Production of a Dendritic Cell-Based Vaccine Containing Inactivated Autologous Virus for Therapy of Patients with Chronic Human Immunodeficiency Virus Type 1 Infection

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Received 19 February 2008/Returned for modification 18 May 2008/Accepted 17 November 2008

In preparation for a pilot clinical trial in patients with chronic human immunodeficiency virus type 1 (HIV-1) infection, a novel dendritic cell (DC)-based vaccine is being manufactured. The trial will test the hypothesis that isolated endogenous virus presented by DCs serves as a potent immunogen for activation of CD8+ and CD4+ T cells specific for a broad range of autologous HIV-1 antigens. Production of the vaccine under good manufacture practice conditions involves (i) autologous virus isolation; (ii) superinfection of CD4+ T cells with the virus; (iii) inactivation of the virus in CD4+ T cells, T-cell apoptosis, and coincubation of T cells with autologous DCs; and (iv) product testing and release. Endogenous virus was isolated from peripheral blood-derived CD4+ T cells of three HIV-1-positive subjects by coincubation with autologous OKT-3-stimulated CD4+ T cells. CD4+ T-cell supernatants were tested for p24 levels by enzyme-linked immunosorbent assay (>$25$ ng/ml) and for the 50% tissue culture infective doses (TCID50; ranged from 4,642 to 46,416/ml on day 19 of culture). Autologous CD4+ T cells that were separated on immunobeads (>95% purity) and superinfected with virus-expressed p24 (28 to 54%) had TCID50 of >400/ml on days 5 to 10. Virus inactivation with psoralen (20 μg/ml) and UVB irradiation (312 nm) reduced the TCID50 of the supernatants from 199,986 to 11/ml (>99%). 7-Amino-actinomycin D-positive, annexin V-positive CD4+ T cells were fed to autologous DCs generated by using the Elutra cell separation system and the Aastrom system. Flow analysis showed that DC loading was complete in 24 h. On the basis of these translational results and experience with the generation of DCs from HIV-1-infected patients in a previous clinical trial, the Investigational New Drug application for clinical vaccination was submitted and approved by the FDA (application no. BB-IND-13137).

Antiretroviral therapy (ART) has been widely used to suppress human immunodeficiency virus type 1 (HIV-1) replication and increase the number of CD4+ T cells in patients with HIV-1 infection. However, in most of these patients, the recovery of anti-HIV-1-specific T-cell function is incomplete. As the complete restoration of T-cell immune function is considered to be necessary for effective control of the viral infection, additional measures aimed at the bolstering of the HIV-1-specific adaptive immunity in patients treated with ART are being evaluated.

Dendritic cells (DCs) are the most potent antigen-presenting cells that can both prime and sustain memory responses (24, 28). DCs have been used increasingly frequently in vaccines against cancer and viral infections (4, 13, 20). Previous studies from our group showed that DCs derived from the blood of subjects with chronic progressive HIV-1 infection and not receiving ART were able to stimulate anti-HIV-1 reactivity (5). HIV-1-reactive CD8+ T cells are detectable in the peripheral circulation of subjects receiving ART following in vitro activation with many types of HIV-1 antigens, including HIV-1 proteins, HIV-1 peptides, and virus-infected apoptotic cell-loaded matured DCs (6, 10, 14, 22, 23, 31). We hypothesized that it may be possible to reconstitute the reactivity of naive and memory virus-specific T cells by delivering to patients autologous DCs engineered ex vivo to express and present known immunodominant peptides of HIV-1. To this end, we have recently completed a phase I clinical protocol in which autologous monocyte-derived DCs were pulsed with a mix of three HIV-1 peptides (Gag, Pol, and Env) and one influenza A virus (matrix) major histocompatibility class I superantigen and delivered as vaccines to 18 HIV-1-infected, ART-treated subjects (5). This vaccination strategy was found to be safe and feasible and resulted in a transient but significant increase in the frequency of CD8+ T cells specific for HIV-1 peptides present in the vaccine (5). On the basis of the results of this trial, we have been considering a strategy of stimulating HIV-1-specific, naïve CD8+ and CD4+ T cells by priming them with DCs engineered to express autologous HIV-1 (19). The rationale for this strategy is that autologous virus represents a large repertoire of the host’s diverse HIV-1 antigen pool and offers the potential to elicit the most specific, broadest, and most effective immune responses for each subject’s quasispecies of HIV-1, thus increasing vaccine efficacy.

In this report, we provide evidence that the production of an antiviral vaccine containing autologous DCs fed with inactivated HIV-1-infected, autologous, apoptotic CD4+ T cells is...
feasible, can be successfully accomplished in a good manufac-
ture practice facility, and can be scaled up for therapeutic
delivery to HIV-positive (HIV-1) patients. The production
process consists of several steps: (i) isolation of autologous
virus from the peripheral blood of HIV-1-infected subjects; (ii)
superinfection of autologous enriched CD4+ CD8- T cells
with viral supernatants; (iii) virus inactivation by psoralen and
UVB irradiation; (iv) testing for p24 levels and the residual
virus from the peripheral blood of HIV-1-infected subjects; (ii)
patients and healthy donors. Four HIV-1 subjects not treated with ART and
with high plasma HIV-1 RNA levels (> 50,000 copies/ml) were recruited as
potential PBMC donors for cell (PBMC) and virus isolation. After they
signed an informed consent approved by the Institutional Review Board (IRB) at
the University of Pittsburgh. These subjects were seen at the HIV clinic and
donated venous blood weekly, so that autologous feeder PBMCs were available
for autologous virus isolation. For development of the assays and to serve as
control cells, PBMCs were also obtained from healthy donors in the form of buffy
coats purchased from the Central Blood Bank of Pittsburgh, PA. For the scale up
of vaccine production, two of the three patients underwent leukapheresis at the
Hillman Cancer Center Pheresis Unit to provide monocytes for DC generation.
IRB approval was obtained, and the patients consented to the procedure.

Cell lines. The TZM-b1 and SE5 cell lines were obtained from the NIH AIDS
Research and Reference Reagent Program. These cell lines were originally gen-
erated by Thomas Folks by infecting parent cell line A03.01 with HIV strain LAV
(later called strain IIIb). The infected cells were treated in such a way (i) that one
donor that had a mutation in the integrated copy of the polymerase gene so that
no infectious virus was produced (only replication-incompetent particles were
released in the supernatant) and (ii) that cells with only one copy of HIV
integrated per cell genome were derived (8). The SE5 cell line was further
subcloned to obtain 2A9 cells, which stably express high levels of HIV-1 p24. The
2A9 subclone was used as a positive control for intracellular HIV p24 staining.

Reagents and antibodies. RPMI 1640 medium, phosphate-buffered saline
(PBS), and Hanks’ balanced salt solution were purchased from Invitrogen (Carls-
bad, CA); and PermForm solution was purchased from Invirion, Inc. (Oakbrook,
IL). Ficol-Hypaque and psoralen were from Sigma-Aldrich (St. Louis, MO). X-Vivo
medium was from BioWhittaker (Walkersville, MD), and DMEM was
purchased from CellGenix (Freiburg, Germany). Human type AB serum was purchased
from Gemini BioProducts (West Sacramento, CA). Anti-CD3/anti-CD28 and
OKT3 monoclonal antibody (MAb) was from OrthoBiotech (Bridgewater,
NJ). HIV-1 core antigen (clone KC57) was from Beckman Coulter (Fullerton,
CA). Anti-CD8 antibody-charged microbeads were from Miltenyi Biotec Inc. (Auburn,
CA). The annexin V and 7-amino-actinomycin D (7-AAD) reagents were pur-
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subcloned to obtain 2A9 cells, which stably express high levels of HIV-1 p24. The
2A9 subclone was used as a positive control for intracellular HIV p24 staining.

Uninfected A03.01 cells were used as a negative control. The cell lines were
subcultured in RPMI 1640 medium supplemented with 10% (vol/vol) human
type AB serum.

Endogenous virus isolation. Peripheral blood isolated from each of the
HIV-1- subjects was centrifuged on Ficoll-Hypaque gradients to recover the
PBMCs. Following a wash in Hanks’ balanced salt solution, the cells were treated
with trypsin blue dye, counted, and resuspended at a concentration of 1 × 10^6
cells/ml in complete RPMI 1640 medium supplemented with 20% (vol/vol) human
type AB serum. Depletion of CD8+ T cells was performed with magnetic
cell separation (MACS) columns by using microbeads charged with anti-CD8
antibodies. CD8-depleted PBMCs (i.e., PBMCs enriched in CD4+ T cells)
were resuspended at a final concentration of 1 × 10^6 cells/ml in a complete RPMI
1640 medium containing 1 μg/ml MAb OKT3. The cells were plated in T25
vented flasks (Corning, Lowell, MA) set upright with no more than 20 ml
of medium per flask. The flask were incubated for 24 h at 37°C in an atmosphere of
5% CO₂ in air. Following incubation, the MAb OKT3-containing medium was
removed by centrifugation, and the cells were resuspended at 1 × 10^6 cells/ml in
fresh RPMI 1640 medium supplemented with 10 IU/ml of IL-2 in T25 flasks. The
flasks were incubated as described above for 3 to 5 days, at which time (day 6 or
7), half the volume of the culture medium was removed and frozen at −20°C in
15-ml conical tubes. The medium that was removed was replaced with an aliquot of
fresh IL-2-containing medium plus freshly prepared autologous feeder cells. These
were PBMCs obtained from 20 ml of freshly drawn heparinized blood
enriched in CD4+ T cells by MACS and stimulated with OKT3, as described
above. In the initial experiments, anti-CD3/CD28 Ab-charged beads were used in
place of MAb OKT3 as an alternative T-cell stimulus. However, the addition of
anti-CD28 Ab did not maximize the virus yields, and subsequent isolations were
performed only with OKT3-charged beads. The cycles of medium harvesting and
addition of fresh autologous feeder cells on every 4th day continued for at least
4 weeks. Culture aliquots (viral supernatants) were tested for the presence of virus
by measuring the p24 levels.

Testing of T cells and culture supernatants for virus. Cells in cultures were
monitored for p24 expression by flow cytometry every 3 to 5 days. A total of 2 × 10^6
cells were microcentrifuged for 30 s. The cells were resuspended in 200 μl wash
medium (PBS [pH 7.4], 2% [vol/vol] heat-inactivated human type AB serum,
0.1% sodium azide), transferred to a well of the 96-well plate (Becton Dickenson,
Franklin Lakes, NJ), centrifuged, and resuspended in 200 μl of 1× PBS wash
medium containing 1% (vol/vol) pararosanilin red. Cells were again washed with
200 μl of wash medium. The cells were resuspended at a 1:240 dilution of antibody to
HIV-1 core antigen and incubated in the dark at 4°C for 1 h. After one more wash, the
cells were fixed in 1% (wt/vol) pararosanilin red and analyzed on a Coulter Epics XL-MCL flow cytometer. The
expression of p24 was routinely determined: it served as a screen because the
optimal time for virus production varied with T cells from different donors.

Once the cultures were positive for p24 expression, the titers in the reserved
supernatants were determined by using a Retro-Tek HIV-1 p24 antigen ELISA kit.
Culture supernatants containing p24 levels of 100 pg/ml or greater were
considered positive and were tested for viral infectivity in a TCID₅₀ assay before
they were used for subsequent superinfection of autologous CD4+ cells.

TCID₅₀ titer measurements. Cells of the TZM-b1 cell-line, which carry a copy of
the HIV promoter terminal repeat in tandem with the β-galactosidase
(β-Gal) enzyme gene, were used as indicator cells. If TZM-b1 cells become
infected with HIV-1, the β-Gal gene is also transcribed and is detected as a color
reaction. The optical density of each well was measured at 570 nm with a microplate
reader. The HIV titer was calculated as the TCID₅₀ by the Spearman-Karber method
(11).

Superinfection of CD4+ -enriched T cells with autologous HIV-1. PBMCs were
obtained from the venous blood of each patient by Ficoll-Hypaque centrifugation
or elutriation (for a large-scale production). Enrichment in CD4+ T cells was
performed by MACS, as described above, or with the ClinMACS system
(Milenyi Biotec).

CD4+ T cells were cultured at 1 × 10⁶ cells/ml in RPMI 1640 complete
medium containing 20% (vol/vol) heat-inactivated human type AB serum and
supplemented with 10 IU/ml human IL-2 and 1-μg/ml MAb OKT3 in upright 75-mm
flasks (Corning) at 37°C in a 5% CO₂ incubator for 24 to 72 h. The cells were
then washed with RPMI 1640 complete medium to remove MAb OKT3, and
HIV-1 superinfection was initiated.

Supernatants with high titers of autologous HIV-1 previously generated and
frozen were used for the superinfection of CD4+ cells. Autologous CD4+ cells
incubated at 37°C in a 5% CO2 incubator for 12 to 24 h. The matured DCs were poly(I-C) were added, as described by Mailliard et al. (16); and the cultures were of DiOC6 in PBS for 30 min at 37°C and were then washed three times in PBS. Cells were stained with 2 with a lipophilic cationic fluorochrome, DiOC 6. Cells were stained with 2 for p24.

Generation of DCs. PBMCs were obtained from each of the patients whose virus was isolated as described above by using heparinized venous blood (90 ml) or leukapheresis products (for large-scale production). Monocytes were separated by adherence to plastic or elutriation by using the Elutrap cell separation system (Gambro BCT, Lakewood, CO). Monocytes were recovered and plated in T162 flasks or cartridges, which are a component of the Aastrom Biosciences, Inc. (Ann Arbor, MI), Replicell system. In this closed computer-monitored system used for the large-scale manufacture of DCs, from 0.5 /H9262 to 2.5 /H9262 monocytes suspended in CellGenix DC medium supplemented with 1,000 U/ml IL-4 and 1,000 U/ml of recombinant human granulocyte-macrophage colony-stimulating factor were cultured for 6 days in a sealed cartridge. The cartridge was placed in a computer-monitored chamber of an incubator, and fresh medium was automatically added as needed. Immature DCs (iDCs) were harvested on day 6 or 7 with the Aastrom Replicell processor; washed in medium; counted; and IL-2 was added to adjust the cell density to 1 x 10^6 cells/ml. The cells were then cultured in upright T25 flasks at 37°C in a 5% CO2 incubator. Aliquots of cells were taken for analysis of p24 levels on days 4 to 10, as described above. The cultures were continued until at least 40 to 65% of the cells became positive for p24.

Psoralen and UVB irradiation treatment of superinfected CD4+ cells. The HIV-1 superinfected CD4+ cells were treated by psoralen and UVB irradiation to induce apoptosis and inactivate the virus. The cells were collected and washed several times with RPMI 1640 medium and were then resuspended in X-Vivo 10 medium containing 20 μg/ml psoralen at 1 x 10^6 to 10 x 10^6 cells/ml. The cells were aliquoted and placed into the wells of six-well tissue culture plates at 0.5 x 10^6 cells/well. With the lid off, the plates were placed 1 in. below a UVB light source (Spectronics, Westbury, NY) inside a biosafety cabinet for 30 min. The plates were rotated every 5 to 10 min. At the end of the irradiation, the cells were combined and washed in RPMI 1640 complete medium three times to remove the psoralen. Next, the cells were incubated in medium at 37°C in a 5% CO2 incubator for 12 to 24 h to allow them to undergo apoptosis. Apoptosis of CD4+ T cells was confirmed by comparing the annexin V binding of untreated and psoralen- and UVB irradiation-treated cells by flow cytometry. Aliquots of psoralen- and UVB irradiation-treated and untreated cells were also analyzed by the TCID50 assay to ensure that the virus had been inactivated.

Coculture of DCs with autologous apoptotic CD4+ T cells. To prepare the vaccine, HIV-1 superinfected apoptotic CD4+ T cells were cocultivated with autologous iDCs at an iDC/CD4+ T-cell ratio of 1:5.1. The cultures were set up in T25 flasks at a concentration of 1 x 10^5 to 1.5 x 10^5 cells/ml of CellGenix DC medium. Maturation cytokines consisting of a mix of 50 ng/ml tumor necrosis factor alpha, 25 ng/ml IL-1β, 1,000 U/ml IFN-γ, 3,000 U/ml IFN-α, and 20 μg/ml poly(I-C) were added, as described by Mailliard et al. (16); and the cultures were incubated at 37°C in a 5% CO2 incubator for 12 to 24 h. The matured DCs were then examined microscopically and by flow cytometry to verify that apoptotic bodies (Abs) were indeed ingested during coculture. Prior to psoralen and UVB irradiation treatment, an aliquot of superinfected CD4+ T cells was stained with a lipophilic cationic fluorochrome, DiOC6. Cells were stained with 2 μg/ml of DiOC6 in PBS for 30 min at 37°C and were then washed three times in PBS. They were resuspended in irradiation medium and subject to the psoralen and UVB irradiation treatment. These labeled CD4+ T cells were cocultured with autologous iDCs as described above and were then stained with anti-CD11c Ab (Beckman Coulter) for determination of the uptake of labeled AbBs by DCs by flow cytometry. An aliquot of the cocultured cells was also placed in a Lab-Tek II chamber slide system (Nalgene Nunc International Corp., Naperville, IL), stained with anti-CD11c Ab, and counterstained with a secondary Cy3-conjugated goat anti-mouse Ab for examination by confocal microscopy.

RESULTS

Cultivation and isolation of autologous HIV-1 from PBMCs. Four HIV-1" subjects not treated with ART were recruited for this study. The subjects donated blood weekly, so that fresh autologous feeder PBMCs were available for culture of enriched CD4+ T cells. The isolation of endogenous virus from the PBMCs of these subjects involved the depletion of CD8+ T cells by negative selection of CD4+ T cells on immune magnetic beads. The CD4+ T-cell-enriched fractions contained from 90 to 95% CD3+ CD4+ T cells, as determined by flow cytometry. In the initial experiments, the CD4+ T cells selected were stimulated with immunobeads coated with anti-CD3 and anti-CD28 MABs or anti-CD3 MAB overnight, centrifuged, washed, and resuspended in medium supplemented with IL-2. They were fed fresh autologous CD4+ T cells, and this process of feeding was repeated at weekly intervals for 4 to 6 weeks. Endogenous virus was successfully isolated from these CD4- cells from three of four subjects, as judged by the p24 levels in culture supernatants measured by ELISA (Table 1). We failed to isolate virus from one subject (subject 4), possibly because of the learning curve: this was the very first subject that we recruited. Stimulation with MAb OKT3 alone in the presence of autologous feeder cells gave better results than stimulation with anti-CD3/CD28 beads (Table 1). Allogeneic feeder cells obtained from healthy donors were not as effective

| subject no. | p24 level (ng/ml) | No. of TCID50/ml |
|-------------|-------------------|-----------------|
| 1           | 337               | 31,623          |
| 2           | 227               | 215             |
| 3           | 39                | 14,678          |
| 4           | 0.2               | 3               |

* The supranatants of cultures established with autologous feeder cells were harvested on days 10 to 19. The TCID50 was measured by colorimetric assays with the TZM-bl indicator cell line, as described in Materials and Methods.

### Table 1. Viral isolation in supernatants of CD4+ T-cell cultures determined by measurement of p24 levels

| HIV-1 subject no. | p24 concn (ng/ml) in the following feeder cells: |
|------------------|-----------------------------------------------|
| Allogeneic cells (healthy donor) | Autologous cells + anti-CD3 beads | Autologous cells + anti-CD3/CD28 beads |
| 1                | 38.0 >50 >50 |
| 2                | 2.7 >50 4.0 |
| 3                | 7.0 >50 6.3 |

* The supranatants of cultures established with various feeder cells (allogeneic cells, autologous cells plus anti-CD3 immunobeads, or autologous cells plus anti-CD3/CD28 immunobeads) were harvested on day 29 and tested for p24 levels by ELISA.
subject's CD4\(^+\) T-cell cultures were separated from the PBMCs of each subject by negative selection on immunobeads. Their own viral supernatants were used for superinfection. The percentage of p24\(^+\) cells was determined by flow cytometry, cell-associated p24 was assayed with lysed CD4\(^+\) T cells, and ELISA was used to measure p24 levels in CD4\(^+\) T-cell supernatants. The TCID\(_{50}\) was measured as described in Materials and Methods. Negative controls were uninfected autologous CD4\(^+\) T cells, and positive controls were cells of a CD4\(^+\) T-cell line infected with HIV-1 (2A9 subclone) in the laboratory.

In subsequent experiments, the viral infectivity of the cultures was determined in TCID\(_{50}\) assays, in addition to by determination of p24 levels (Table 2). The culture supernatants were repeatedly tested on various days (days 4 to 37), and the highest titers were obtained on days 10 to 19 (Table 2). One subject's CD4\(^+\) T-cell cultures (subject 4) were negative for p24 and infectious virus. On the basis of the data obtained with the cells of subject 3 (Table 2), the supernatant of which had a p24 level of 39 ng/ml and over 14,000 TCID\(_{50}/\)ml, it was arbitrarily determined that a level of >25 ng/ml p24 in the supernatant likely reflected successful viral isolation and, therefore, that the culture supernatant could be used for the superinfection of autologous CD4\(^+\) CD8\(^-\) T cells. Consequently, the viral supernatants of cell cultures for subjects 1, 2, and 3 were aliquoted and frozen for use in the superinfection experiments.

### TABLE 3. Superinfection of autologous CD4\(^+\) CD8\(^-\) T cells with viral supernatants obtained from three HIV-1 subjects\(^a\)

| Subject (day) | % p24\(^+\) cells | p24 concn | No. of TCID\(_{50}\)/ml |
|--------------|-------------------|-----------|------------------------|
| 1 (5)        | 30                | 1.7       | 55                     | 31,623                  |
| 2 (10)       | 5                 | 0.8       | 15                     | 464                     |
| 3 (6)        | 18                | 2.3       | 0.1                    | 10,000                  |
| Positive controls | 52                | 3.6       | 17                     | 3,162                   |
| Negative controls | 0                 | 0         | 0                      | 0                       |

\(^a\) Autologous CD4\(^+\) T cells were cultured in the presence of viral supernatants for 5 to 10 days, and the percentage of p24-positive (p24\(^+\)) cells as well as p24 levels in cells and culture supernatants were measured each day. The highest values obtained are presented. CD4\(^+\) T cells were separated from the PBMCs of each subject by negative selection on immunobeads. Their own viral supernatants were used for superinfection.

**Superinfection of CD4\(^+\) CD8\(^-\) T cells with autologous virus.** Experiments were initially performed with the preparations of enriched CD4\(^+\) T cells obtained from the PBMCs of a healthy donor by negative selection on immunobeads. These cells were incubated in the presence of the cryopreserved and thawed viral culture supernatants. The results indicated that superinfection of CD4\(^+\) T cells from healthy subjects with the viral supernatants of HIV-1 subjects was successful. For example, on day 5 of culture with the viral supernatant of healthy subject 1, 28% of CD4\(^+\) T cells were p24 positive, as indicated by intracytoplasmic staining for p24 expression by flow cytometry.

The level of p24 in the supernatant was 20 ng/ml for this subject. Therefore, the conditions established for superinfection of CD4\(^+\) T cells from healthy donors were next used for infection of freshly harvested CD4\(^+\) CD8\(^-\) T cells from the three HIV-1 subjects who had donated PBMCs for viral isolation and whose culture supernatants were positive for HIV-1, as shown in Table 1. The subjects' enriched CD4\(^+\) T cells (98% purity) were cultured with the individual viral culture supernatants, which were thawed and added to the cells. Superinfection was evaluated by intracytoplasmic p24 staining and flow cytometry and by testing lysed CD4\(^+\) T cells and their supernatants for p24 by ELISA daily for 4 to 10 days after infection. Table 3 presents the highest values obtained for the cells and infectious supernatant of all three HIV-1 subjects on the indicated day. In aggregate, the results of these experiments confirmed that substantial levels of p24 can be detected in superinfected CD4\(^+\) T cells and their supernatants following 5 to 10 days of incubation with cryopreserved and thawed autologous viral culture supernatants.

**Psoralen and UVB light inactivation of autologous virus.** To inactivate the virus, we used UVB light (3 mW/cm\(^2\)) in the presence of psoralen (20 \(\mu\)g/ml) and monitored viral infectivity by use of the TCID\(_{50}\) assay. The experiments were first performed with primary CD4\(^+\) T cells expressing high titers of HIV IIb (Fig. 1). Having established the conditions for virus inactivation that resulted in a 99.99% decrease in the viral titer (4 log\(_{10}\) units), we next applied the procedure to HIV-1 superinfected CD4\(^+\) T cells from the three subjects included in the experiments.

![FIG. 1.](http://cvi.asm.org/Downloaded from http://cvi.asm.org/ on April 27, 2019 by guest)
study. Table 4 summarizes the results of these experiments and shows that the treatment consistently reduced the viral titers in the treated CD4\(^+\)/H11001 T cells to essentially undetectable levels of infectious virus.

**Apoptosis of CD4\(^+\)/CD8\(^-\) T cells after psoralen and UVB light treatment.** We have previously shown that psoralen and UV light treatment result in the apoptosis of HIV-1-infected CD4\(^+\)/H11001 T cells that can serve as a source of antigen (30). In the present experiments, we determined the effects of psoralen and UVB light treatment on CD4\(^+\)/H11001 T-cell viability and apoptosis. As illustrated in Fig. 2, after only 30 min of irradiation in the presence of psoralen, 95% of the cells were annexin V positive and almost 75% were also 7-AAD positive, suggesting apoptosis and/or necrosis of the virally infected CD4\(^+\)/H11001 T cells. This was a desirable result, because the vaccine was to be prepared with DC-fed ApBs of autologous CD4\(^+\)/H11001 T cells containing inactivated endogenous HIV-1.

**Loading of DCs with endogenous virus-infected, inactivated CD4\(^+\)/CD8\(^-\) T cells.** After the psoralen and UVB light treatment, ApBs of CD4\(^+\)/CD8\(^-\) T cells were fed to autologous iDCs. The uptake of ApB by iDCs was monitored by flow cytometry by using aliquots of superinfected CD4\(^+\)/CD8\(^-\) T cells labeled with DiOC\(_6\) prior to the psoralen and UVB light treatment. As shown in Fig. 3, coincubation of the iDCs with labeled autologous CD4\(^+\)/H11001 T cells in the presence of maturation cytokines for 12 h resulted in the uptake of ApBs by the majority of DCs.

**Generation of monocyte-derived DCs from HIV-1\(^+\) subjects.** We have previously reported that monocyte-derived DCs can be successfully obtained from subjects with HIV-1 infection (7). However, those studies used different techniques for the generation of DCs or the subjects had undergone ART and their viral titers were reduced (3). As it was not certain that DCs can be reliably generated by our new procedures from HIV-1\(^+\) subjects who were not on ART, we proceeded to generate DCs from two of the three patients on a therapeutic scale, using leukapheresis as a source of peripheral blood monocytes. The subjects signed informed consent and agreed to undergo leukapheresis. Elutriation was used for the isolation of monocytes from PBMCs, and separation of CD4\(^+\)/CD8\(^-\) T cells was performed on a CliniMACS instrument. The method selected for DC maturation involved the mix of cytokines made up to induce the polarization of DC to DC1, as reported previously (16). The phenotypic characteristics of DCs of one of the patients are summarized in Table 5. In aggregate, the data from two scale-up experiments showed that the DCs generated from the monocytes of untreated subjects with chronic HIV-1 infection have the phenotypic and functional properties (i.e., IL-12 p70 production) similar to those previously generated from healthy donors or HIV-1-infected subjects receiving ART therapy (3, 5).

The ApB-loaded autologous DCs were shown to meet the release criteria established by our laboratory for therapeutic DC product release (i.e., 14-day sterility, negative results; gram stain, negative for mycoplasmas and endotoxin; viability, >75%; purity, >75%; stability, >4 h at room temperature) (Table 6).

A summary of the process of manufacturing a vaccine containing autologous DCs loaded with ApBs of inactivated endogenous HIV-1 is shown in Table 7.

### Table 4. HIV-1 inactivation in CD4\(^+\)/CD8\(^-\) T cells by UVB light and psoralen\(^a\)

| Cell treatment       | TCID\(_{50}\)/ml for subject no.: |
|----------------------|----------------------------------|
|                      | 1      | 2      | 3      |
| Untreated            | 31,623 | 2,154  | 14,678 |
| Treated              | 22     | 2      | 3      |
| Positive control (not treated) | 681    | 3,162  | 3,162  |

\(^a\) CD4\(^+\)/CD8\(^-\) T cells obtained from all three subjects were superinfected with the autologous virus supernatants and were treated with UVB light (3 mW/m\(^2\)) and psoralen (20 \(\mu\)g/ml) for 30 min to inactivate the virus. Supernatants were tested for infectivity in TCID\(_{50}\) assays prior to and after treatment. HIV IIIb-infected cells were used as a positive control.
DISCUSSION

The ex vivo loading of DCs with ApBs of autologous CD4<sup>+</sup>-CD8<sup>+</sup>-T cells which had been superinfected with endogenous inactivated HIV-1 could potentially produce an anti-HIV-1 vaccine that would be effective in the control of chronic HIV-1 infection. The rationale for selecting this strategy is based on evidence that DCs loaded with HIV-1-infected apoptotic cells can stimulate both anti-HIV-1 CD8<sup>+</sup> and anti-HIV-1 CD4<sup>+</sup>-T-cell responses in vitro (12, 31). Furthermore, there is well-documented evidence for the existence of HIV-1 variants or quasispecies even in subjects treated with ART (2). Moreover, it has been established that infectious viruses can be isolated from PBMCs for several years while the individuals are on ART, even when virus RNA is not detectable in plasma (25). The diversity of viral quasispecies is very high due to rapid viral replication. The presence and extent of the diversity of quasispecies is expected to have a strong impact on antiviral immune responses (2). Immune responses are broadly targeted and are likely to be less effective in the presence of rapidly arising and highly diverse viral quasispecies (29). The recognition of viral proteins by T cells is highly specific, and the diversity of the quasispecies requires the continuous production of new effector T cells in a futile attempt to control infection. Immunotherapy as an adjunct to ART is expected to help the immune system control HIV-1; and DC-based vaccines targeting endogenous HIV-1, presumably including its multiple variants, might be particularly efficacious. The advantage of targeting autologous virus representing a large reper-

TABLE 5. Characteristics of DCs generated from monocytes of an HIV-1<sup>+</sup> subject

| Phenotype | iDCs | αDC1 cells alone<sup>a</sup> | αDC1 cells + ApBs |
|-----------|-----|---------------------------|-------------------|
| HLA-DR    | 86  | 94                        | 97                |
| CD80      | 50  | 94                        | 93                |
| CD83      | 4   | 60                        | 49                |
| CD86      | 19  | 86                        | 91                |
| CD11c     | 97  | 96                        | 96                |
| CD40      | 86  | 96                        | 97                |
| CCR7      | 10  | 45                        | 42                |

<sup>a</sup> DC characteristics before and after they were loaded with ApBs of autologous CD4<sup>+</sup>-CD8<sup>+</sup>-T cells superinfected with endogenous virus.

TABLE 6. Criteria established for therapeutic DC product release<sup>a</sup>

| Parameter | Cell count | % Purity<sup>b</sup> |
|-----------|------------|-----------------------|
| Initial white blood cell count | 3.9 x 10<sup>10</sup> |  |
| Elutra fraction 2 (CD4<sup>+</sup>CD14<sup>-</sup>) | 1.2 x 10<sup>10</sup> | 99 |
| Elutra fraction 5 (CD4<sup>+</sup>CD14<sup>-</sup>) | 5.6 x 10<sup>9</sup> | 77 |
| Post-CliniMACS CD4<sup>+</sup>CD8<sup>-</sup>T cells | 9.8 x 10<sup>8</sup> | 95 |

<sup>a</sup> The criteria established by our laboratory for therapeutic DC product release are as follows: recovery, 57%; viability, 87% after coincubation with apoptotic CD4<sup>+</sup>-CD8<sup>-</sup>-T cells; sterility, mycoplasma and endotoxin negative at 14 days; level of IL-12 p70 production, 473 pg/ml; TCID<sub>50</sub>/ml for untreated CD4<sup>+</sup>-CD8<sup>-</sup>-T cells, 1,467,799; TCID<sub>50</sub>/ml for mDCs plus ApBs, 3; and stability at room temperature (on the basis of the DC viability and phenotype), 4 h.

<sup>b</sup> The purity of DCs was determined by flow cytometry, as described in Materials and Methods.
Separate CD4+ monocytes and T cells by using the Elutra system.

Proceed with leukapheresis of the subject.

Freeze virus-positive supernatants obtained from patients with cancer or HIV-1 infection in our laboratory and has been broadly used with peripheral blood products in clinical use. Large-scale, clinical-grade DC preparation is routinely performed on a small scale, to demonstrate that viral isolation, inactivation of the virus, and loading of the inactivated virus into autologous DCs can be accomplished in a research laboratory. With the successful production of viral supernatants in three of four HIV-1+ subjects, the procedure was scaled up for clinical use. Large-scale, clinical-grade DC preparation is routine and has been broadly used with peripheral blood products obtained from patients with cancer or HIV-1 infection in our and other laboratories (5, 17, 18, 21, 26, 30). Although the entire HIV-1 vaccine production process is complex, it was shown to be feasible and applicable to the controlled good manufacturing practice setting. The multiple steps involved in the production of the vaccine can be timed to accommodate the anticipated therapy with ART, followed by treatment interruption prior to DC generation and therapeutic vaccine delivery.

The preclinical feasibility studies described here indicate that the autologous virus-based vaccine can now be routinely produced for a clinical trial. The numbers, viability, phenotypic characteristics, and stability of the DC-fed ApBs of CD4+ T cells infected with endogenous virus will be considered in defining the release criteria for the vaccine. On the basis of the reported data, we have initiated a phase I clinical vaccination trial under Investigational New Drug application no. 13137 and Institutional Review Board approval no. 0702006 for patients with chronic HIV-1 infection at the University of Pittsburgh Medical Center. It remains to be determined whether this strategy will prove to be clinically effective.

ACKNOWLEDGMENTS

This study was supported in part by Production Assistance for Cellular Therapies (PACT) under contract N01-HB-37165 from the National Heart, Lung, and Blood Institute to T.L.W. and by NIH grant U19 AI055794 to C.R.R.

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