Memory Cytotoxic T Lymphocyte Responses in Human Immunodeficiency Virus Type 1 (HIV-1)–Negative Volunteers Immunized with a Recombinant Canarypox Expressing gp160 of HIV-1 and Boosted with a Recombinant gp160

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A vaccine against human immunodeficiency virus (HIV) should induce virus-specific cytotoxic T lymphocyte (CTL) activity. Immunization of uninfected volunteers with a canarypox virus expressing HIV envelope was carried out in a phase I trial. Two injections of canarypox expressing HIV-1MN gp160 (months 0 and 1) were followed by two boosts of recombinant envelope protein (months 3 and 6). HIV envelope-specific CTL were detected in peripheral blood mononuclear cells stimulated with autologous HIV-1-infected blast cells. T cell lines were obtained from 18 of 20 donors: CTL were detected at least once following immunization in 7 (39%) of these 18. This activity was mediated by major histocompatibility complex class I–restricted CD3+CD8+ T cells. For two subjects, this activity was still present 2 years after the initial immunization. The CTL responses with this prime-boost regimen are the best observed with any HIV vaccine tested in humans.

Vaccination against the human immunodeficiency virus type 1 (HIV-1) is a key strategy for the eventual control of the AIDS pandemic. Since the discovery of the etiologic agent of AIDS, different candidate vaccines have been designed and developed, including whole killed virus, live attenuated virus, virus-like particles including pseudovirions, protein subunits based on HIV structural genes, peptides based on selected immunoreactive parts of the viral proteins, live recombinant viral or bacterial vectors, and DNA-based immunogens encoding one or more HIV proteins [1–3]. Live recombinant vaccinia viruses have been used successfully to induce protective immunity against animal infectious diseases such as rabies [4], but because the use of vaccinia virus for human vaccination against smallpox has been shown to induce rare but serious complications [5], other recombinant poxviruses such as avian poxviruses have been developed [6, 7]. As was the case for the most live expression vectors in AIDS vaccine studies, initial efforts have focused on products based on the HIV-1 envelope protein, since several epitopes of env have been described to induce neutralizing antibodies and cell-mediated immune responses, including cytotoxic T lymphocytes (CTL). In HIV-seronegative volunteers at low risk of HIV infection, the safety and immunogenicity of a combined vaccine regimen consisting of priming with a recombinant canarypox virus expressing the HIV-1MN env gene (ALVAC-HIV, vCP125) followed by booster immunizations with a soluble recombinant envelope glycoprotein gp160MN/LAI has been reported [8]. The characterization of the CD8-mediated, major histocompatibility complex (MHC) class I–restricted cytolytic response elicited in 39% of the vaccinees is presented here.

Materials and Methods

Study Population

Twenty volunteers, 10 men and 10 women, were enrolled into the study as described [8]. All were healthy HIV-1–seronegative adults with low risk for HIV-1 exposure.

Vaccine Products and Protocol Design

The recombinant canarypox expressing the HIV-1 gp160 gene from the MN isolate (ALVAC-HIV; vCP125; Virogenetics, Troy, NY) and the soluble recombinant hybrid envelope MN/LAI (rgp160; Transgène, Strasbourg, France) were previously described in detail together with the results obtained on the safety of this phase I, randomized, open-labeled trial [8]. All subjects received 10⁶ TCID₅₀ of vCP125 at months 0 and 1 followed by booster injections of 176 μg of rgp160 formulated in alum (n = 10) or in incomplete Freund’s adjuvant (Montanide ISA 51; Seppic, Paris) (n = 10) at months 3 and 6 according to random assignment.

Cytotoxic Assays

Target cells. For each patient, an autologous Epstein-Barr virus (EBV)–transformed B cell line was obtained by infection of peripheral blood mononuclear cells (PBMC) with supernatant from...
the EBV-producing cell line B95-8 (ATCC CRL 1612). EBV-
transformed B cells (3 × 10^6) were infected with the Copenhagen
strain of wild type vaccinia virus (vvWT) or with the various
recombinant vaccinia viruses at an MOI of 10 pfu/cell, for 1 h at
37°C, then washed and cultured for 16 h at 37°C in 5% CO₂ before
their use in the cytotoxic assays. Recombinant vaccinia viruses
vvTG1139 and vvTG5167, encoding the HIV-1,LA1 and the HIV-
1,MIN env proteins, respectively, have been described [9]. Where
cited, recombinant vaccinia viruses encoding signal peptide dele-
tions of HIV-1,LA1 or HIV-1,MIN env proteins (vvTG1383 and
vvTG6110, respectively) were used [9, 10]. The human K562 cell
line (ATCC CCL243) was used as a target to detect NK activity.

Effectors cells. PBMC were isolated from heparinized blood
density gradient centrifugation on Ficoll-Paque (Pharmacia, Les
Ulis, France). PBMC collected at different times after immuniza-
tion were aliquoted and frozen in liquid nitrogen. CTL lines were
generated by two different procedures using either nonspecific or
HIV-specific stimulation. Nonspecific stimulation was done after
in vitro activation of thawed PBMC with an anti-CD3 monoclonal
antibody (OKT3; Ortho Diagnostics System, Roissy, France; 25
ng/mL) in RPMIc: RPMI 1640 (reference catalog 12-167B; Whit-
taker, Gagny, France) supplemented with 5% heat-inactivated hu-
an AB serum (ETS, Les Ulis, France), 1-glutamine (2 mM, refer-
ence 043-05030H; GIBCO, Cergy Pontoise, France), sodium
pyruvate (1 mM, reference 043-01360H; GIBCO), and nonessent-
ial amino acids (1X; reference 043-01140H; GIBCO). HIV-spe-
cific stimulation was done by in vitro activation of thawed PBMC
with autologous irradiated phytohemagglutinin (PHA)-stimulated
blasts (2 µg/mL; Difco, Paris) infected 6 or 7 days previously with
HIV-1,LA1 as stimulator cells (responder/stimulator ratio = 5/1) in
RPMIc. After 3 days and for both nonspecific and HIV-specific
stimulation protocols, the cells were washed and resuspended (10^6/
ML) in RPMIc supplemented with 10% T cell growth factor
(Lymphocult-T-LF; Biotest, Buc, France), sodium pyruvate
(1 mM, reference 043-01360H; GIBCO), and nonessential
amino acids (1X; reference 043-01140H; GIBCO). HIV-spe-
cific stimulation was done by in vitro activation of thawed PBMC
with autologous irradiated phytohemagglutinin (PHA)-stimulated
blasts (2 µg/mL; Difco, Paris) infected 6 or 7 days previously with
HIV-1,LA1 as stimulator cells (responder/stimulator ratio = 5/1) in
RPMIc. After 3 days and for both nonspecific and HIV-specific
stimulation protocols, the cells were washed and resuspended (10^6/
ML) in RPMIc supplemented with 10% T cell growth factor
(Lymphocult-T-LF; Biotest, Buc, France). Four days later, recom-
binant human interleukin-2 (100 IU/mL, gift of D. Lando, Rousset
Uclaf, Romainville, France) was added to the cultures. The cultures
were fed every 3–5 days for 2–6 weeks. The cytolytic activity
and the characteristics of the T cell lines mediating this activity
were evaluated from day 12 to day 35 after in vitro stimulation
[9–11].

Chromium release assays. Cytotoxic assays were conventional
4-h ⁵¹Cr release assays as described [12]. Briefly, 10⁵ target cells
were labeled for 1 h with 3.7 MBq of [⁵¹Cr]Na₂CrO₄ (reference
62014; ICN, Orsay, France), washed three times, and distributed at
5 × 10⁶ cells/well in 0.1 mL of medium in round-bottom 96-
well microtiter plates (Costar; OSI, Maurepas, France). Various
concentrations of effector cells in 0.1 mL (in triplicate or quadru-
plicate) were added. The plates were incubated at 37°C in 5% CO₂
for 4 h, then centrifuged at 150 g for 5 min. Fifty microliters of
supernatant from each well was transferred together with 100 µL
of scintillation liquid (Optiphase Hisafe 3; EGG, Evry, France)
into a 96-well harvesting plate (reference 81150401; EGG), and the
radioactivity was measured in a beta counter (Microbeta 1450; EGG).
The percentage of specific lysis was calculated as 100 × ([experimental release – spontaneous release]/(maximal release –
spontaneous release)). Maximal release was obtained from targets
lysed by 5% Triton X-100 with 1% SDS. Spontaneous release was
obtained from targets incubated for 4 h in medium alone. Assays
in which spontaneous ⁵¹Cr release exceeded 35% were discarded.

if they exceeded the mean of vvWT-specific lysis by 3 SD and by
10%.

**Table 1.** Longitudinal analysis of envelope-specific CTL activity in
volunteers primed with canarypox virus expressing HIV envelope and
boosted with recombinant gp160.

<table>
<thead>
<tr>
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<th>12</th>
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</table>
| **Note.** Subjects received 10⁵ TCID₅₀ of cCPI25 at months 0 and 1,
followed by booster injections of 176 µg of gp160 formulated in alum or in
incomplete Freund's adjuvant (n = 10 in each group) at months 3 and 6
according to random assignment. Boldface indicates subjects with env-specific
lytic activity. *, no specific lysis; +, specific lysis (results were derived from cytotoxic
assays in which 3 consecutive effector-to-target ratios, routinely 60/1, 20/1,
and 7/1, were used); ND, not done; NG, no growth.

* After first immunization.

Results

**Envelope-specific cytolytic activity is detected after immunization.** T cell lines were obtained from 18 of 20 donors; for
2 donors, it was not possible to grow the PBMC after in vitro
stimulation. Nonspecific stimulation with anti-CD3 monoclonal
antibodies did not provide detectable env-specific CTL activity
(not shown). On the contrary, HIV-1 envelope–specific cyto-
lytic activity was only observed when the T cell lines were
grown in response to recombinant gp160 (CD3), phycoerythrin-conjugated anti–Leu-1c (CD16)
(Perilyte Dickinson, Becton Dickinson, Le Pont de Clai, France), and phycoerythrin-con-
jugated OKT4 (Ortho). Samples were analyzed with a FACS-
can flow cytometer (Becton Dickinson).

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immunization). The cytolytic activity was tested for from days 13 to 35 after the initiation of the culture and was found from days 21 to 35. For each postimmunization time, the results generally agree: for example, donors 102, 106, 108, 110, and 117 were tested three or four different times after immunization and were negative each time. For 6 of the 7 donors who had an env-specific CTL response, this activity was detectable at two different times following immunization.

For 3 subjects (101, 105, and 107), the cytolytic activity was detected at month 2, 1 month after the second ALVAC-HIV administration. The cytolytic activity was still present at months 7 or 12, 1-6 months after the second immunization with rgp160. Four other subjects were positive: at months 4 and 7 for subject 112, at months 7 and 24 for subjects 113 and 116, and at month 12 for subject 115. No significant difference was observed between the alum versus incomplete Freund's adjuvant groups. As shown in figure 1 and table 1, envelope-specific cytolytic activity was detected from PBMC collected from donor 113 at month 7, but no envelope-specific cytolytic activity was detected from the T cell lines derived from preimmune PBMC.

**Envelope-specific cytolytic activity is mediated by CD3⁺CD8⁺ MHC class I-restricted T cells.** Most of the cell lines mediating env-specific cytolytic activity were CD8⁺ T cells (64%-90%) with <4% CD4 cells as shown by cell cytometric analysis. As an example, envelope-specific effector cells from subject 107, measured 28 days after HIV-specific in vitro stimulation of the PBMC collected at month 12, were 97% CD8⁺ T cells, and the cytolytic activity was directed against the envelope of both MN and LAI isolates (table 2). In this experiment, the target cells were infected with vvTG 3183 and vvTG 6110, which encode the signal peptide deletion mutant of HIV-1 env.

**Table 2.** HIV envelope-specific cytotoxicity is mediated by CD8⁺ T cells.

<table>
<thead>
<tr>
<th>Target/Effector</th>
<th>Effector-to-target ratio</th>
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<tbody>
<tr>
<td></td>
<td>60/1</td>
</tr>
<tr>
<td>vvWT/M0</td>
<td>18 (1.4)</td>
</tr>
<tr>
<td>vvWT/M7</td>
<td>vvTG3183</td>
</tr>
<tr>
<td>vv6110/M0</td>
<td>vvTG6110</td>
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</tbody>
</table>

NOTE. Effectors were derived from peripheral blood mononuclear cells of volunteer 107, at month 12, and stimulated in vitro with autologous HIV-1-infected blasts. Targets were autologous Epstein-Barr virus–transformed B cells infected 16 h before with wild type vaccinia virus (vvWT) or with recombinant vaccinia virus encoding for signal peptide–deleted env gene of HIV-1 (vvTG3183) or HIV-1Δen (vvTG6110). Data are mean % specific lysis of triplicate (SD). Positive results are indicated in boldface type.

Figure 1. Cytotoxic activity is envelope-specific and is detected after immunization. Targets: Epstein-Barr virus–transformed B cells infected with vvWT (wild type vaccinia virus) or vv5167 (recombinant vaccinia virus expressing env gene of HIV-1Δen). Effectors: cell lines derived from peripheral blood mononuclear cells from subject 113, collected at months (M) 0 and 7. CTL activity was tested 21 days after initiation of culture after in vitro stimulation with autologous HIV-1-infected blasts.

Figure 2. Envelope-specific cytotoxic activity is major histocompatibility complex class I-restricted. Targets: autologous (auto) or heterologous (hetero) Epstein-Barr virus–transformed B cells infected with vvWT (wild type vaccinia virus) or vv6110 (recombinant vaccinia virus synthesizing the env protein of HIV-1Δen). Effectors: cell line derived from peripheral blood mononuclear cells from subject 105, collected at month 2. CTL activity was tested 29 days after initiation of in vitro stimulation with autologous HIV-1-infected blasts.
after the last rgp160 boost (month 12). Three subjects who had env-specific CTL activity at one or more of these times (112, 113, and 116) were tested 18 months after the last rgp160 boost, 2 years after the first ALVAC-HIV immunization. Three weeks after in vitro stimulation with autologous HIV-1-infected blasts, 2 of the 3 cultures had env-specific cytolytic activity (table 1). The results of a positive T cell line from subject 116 are shown in figure 3. At day 21 after in vitro stimulation with autologous HIV-1-infected blasts, there was no detectable env-specific CTL activity with PBMC collected at month 0, but env-specific activity was observed with the PBMC collected at months 7 and 24. The T cell line was further restimulated with PHA (2 μg/mL), and the cells mediating the env-specific cytolytic activity were found to be 94% CD3+CD8+ by cell cytometric analysis. Further, the activity was shown to be MHC class I-restricted. A panel of allogeneic target cells matched at one or more HLA class I alleles infected with a recombinant vaccinia virus encoding HIV-1, env (vv-env) were used as target for this CTL line. In addition to the autologous vv-env-infected targets, one EBV target (target 1) was also lysed when infected with vv-env (table 3). This target and autologous target share HLA A32 and C7. Target 4, sharing HLA A29 and C7, was not lysed; the HLA-restricting antigen was thus demonstrated to be A32.

Discussion

This report shows that immunization with a live recombinant canarypox virus vector (ALVAC) expressing the HIV-1, env gene (vCP125) induced HIV-1 envelope-specific CTL activity in 7 (39%) of the 18 HIV-1-seronegative human volunteers tested. For 6 of 7 donors, cytolytic activity was detected at various times after immunization (subjects 101, 105, 107, 112, 113, and 116; table 1). In some cases, the cytotoxic response was transient (table 1). For example, the cytotoxic response of donor 112 was present at months 4 and 7 but not at months 12 and 24; this might be due to the loss of memory T cells in this donor. Consistent with this hypothesis, 9 of the 11 donors tested for CTL activity at month 12 were negative. For donor 101, the cytotoxic response was present at months 2 and 7 not at months 4 and 12. In this case, the absence of detectable CTL activity at month 4 might be due to fluctuations in the kinetics of the CTL response in vitro, thus reflecting the tremendous difficulty in assessing the capacity of vaccine candidates to induce CTL. These results reinforce the need to establish a consensus for techniques to detect CTL in vaccinees.

PBMC from 3 of 10 subjects tested at month 2 were found to contain envelope-specific cytolytic activity, showing that the recombinant canarypox per se was sufficient for CTL induction, despite the absence of detectable HIV-specific antibodies by ELISA or Western blot [8]. Five of the 11 subjects tested at month 7 and 4 of the 13 tested at month 12 or 24 were positive for envelope cytolytic activity. There were no major differences in the frequency of donors with a positive CTL response after rgp160 boost, but there was a significant increase, for all subjects, in anti-gp160 and anti-V3 antibodies after each rgp160 boost [8]. The response with the preimmune PBMC was negative. No significant difference could be attributed to the specific adjuvant used; in fact, the overall frequency of responses was 4 of 9 for subjects who had received

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**Table 3.** CTL line 116 is restricted by HLA A32.

<table>
<thead>
<tr>
<th>HLA alleles,* targets</th>
<th>Effector-to-target ratio</th>
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<tr>
<td></td>
<td>30/1</td>
</tr>
<tr>
<td>A29,32 B7,17 C7 (autologous) vvWT</td>
<td>4.5 (3.5)</td>
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<tr>
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<td>vvTG6110</td>
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<tr>
<td>A32,2 B8,51 C1,7 (target 1) vvWT</td>
<td>1.2 (1.6)</td>
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<td>A28,30 B7,55 C2,6 (target 2) vvWT</td>
<td>35.8 (6.2)</td>
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<tr>
<td>A30,68 B53 C4 (target 3) vvWT</td>
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<tr>
<td>A1,29 B8,44 C5,7 (target 4) vvWT</td>
<td>1.9 (0.8)</td>
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<td>vvTG6110</td>
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NOTE. Effectors were CTL line from month 24 peripheral blood mononuclear cells from volunteer 116, stimulated with autologous HIV-1-infected blasts at day 0 and restimulated with phytohemagglutinin (2 μg/mL) at day 23; at day 13 of restimulation, cell line was 94% CD3 CD8 by cell cytometry. Chromium release assay was done 18 days after initiation of restimulation. vvWT, wild type vaccinia virus; vvTG6110, recombinant vaccinia virus encoding for signal peptide-deleted env gene of HIV-1. Data are mean % specific lysis of triplicate (SD). Positive results are indicated in boldface type.

* HLA alleles are in boldface type when they match with effector cells.

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**Figure 3.** Envelope-specific cytotoxic activity is present 2 years after first canarypox injection. Targets: autologous Epstein-Barr virus-transformed B cells infected with vvWT (wild type vaccinia virus) or vv5167 (recombinant vaccinia virus expressing env gene of HIV-1). Effectors: cell lines derived from peripheral blood mononuclear cells from subject 116, collected at month (M) 0, 7, or 24. CTL activity was tested 21 days after initiation of in vitro stimulation with autologous HIV-1-infected blasts.
alum-adjuvanted rgp160 and 3 of 9 for those who had received the subunit in incomplete Freund’s adjuvant.

PBMC from 3 subjects were assessed for HIV-specific CTL activity 2 years after the first ALVAC-HIV immunization. These 3 subjects were previously positive for env-specific cytotoxic activity at month 7. For 2 of them, CTL activity was still present in in vitro-stimulated PBMC cultures at this 2-year time point. This result indicates the persistence of memory CTL activity 18 months after the last immunization. There has been much interest in understanding how T cell memory is maintained, the dogma being that long-term T cell memory is dependent on persistent antigenic stimulation and presumably that the chronic stimulus originates from antigen persistence on follicular dendritic cells [14]. Recent experiments, however, describing data from three murine models of viral infection have challenged the current dogma and suggested that memory T cells generated in these experimental models did not require specific antigen for their persistence [15–17]. Because avian poxviruses undergo abortive replicative cycles in mammalian cells, it is unlikely that there exists a persistent reservoir of immunogen in the host. Thus, our data support models in which long-term memory CD8 CTL persist in the absence of the priming antigen, indicating that T cell memory might be independent of continuous antigenic exposure.

Induction of envelope-specific CTL activity has been reported from PBMC of HIV-1–seronegative human subjects immunized with HIV-1 gp160–based candidate vaccine [1]. The combination consisting of priming with a live recombinant vaccinia virus expressing HIV-1 envelope and boosting with a recombinant soluble gp160 envelope glycoprotein has been shown to enhance humoral antibodies and cytolytic responses compared with those induced by the rgp160 or recombinant vaccinia alone [18]. Recently, induction of HIV-1 CTL responses in seronegative adults by the same canarypox construct has been shown by Egan et al. [19]. These investigators described envelope-specific CTL detection in 3 (25%) of 12 subjects immunized with two or three injections of vCP125. The overall frequency of CTL responses reported herein was higher (7/18, 39%). This difference may be linked to the different stimulation protocols used (autologous EBV-transformed B cells infected with a recombinant vaccinia virus encoding the HIV-1 env gene, treated with psoralen and UV-light inactivated [19], vs. autologous irradiated HIV-1–infected PBMC in our experiments) or a unique assay at day 9 after in vitro stimulation for CTL detection [19] versus at least two assays between day 13 and 35 after in vitro stimulation in our experiments.

The CTL responses reported with this vaccine approach using a nonreplicating recombinant live vector and a boost with rgp160 are the best observed for any AIDS vaccine regimen tested, to date, in humans [1–3, 18, 19].

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References


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