on the night prior to infection. Infections were performed when the cells were noted to be ~80% confluent on the dish. An accurate count of the number of cells present per dish was achieved by counting the cells from 1 100-mm² dish on the morning of infection (~1.2 × 10⁷ CEF cells or 0.8 × 10⁷ BSC-40 cells at 80% confluence). The cells were infected with vCP205 at an MOI of 10 in 2 mL of complete nutrient media, after which an additional 8 mL of media was added to each dish. For time-course experiments, 10 100-mm² dishes were infected simultaneously and supernatants and cells harvested from one plate at each of the following time points: 0, 2, 4, 8, 10, 12, 24, 36, 48, and 72 h. Cells were lysed by detergent lysis in PBS containing 0.75% agar. Plaques were prepared from vCP205-infected CEF and BSC-40 cells infected with pseudovirions. The p24 content of cells and supernatants was determined by using a standard p24 antigen capture ELISA assay (Organon-Teknika, Durham, NC).

Sucrose gradient analysis of Gag-Env pseudovirions. Supernatants from 10 100-mm² dishes of CEF or BSC-40 cells infected with vCP205 were harvested at 24 h postinfection and filtered through a 0.45 μm filter. Particles were then pelleted via centrifugation through a 5 mL 20% sucrose cushion at 100,000 rpm in a Beckman SW28 rotor. The resulting pellet was resuspended in PBS containing 1% NP-40 and nuclei separated via centrifugation at 1000 g for 10 minutes. The p24 content of cells and supernatants was determined by using a standard p24 antigen capture ELISA assay (Organon-Teknika, Durham, NC).

Analysis of Gag and Env protein content of vCP205-produced pseudovirions. Proteins produced from 5 plates of infected BSC-40 cells were radio labelled with 35S-cysteine/methionine in cysteine/methionine-deficient media overnight. Labeled supernatants were pooled, filtered, and subjected to sedimentation through a sucrose cushion as already described. The resulting pellet was analyzed via immunoprecipitation with pooled HIV patients’ sera, followed by Western blot analysis by use of a polyclonal rabbit anti-p24 antiserum (a gift from Lee Ratner, Washington University, St. Louis).

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Electron microscopic analysis of Gag-Env pseudovirions. Cell pellets were prepared from vCP205-infected CEF and BSC-40 cells 24 h postinfection via low-speed centrifugation in a microfuge tube.
Cell pellets were fixed in 2% glutaraldehyde in phosphate buffer, postfixed with 1% osmium tetroxide, stained with 1% uranyl acetate, dehydrated in ethanol, and embedded with propylene oxide and Spurr resin. Thin sections were cut with a diamond knife and grids examined on a Phillips model 3000 electron microscope. Control, uninfected BSC-40 and CEFs were included in this analysis.

Analysis of particle production in a panel of avian and mammalian cell lines. The release of p24 over time into the supernatants of a panel of 10 cell lines was measured by use of methods described above. For infections, the number of cells was adjusted to an 80% confluent monolayer in a 100 mm² dish. The precise number of cells required to achieve 80% confluence was determined and final results calculated as total p24 release per million cells plated at the outset of the experiment. The cells in this panel included: (1) primary CEF; (2) QT-6, a quail cell line (ATCC CRL-1078); (3) BSC-40 cells (African green monkey kidney); (4) activated human peripheral blood mononuclear cells (PBMC) from a healthy donor; (5) MRC-5, of human embryonic lung origin (ATCC No. CCL-171); (6) Vero cells, a line from African green monkey kidney (ATCC CCL-81); (7) HEP-2, derived from a human epidermoid carcinoma (ATCC CCL-23); (8) HeLa, derived from a human cervical carcinoma (ATCC CCL-2); (9) CHO-K1, a Chinese hamster ovary cell line (ATCC CCL-61); and (10) C2C12, a murine muscle myoblast cell line (ATCC CRL-1772).

Gag protein expression via the vaccinia virus/T7 polymerase system and subcellular fractionation. The production of Gag protein by use of this expression system has been described elsewhere [14, 16, 17]. A modification of this expression system to incorporate Gag-GFP fusion constructs has recently been reported [18]. vTF 7–3 is a recombinant vaccinia virus that expresses bacteriophage T7 RNA polymerase under the control of a vaccinia virus promoter, allowing high-level expression of target genes placed under the control of the T7 promoter [19]. Cells were infected with vTF 7–3 at an MOI of 10 when 80% confluent on 10-cm² dishes, then transfected with 10 μg of 55Gag/GFP [18] or with pTM/Gag (described later) via lipofection. p24 antigen detection in supernatants and cellular lysates was performed 24 h posttransfection. Subcellular fractionation was done as described elsewhere [14]. Briefly, cells were harvested at 24 h, washed in NTE buffer (150 mM NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA), then exposed to hypotonic buffer (10 mM Tris pH 7.4, 1 mM MgCl₂, and protease inhibitors) for 15 min on ice. Cells were broken by dounce homogenization, NaCl added to achieve a 0.15 M concentration, and nuclei and unbroken cells removed via centrifugation at 1000 g. The remaining cell lysate was subjected to ultracentrifugation in microcentrifuge tubes in a Sorvall RCM120-EX ultracentrifuge (Sorvall, Newtown, CT) at 100,000 g for 30 min. Membrane-enriched pellets (1000 fraction) and supernatants (S100 fraction) were analyzed for p24 content via antigen capture ELISA or for fluorescence in experiments employing Gag-GFP by microplate fluorometry. For quantitation via fluorescence, a standard curve generated by use of recombinant enhanced green fluorescent protein (Clontech, Palo Alto, CA) was used. The construction of Gag expression vector pTM/Gag has not been previously described. pTM/Gag includes an Nco1-BamH1 fragment encoding the HXB2 Gag sequence that was generated by polymerase chain reaction (PCR) by use of the HXB2gpt2 template and the following oligonucleotide primers: forward primer, AGAGCCATGGGTGCGAGAGCGTCAGTA; reverse primer, CGGGATCCTATTGTGACGAGGGGTTCG. The resulting fragment was digested with Nco1 and BamH1 and ligated into pTM1 by use of the Nco1 and BamH1 sites. The entire gag gene insert in this vector was sequenced by automated methods to confirm the absence of PCR-induced changes. This expression vector encodes a full-length Gag protein under the control of the T7 promoter.

Results

p24 production and release from vCP205-infected avian and mammalian cell lines. Canarypox viruses are unable to support a productive infection in mammalian cells. The canarypox vaccine candidate used in this study, vCP205, expresses HIV Gag, protease, and Env proteins and was postulated to be capable of forming pseudovirions in human cells. To test whether the production of HIV antigens by this vaccine vector was adequate for the formation of pseudovirions, CEF and BSC-40 cells were infected with vCP205 at an MOI of 10. Ten 10-cm² dishes were infected simultaneously and supernatants and cellular lysates collected for measurement of p24 content by antigen-capture ELISA at multiple time points within 72 h of infection. p24 production within CEF cells peaked at 10–12 h postinfection and remained stable over the following 60 h (figure 1A). Release of p24 into the supernatant was minimal at 10–12 h and increased steadily over the next 60 h. The production and release of p24 in BSC-40 cells was markedly different (figure 1B). The peak production of p24 in these cells was higher than in CEFs, although it was reached at the same 10–12 h time point. A biphasic pattern of p24 release was noted, with a rapid release up to 12 h followed by a slower release continuing to 36 h. At 48 h postinfection, the release of p24 antigen was >40% of the total p24 produced in the culture, whereas <12% of the p24 produced in CEFs was released into the media. The data shown are representative of 3 independent experiments. Differences noted for p24 release were not caused by lysis of BSC-40 cells, as these cells did not show significant cytopathology upon infection with vCP205 during the time points studied (data not shown).

Sucrose density gradient analysis of Gag-Pro-Env pseudovirions. The release of p24 from avian and mammalian cells measured in time-course experiments may have been caused by pseudovirion formation or the release of soluble, nonparticulate p24 from infected cells. To determine whether HIV-like particles were indeed released from infected cells, p24 antigen in cellular supernatants was examined by sedimentation through a sucrose linear gradient. Equal fractions of the gradients were collected and assayed for p24 content by ELISA and Western blot analysis. p24 released from CEFs was shown to band at a density of 1.16–1.18 g/mL, a value identical to that of HIV particles (figure 1C). Western blotting of the fractions from this gradient with a polyclonal anti-p24 antiserum confirmed the measurements obtained by p24 ELISA (figure 1C, bottom panel).
Figure 1. Pseudovirion particle release from chick embryo fibroblasts (CEFs) and BSC-40 cells infected with vCP205. A. Ten 10-cm² dishes of CEFs were infected simultaneously with vCP205 at an MOI of 10. Cell lysates and supernatants harvested at 0, 2, 4, 8, 12, 24, 36, 48, and 72 h postinfection were analyzed for p24 content by use of a p24 antigen-capture ELISA. Shown is total intracellular p24 at each time point (□) vs. p24 release into the supernatant (○). B. Intracellular vs. supernatant p24 from BSC-40 cells; time points and labels are as in A. C. Analysis of pseudovirion formation through equilibrium density centrifugation on sucrose gradients. Supernatants from 10 10-cm² dishes of CEFs were harvested 24 h after infection with vCP205, filtered through a 0.45 μm filter, and pelleted through a 20% sucrose cushion. The pseudovirion pellet was resuspended, layered on top of a linear 20%-60% sucrose gradient, and subjected to centrifugation at 35,000 rpm in an SW41 rotor for 18 h at 4°C. Fractions were analyzed by p24 ELISA and Western blotting by use of a polyclonal rabbit anti-p24 antibody. Top: gradient density (lit) vs. p24 content. Bottom: Western blot of corresponding fractions from gradient. D, Gag-Env pseudovirion formation, BSC-40 cells. Methods were identical to those in C.

panel). Similarly, released p24 from the mammalian cell line examined in an identical manner yielded particles of 1.16–1.18 g/mL density (figure 1D). The production of pseudovirions by the mammalian cell line was much higher than the production from the avian cells, as illustrated by >250 ng/mL in the peak gradient fraction from BSC-40 cells versus 35 ng/mL in the peak gradient fraction from CEFs (figure 1C and 1D, compare fraction 3). The percentage of total p24 protein released into the media in particulate form (as pseudovirions) was calculated by comparing the total p24 content of supernatants to the amount of p24 recovered after pelleting through the sucrose cushion. The mean percentage of p24 that pelleted in 2 independent experiments was 86.5%. Thus, the great majority of released p24 in these experiments was found in the form of pseudovirions.

**Analysis of Gag and Env proteins in pseudovirion particles.** The design of the vCP205 vaccine product includes an env gene, which consists of HIV-1 gp120 (SU) fused to the 28-amino acid membrane-spanning domain of HIV-1 gp41 (TM). To determine whether the released Gag proteins (pseudovirions) incorporated this truncated Env molecule, pseudovirion particles released from [35S-cysteine/methionine-labeled and vCP205-infected BSC-40 cells were centrifuged through a 20% sucrose cushion and analyzed via immunoprecipitation with pooled
HIV patient sera, followed by SDS-PAGE and autoradiography. A band of 120–130 kD molecular mass corresponding to Env was noted in the pseudovirion pellet (figure 2A). In addition, a significant amount of uncleaved Pr55\text{Gag}\text{Cleaved} precursor protein was noted. To verify the presence of envelope protein in the pseudovirion preparation, Western blot analysis was performed by use of a monoclonal antibody directed against gp120. A band that comigrated on SDS-PAGE with recombinant gp120 and was recognized by the gp120 monoclonal antibody was identified in the pseudovirion pellet (figure 2B). The pseudovirion pellet was also analyzed by Western blot by use of a polyclonal anti-p24 antisera, to further examine the Gag precursor polyprotein and its cleavage products. A large amount of uncleaved Pr55\text{Cleaved}, as well as a 41-kD intermediate cleavage product, was shown (figure 2C), indicating incomplete cleavage of Gag by the HIV protease encoded in vCP205. The inefficient cleavage of Gag polyprotein in the released pseudovirions was not caused by factors specific to BSC-40 cells, as the cleavage of Pr55\text{Gag} produced in BSC-40 cells from an HIV-1 Gag and Pol expression plasmid normally results in completely cleaved Pr55\text{Gag} [14], and inefficient cleavage was also noted in particles purified from vCP205-infected CEFs (data not shown).

**Electron microscopic analysis of vCP205-infected avian and mammalian cells.** To confirm the observation that vCP205 infection of both avian and mammalian cells elicits the production of Gag-Env pseudovirions, cells were pelleted and examined by transmission electron microscopic techniques 24 h after infection with vCP205. CEFs were found to produce immature HIV-like particles on the cell surface (figure 3A). The particles observed in sections from CEFs were of the appropriate size and morphology for HIV Gag-Env pseudovirions, with a diameter of 110–130 nm. BSC-40 cells examined at 24 h showed numerous Gag-Env pseudovirions, some budding from the cell surface (figure 3B). The pseudovirion particles from BSC-40 cell preparations were noted to be much more numerous than in comparable fields of infected CEFs, although precise quantitation from electron microscopy was not performed. The particles were identical in size to HIV virions (110–130 nm). In a few fields, indentations on the plasma membrane of cells appeared to be releasing pockets or vacuoles full of Gag pseudovirions (figure 3C). Also indicated in figure 3C is one of the input canarypox virions. The vast majority of pseudovirions observed were of immature morphology (figure 3A–D), confirming the results of radioimmunoprecipitation and Western blot analysis described above. Occasional particles

![Figure 2](https://academic.oup.com/jid/article-abstract/180/4/1122/498343)
Figure 3. Transmission electron microscopy of Gag-Env pseudovirions produced by vCP205. Chick embryo fibroblasts (CEFs) or BSC-40 cells were infected with vCP205 at an MOI of 10 for 24 h prior to harvesting and fixation. A, Pseudovirion particle from CEF cell; magnification 99,750, bar represents 100 nm. B, Budding pseudovirion particle from BSC-40 cell. Magnification 94,125; bar represents 159 nm. C, Multiple pseudovirion particles in cleft on BSC-40 cell. Also pictured is 1 canarypox virion particle (CP). Magnification 62,750; bar represents 159 nm. D) Dense core formation in pseudovirion particle from infected BSC-40 cell. Magnification 94,125; bar represents 159 nm.

contained some condensed Gag protein in the center of an immature core (figure 3D). In addition, long extensions radiating from the immature Gag-Env particles were noted (figure 3D).

Cell type-dependence of HIV Gag-Env pseudovirion formation. The production of pseudovirions by BSC-40 cells, a mammalian cell line, was much more efficient than that of the avian CEF cells (described earlier). To determine whether this property was a general property of mammalian versus avian cells or the efficiency of production was limited to a subset of mammalian cells, 8 additional cell lines were evaluated for p24 release on infection by vCP205. An immortalized avian cell
line, QT-6, showed low p24 release over time, compared with BSC-40 cells (figure 4). The production and release of p24 over time from this cell line paralleled that of CEFs (data not shown). However, several mammalian cell lines also showed low/slow particle release, including MRC-5, HeLa, Hep-2, CHO, and phytohemagglutinin-stimulated human PBMC. It should be noted that none of these cell lines failed to release p24 antigen but that the efficiency of release was low and similar in kinetics to that already outlined for CEFs (plotted as nanograms of p24 released per million infected cells; figure 4). Two additional efficient or high-release mammalian cell lines were identified in this survey. One of these, Vero cells, is an African green monkey kidney cell line similar to BSC-40. Interestingly, the most efficient pseudovirion-producing cell line identified in this study was the murine skeletal muscle cell line C2C12. This cell line showed a rapid rate of particle release that continued up to 36 h, in contrast to the 10–12 h peak of the rapid particle release previously noted for BSC-40 cells.

The differences in pseudovirion output between high- and low-producing cell lines could be explained by cell line–specific defects in assembly and release of HIV pseudovirion particles, or by limitations to antigen production within cells related to poxvirus-cell interactions. An essential step in the assembly of HIV pseudovirions is the transport and binding of Gag proteins to cellular membranes, predominantly the plasma membrane. To determine whether low-producer cells were defective in Gag protein membrane binding, subcellular fractionation was performed in a high-producing cell line (BSC-40) and in a low-producing cell line (CHO) infected with vCP205. Analysis of p24 antigen (which reflects both cleaved and uncleaved Pr55\(^{\text{Gag}}\) in this experiment) in membrane-enriched and cytosolic fractions revealed little difference in the percentage of membrane-associated Gag protein between CHO and BSC-40 cells (figure 5A). To further examine the hypothesis that low-producing cell lines may exhibit defects in Gag protein membrane binding, an alternative method of Gag protein production was employed. The vaccinia virus/T7 polymerase expression system has previously been used as an efficient means of producing HIV Gag proteins and pseudovirions [14, 16, 17]. Two high-producing cell lines (based upon the canarypox infection data above) and 2 low-producing cell lines were examined. To facilitate quantitation of Gag, we employed a Gag-GFP fusion construct, which allows accurate and rapid quantitation of Gag in membrane and cytosolic fractions [15]. No difference in the percentage of membrane-bound uncleaved gag-GFP protein was noted between high- (BSC-40, Vero) and low- (CHO, HeLa) producer cells (figure 5B). The higher percentage of membrane-bound Gag protein shown in this assay versus figure 5A likely reflects the cytosolic p24 present in the vCP205-infected cells caused by expression of HIV protease and is consistent with previously published results [15]. The protease gene was not included in the Gag-GFP expression construct. Results in these assays represent the mean of two independent experiments.

To determine whether high- and low-producer cell lines exhibit differences in the efficiency of release of HIV antigens as pseudovirions, the amount of Gag protein released from cells was compared with total p24 antigen production for each of

![Figure 4](https://academic.oup.com/jid/article-abstract/180/4/1122/840843/figure-4)

**Figure 4.** p24 release from ten infected cell lines over time. Data is plotted as nanograms of released p24 per 1 × 10\(^4\) infected cells. Cells were infected in 10-cm\(^2\) tissue culture dishes at a number approximating 80% confluence of the plate by use of an MOI of 10.
Figure 5. Membrane-binding and particle release in high- and low-producer cell lines. Experiments performed in cells infected with vCP205 are indicated in stippled bars; those employing antigen production through the vaccinia/T7 expression system are indicated in dark gray bars. Results of A–C represent the mean of two independent experiments, and 1 SD is indicated. Columns lacking error bars in A–C represent <1% deviation between the two experiments. A, Differential sedimentation results in vCP205-infected cells. p24 antigen in the membrane-enriched pellet is shown as a percentage of total p24 production. B, Differential sedimentation results in cells infected with vTF 7-3 and transfected with 55Gag/GFP. The quantity of Gag-GFP protein in the membrane and cytosol was determined by fluorometry and results plotted as Gag-GFP in membrane/membrane + cytosolic Gag-GFP. C, The quantity of p24 in the cellular supernatant and in the cells was determined 24 h postinfection with vCP205 and is presented as p24 in supernatant/p24 in cell + p24 in supernatant. D, Total p24 production (cellular and supernatant) per 1 × 10⁶ cells at 24 h postinfection with vCP205.

the 4 cell lines previously examined for membrane binding (BSC-40, Vero, CHO, and HeLa). This analysis was performed on cellular supernatants and cell lysates collected at 24 h postinfection, a time when the separation into high- and low-producer cells is apparent (figure 4). The two low-producer lines yielded the most inefficient particle release in canarypox–infected cells (figure 5C). However, the striking differences in particle release from vCP205-infected cell lines were not observed when Pr55Gag was expressed in the same cells by use of the vaccinia virus/T7 polymerase expression system (data not shown). The most striking discrepancy in particle release between the two expression systems was noted in HeLa cells. vCP205-infected HeLa cells showed a low efficiency of particle release (figure 5C), whereas expression via the vaccinia/T7 system allowed release from HeLa cells at levels approaching that of BSC-40 cells. Although significant differences in particle re-
lease were seen in vCP205-infected cells, more striking differences were noted when the total amount of Gag antigen produced in the 4 cell lines was examined (figure 5D). The total production of antigen in the two high-producing cells (BSC-40, Vero) greatly exceeded that of the low-producing cells (CHO, HeLa). Thus, the differences seen in the efficiency of assembly of pseudovirions in vCP205-infected cells were specific to the interactions of this canarypox virus with each cell line were not consistent with a cellular assembly defect as assessed by alternative expression of Gag and correlated best with the total amount of Gag antigen produced in infected cell cultures.

Discussion

Future generations of HIV vaccine candidates may best be designed through a comprehensive understanding of the factors that have led to some measure of success in the current generation of vaccines. To achieve this goal, it will be useful to examine the production of viral antigen by current vaccine vectors at the molecular and cellular level and determine which specific aspects of each vaccine candidate may contribute to the induction of an immune response. In this regard, the canarypox HIV vectors have thus far been the most successful family of vectors in eliciting HIV-specific CD8+ CTLs in human volunteers. The reasons for this relative success in comparison with other strategies are not certain. The present study was conducted to test the hypothesis that a canarypox gag/pro/env vector is capable of eliciting pseudovirion formation in mammalian cells. Although the significance of pseudovirion formation to the induction of an immune response has not been fully established, particle production and release by a host cell might lead to enhanced uptake and processing of particulate antigen by professional antigen-presenting cells. This may be especially relevant to the production of pseudovirions from products such as vCP205, which are administered intramuscularly, as muscle cells themselves may be inefficient at presenting antigen to cells of the immune system. Pseudovirion production may then lead to the generation of an enhanced CTL response. In support of this hypothesis, it has been shown that SIV-like particles can elicit a potent recall CTL response in monkeys previously vaccinated with a recombinant vaccinia-SIV virus [20]. As an extension of this hypothesis, it may be possible to enhance the HIV-specific cellular immune response by employing live vaccine vectors that efficiently elicit the production of pseudovirions from the infected host cells.

In this study, we found that vCP205 elicits pseudovirion formation from avian cells and from a variety of mammalian cell lines. The abortive replication of canarypox recombinants in mammalian cells thus does not limit the ability of these vectors to produce pseudovirions. The production of pseudovirions by avipox recombinants shown in this study is consistent with a previous report in which SIV pseudovirions were released from avian and mammalian cells infected with a recombinant fowl-

The majority of pseudovirions produced by vCP205 were of immature morphology. This result was confirmed by Western blot analysis of purified pseudovirions, which showed inefficient cleavage of Pr55Gag. This inefficiency was not caused by any host cell-dependent factor, as Gag-Pol particles produced in BSC-40 cells from expression vectors have been shown to be efficiently cleaved by HIV protease [14]. Although this is likely an unintentional result of the construction of this vaccine vector, it is not clear whether the presence of uncleaved Gag precursor polyprotein represents an advantage or a disadvantage for future vaccine design. Immature viral cores of both HIV-1 and Rous sarcoma virus are more stable to detergent than fully-cleaved cores [22, 23], implying that the pseudovirions produced with incompletely cleaved Gag proteins may have improved stability over completely cleaved particles. In addition, the production of excessive amounts of protease within a host cell results may result in prematurely-cleaved Gag protein with an accompanying decrease in particle production [24]. Although in this aspect the canarypox-produced pseudovirions do not accurately mimic HIV particles, it remains to be tested whether alterations in the degree of proteolytic cleavage of Gag protein within pseudovirions will alter the magnitude or character of the resulting immune response.

Host cell factors may influence the efficiency of pseudovirion production elicited by recombinant viral vectors. We found that the efficiency of particle release from three mammalian cell lines tested was markedly higher than that from avian cells or from a panel of additional mammalian cells. The initial rate of particle release from BSC-40 cells (prior to 12 h) was markedly different than the slow, steady release that was seen in CEFs (figure 1). A murine muscle cell line showed an even more prolonged, high rate of particle release (C2C12, figure 4). The efficiency of release of Gag-Env pseudovirions (as measured by the ratio of p24 released to total p24 produced) from BSC-40 cells remained higher than release from avian cells throughout the first 48h postinfection (figure 1, A and B). A previous study documented cell line-dependent release of Gag particles by recombinant vaccinia viruses [25]. In this study, Vero cells were shown to be defective in the release of particles...
compared with mouse 1D cells or African green monkey kidney CV-1 cells. We show in the present study that Vero cells infected by vCP205 efficiently produce Gag-Env pseudovirions, although the efficiency of release was less than that seen with BSC-40 cells (figure 5C). The percentage of Gag protein released by vCP205 was dramatically lower in the subset of low-pseudovirion producer cells (CHO and HeLa, figure 5C). These data indicate that differences in individual cell lines influence the amount of pseudovirion particles formed on infection with recombinant HIV Gag-Env poxvirus vectors. It will be desirable in the future to define the cellular factors involved in efficient particle formation and to characterize the human cell types that are most proficient in pseudovirion formation and release. Such studies may be of value for enhancing the production of pseudovirions in vitro for use as injected vaccine products and for improvements in the design of live vector vaccines that are capable of eliciting pseudovirion formation directly from vaccine cells. If subsets of human cells are identified as efficient pseudovirion-producers, then delivery of recombinant vaccine might be targeted specifically to those cells in future trials.

The mechanism that separates cells into efficient or inefficient producers of pseudovirions upon infection with vCP205 may be multifactorial. Interactions between the recombinant canarypox and the cell may alter the late stages of HIV pseudovirion production, as indicated by the inefficient release of HIV particles in low-producer cell lines (figure 5C). This difference is clearly not caused by differences in the efficiency of Gag protein membrane binding between low- and high-producer cell lines. However, the most striking difference between high- and low-producer cell lines was noted in the total p24 antigen production per cell (figure 5D). This indicates that the major limitation to pseudovirion production in low-producer mammalian cell lines lies within or prior to translation of the gag-pro messenger RNA rather than in specific assembly defects. Furthermore, the limited antigen production and release demonstrated in low-producer mammalian cells by vCP205 was not shown when antigen expression was elicited by an alternative method (the vaccinia/T7 expression system), suggesting that the limitation on particle production in low-producer cells occurs at the level of canarypox gene expression or as a result of canarypox-mediated cellular toxicity. Efforts to improve the immunogenicity of these candidate vaccine vectors in human cells may benefit from modifications that enhance gene expression in this subset of cells or reduce adverse effects of virus infection on the cell.

The ability of vCP205 to efficiently produce pseudovirions from several mammalian cell lines, including a murine muscle cell line, suggests that pseudovirions may be produced and released from cells of human canarypox vaccine recipients. The importance of this finding to the immune response has not yet been addressed and will require further studies. If pseudovirion formation itself contributes to the successful induction of a CTL response, then efforts directed at improving the amount of antigen produced and at enhancing particle release can be pursued as a valid vaccine strategy. When combined with future generations of vaccine constructs, which will include strategies designed to elicit more effective neutralizing antibody responses, the goal of producing an HIV vaccine that elicits significant cell-mediated and humoral immunity may be realized.

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References


