Full Title: HIV-1 virion production from single inducible proviruses following T-cell activation ex vivo

John K Bui, 1 John W Mellors, 1 Anthony R Cillo*#  

Division of Infectious Diseases, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

Running Head (<54 characters): Characterization of induced HIV-1 virion production  

Abstract Word Count: 75 of 75  

Text word count: 996 of 1,000  

#Address correspondence to Anthony R. Cillo (arc85@pitt.edu) or John K. Bui (JOB74@pitt.edu)
Abstract (< 75 words)

Quantifying induced virion production from single proviruses is important for assessing the effects HIV-1 latency reversal agents. Limiting dilution ex vivo cultures of resting CD4+ T-cells from 14 HIV-positive volunteers revealed that virion production after T-cell activation from individual proviruses varies by 10,000 to 100,000-fold. High-producing proviruses were associated with increases in cell-associated HIV-1 DNA, suggesting that reactivated proviruses proliferate. Single-cell analyses are needed to investigate variation in proviral expansion and virus production following latency reversal.
HIV-1 virion production from resting CD4+ T-cells (rCD4) is commonly measured to assess the size of the latent reservoir and the effectiveness of latency reversal agents (1-4). Prior \textit{in vitro} and \textit{in vivo} studies have estimated the average viral burst size, defined as the total number of virions produced by an HIV-1-producing cell over its lifetime, to be 3–4 log_{10} virions/cell (5-9). However, most of these values were derived using parameters estimated from bulk proviral populations. No studies have quantified the distribution of virion production \textit{ex vivo} from individual reactivated proviruses.

To better understand latency reversal at the single proviral level, we isolated peripheral blood rCD4 from 14 HIV-1 infected participants on suppressive ART for \geq 2 years by negative selection as described (1). The study was approved by the University of Pittsburgh Institutional Review Board and all blood donors gave written informed consent. The rCD4 were serially diluted and stimulated for 7 days with anti-CD3/CD28 beads (Life Technologies) at 3 beads/cell in the presence of 300nM efavirenz and 100nM raltegravir, which blocks viral replication as determined by single genome sequencing analysis of supernatant HIV-1 RNA (data not shown). HIV-1 virion production was measured using Roche COBAS AmpliPrep/TaqMan v2.0 (1). Preliminary experiments demonstrated that virion production peaked after 7 days of stimulation with high cellular viability (data not shown). Using Poisson’s distribution, we identified 19 wells with \geq 96% chance of containing only 1 reactivated provirus. Virus production by these single proviruses varied by \sim 4 log_{10} HIV-1 RNA copies/provirus (range: 42–42,456) (Fig. 1).

To perform a more detailed analysis of single reactivated proviruses, rCD4 were isolated from a participant and cultured in 352 wells of 96-well plates at 74,000 cells/well (a concentration empirically determined to identify individual reactivated proviruses). The rCD4 were stimulated for 7 days with 50ng/mL phorbol 12-myristate 13-acetate (PMA) and 500ng/mL of ionomycin, in the presence of 300nM efavirenz and 100nM raltegravir.
To characterize the upper limit of virion production by individual reactivated proviruses, a screening method was developed to identify wells with high virion production. Aliquots of supernatant from wells in each plate row were pooled (12 wells/row, 32 rows total) and HIV-1 RNA was quantified using an integrase single-copy assay (iSCA) (10) that was modified to centrifuge supernatants at 21,000xg for 1 hour at 4°C. We identified six rows containing at least 1 provirus producing ≥2,000 HIV-1 RNA copies/well. For these six rows, the remaining supernatant from each well was extracted and HIV-1 RNA was quantified using the modified iSCA to identify wells containing single reactivated proviruses (Fig. 2). Virion production among individual reactivated proviruses from this donor spanned ~5 log_{10} HIV-1 RNA copies/provirus (range: 1–296,759).

Approximately 47% of the wells in the positively screened rows had detectable HIV-1 virion production following stimulation. According to Poisson’s distribution, ~80% of wells with detectable HIV-1 RNA are expected to contain a single expressing HIV-1 provirus. Single-genome sequencing (SGS) (11, 12) revealed that three of four wells had monotypic sequences with infrequent single base pair differences within the known error rate of SGS (~1.1x10^{-4} errors/nucleotide) (Fig. 3) (11), confirming that most wells with detectable HIV-1 RNA contained a single expressing provirus. The diversity in the fourth well (Fig. 3D) is likely a result of >1 reactivated proviruses rather than viral replication, which is blocked by 300nM efavirenz and 100nM raltegravir as described above.

We next quantified cell-associated HIV-1 DNA (CA-DNA) (1, 13) in 14 culture wells with >200 HIV-1 RNA copies/well and in 9 wells that produced <200 HIV-1 RNA copies/well. A statistically significant, positive correlation was found between the increase in CA-DNA in wells and HIV-1 RNA production (Spearman ρ=0.476, P=0.0338) (Fig. 4A). When grouped by the number of virions produced, wells with higher virion production (≥200 HIV-1 RNA copies/well) had greater CA-DNA than the lower producers (<200 HIV-1 RNA copies/well) and non-
producers (<1 HIV-1 RNA copy/well) (P=0.0186, P=0.0075, respectively, by Mann-Whitney U
with Bonferroni adjustment), suggesting that expansion and survival of virus-producing cells
may contribute to the higher virion production observed for some proviruses (Fig. 4B).

Interpretation of changes in CA-DNA is complicated, however, because wells with single
induced proviruses contained multiple non-induced proviruses. Specifically, an average of 144
HIV-1 DNA copies were seeded per well. Although the majority of proviruses (up to 98.5%) are
not inducible (1,14), both induced and non-induced proviruses can expand following activation.
Hence, the association between CA-DNA increases and higher virion production may arise
from: 1) selective proliferation of induced proviruses, 2) proliferation of non-induced proviruses,
or 3) proliferation of both induced and non-induced proviruses. Because CA-DNA increases
were only observed in wells with high virion production (Fig. 4B), proliferation of induced
proviruses could well have played a role.

Variable expansion of proviruses may be attributable to differences in proviral integration
sites and in infected T-cell subsets. Certain integration sites may promote survival and cellular
proliferation, as observed in vivo (15, 16). In addition, HIV-1 proviruses can be found across
many T-cell subsets (17, 18), which vary in proliferative and apoptotic potential (19). Virion
production may also vary as a result of differences in proviral transcription. Integrations in
inducible genes could contribute to higher levels of virion production, but could also lead to
lower virion production from transcriptional interference (15, 20, 21). Epigenetic modifications
that promote or inhibit HIV-1 transcription (22) may differ between individual infected cells.
Cells may also possess variable amounts of key transcription factors (e.g. P-TEFB, NF-kB) (23).
Finally, differential expression of inhibitory receptors (24) and cytokines (25) may limit T-cell
activation and HIV-1 transcription. Detailed single cell analyses are required to differentiate
among these mechanisms of cell proliferation and virion production.
In summary, analysis of single inducible proviruses reveals that induced virion production can vary by 100,000-fold. Given the wide range of virion production following latency reversal, results obtained from bulk cell cultures should be interpreted with caution. Detailed single cell analyses are needed to investigate the mechanisms that contribute to the wide variation in virion production and cellular proliferation following activation.

Acknowledgements

We thank the volunteers for participating in this study. We thank Roche Diagnostics for providing COBAS AmpliPrep/TaqMan kits. Funding was provided by the Pitt AIDS Research Training Program 5 T32 AI065380-08, the Howard Hughes Medical Institute Medical Research Fellows Program, and the Bill and Melinda Gates Foundation.
References


6. O'Connell KA, Rabi SA, Siliciano RF, Blankson JN. 2011. CD4+ T cells from elite suppressors are more susceptible to HIV-1 but produce fewer virions than cells from chronic progressors. Proc Natl Acad Sci U S A 108:E689-698.


Figures

Figure 1. (A) Schematic of theoretical results using limiting dilution method and use of Poisson statistics to calculate the probability that a culture with detectable HIV-1 virion production was produced by a single HIV-1 provirus. Wells with detectable HIV-1 virion production are represented by ‘+’ and the wells without detectable HIV-1 virion production are represented by ‘-.’ The Poisson statistics for each row are indicated in the table. $\lambda$ is the average rate of success, which is the fraction of wells with detectable HIV-1 virion production at a given dilution. $P(x\leq1)$ is the cumulative probability that a given well contains one or fewer expressing proviruses. (B) Distribution of induced HIV-1 virion production from individual proviruses in resting CD4+T-cells from 14-HIV infected donors. Purified resting CD4+T-cells were serially diluted and stimulated with anti-CD3/CD28 beads for 7 days. HIV-1 RNA in supernatant was quantified using the Roche COBAS AmpliPrep/TaqMan v2.0, and Poisson statistics were used to identify 19 wells in which single inducible proviruses were present. Only data from wells with detectable HIV-1 RNA are shown.

Figure 2. Detailed analysis of HIV-1 virion production from single inducible proviruses. Resting CD4+T-cells from a donor were serially diluted, maximally stimulated with PMA/ionomycin for 7 days, and then assayed for HIV-1 RNA in the supernatant using qRT-PCR. Data is shown from the 6 plate rows (72 wells total) that were positively screened to contain at least 1 provirus that produces ≥2,000 HIV-1 RNA cp/mL.

Figure 3. Single genome sequence and phylogenetic analysis of virions induced from proviruses at the limiting dilution endpoint. Closed symbols represent supernatant RNA sequences and open symbols represent a consensus HIV-1 subtype B sequence to which each tree was rooted. The single nucleotide differences of sequences in Panels A-C are within the
expected error rate of SGS (~1.1 x 10^{-4} errors per base sequenced or ~1-2 errors per 10^2 sequences) and are thus consistent with virus production from single proviruses. The sequences in Figure 3D show multiple nucleotide differences and therefore are likely derived from more than one reactivated provirus in that culture.

**Figure 4.** Higher virion production is associated with greater cell-associated HIV-1 DNA (CA-DNA) after activation of resting CD4^+ T-cells. Changes in CA-DNA after activation are relative to before activation. (A) Spearman correlation between HIV-1 virion production and change in CA-DNA in wells with detectable HIV-1 RNA (ρ = 0.476; p-value = 0.03). (B) Change in CA-DNA for non-producing wells (<1 copy HIV-1 RNA), low-producing wells (<200 copies HIV-1 RNA), and high-producing wells (>200 copies HIV-1 RNA); horizontal lines reflect the median; statistical significance was tested with the Mann-Whitney U test with a Bonferroni correction; NS = not significant, *P < 0.05, **P < 0.01.
Figure 1

A) 

B) 

<table>
<thead>
<tr>
<th>λ</th>
<th>P(x≤1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1.0</td>
<td>≤0.74</td>
</tr>
<tr>
<td>≥1.0</td>
<td>≤0.74</td>
</tr>
<tr>
<td>0.8</td>
<td>0.809</td>
</tr>
<tr>
<td>0.3</td>
<td>0.963</td>
</tr>
<tr>
<td>0.1</td>
<td>0.995</td>
</tr>
<tr>
<td>0.1</td>
<td>0.995</td>
</tr>
<tr>
<td>0</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 2

HIV-1 RNA Copies Produced (copies/well)

Frequency of Proviruses (%)
Figure 3

A)  

B)  

C)  

D)
Figure 4

A) 

B) 

Change in Cell-Associated HIV-1 DNA (copies / 74,000 rCD4 cells)