Characterization of Elite Suppressors
Cell-Associated HIV-1 mRNA at Baseline and with T Cell Activation

Christopher W. Pohlmyer1, C. Korin Bullen1, Alyssa R. Martin1, Gregory M. Laird1, Stanley U. Chioma, Victoria E.K. Walker-Sperling, and Joel N. Blankson*

Center for AIDS Research, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD

Objective: Elite Controllers or Suppressors (ES†) are patients who control HIV replication without antiretroviral therapy. In this study, we compared baseline and inducible HIV-1 mRNA levels in CD4+ T cells from ES and chronic progressors (CPs) receiving suppressive antiretroviral therapy. Methods: We quantified basal levels of cell associated HIV-1 mRNA in CD4+ T cells isolated from CPs and ES. Additionally, we measured the fold upregulation of intracellular HIV-mRNA after stimulation of CD4+ T cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin, and quantified the amount of HIV-mRNA levels released into culture supernatant. Results: ES have significantly less cell associated HIV-mRNA per 5x10^6 cells (p = 0.003); 8 of 10 CPs had quantifiable HIV-1 mRNA at baseline, whereas this was present in only 2 of 10 ES. Upon stimulation with PMA and ionomycin, 4 of 5 CPs and 7 of 9 ES showed increased cell associated HIV-mRNA. Interestingly, released HIV-1 mRNA could be detected in supernatants of CD4+ T cells stimulated with PMA/ionomycin from 5 of 8 ES. Conclusion: Our results demonstrate that while the baseline levels of cell associated HIV-1 mRNA are significantly lower in ES compared to CPs, stimulation of CD4+ T cells results in a comparable relative upregulation of viral transcription.

INTRODUCTION

Elite controllers or suppressors (ES) are individuals infected with HIV that maintain undetectable viral loads without receiving antiretroviral therapy (ART). Prior studies have shown that replication-competent virus can be cultured from CD4+ T cells from some ES [1-7] and full genome sequence analysis of replication competent virus has not revealed the presence of mutations associated with attenuation [1,2]. Furthermore, a recent study demonstrated that HIV-1 isolates from ES replicate vigorously and cause CD4+ T cell depletion in humanized mice [2]. Low levels of HIV-1 RNA can be detected in the plasma of most ES with ultrasensitive PCR assays [8-11], and sequence analysis has revealed evolution of plasma virus present in these patients [12-16]. These studies suggest that there is ongoing low-level replication in these patients, and yet in spite of this, ES maintain very small latent reservoirs as measured by total [5,17,18] and integrated DNA [19] as well as by the quantitative vi-
ral outgrowth assay [1] and the mouse viral outgrowth assay [20]. Despite the small reservoirs present in these patients, we hypothesized that ES would have relatively high levels of cell associated mRNA as a result of ongoing viral replication.

Hatano et al. have measured cell-associated in PB-MCs isolated from ES [11] and have compared cell associated RNA present in CD4+ T cells of ES and CPs isolated from gut-associated lymphoid tissue [21]. These studies, however, did not specifically quantify HIV-1 mRNA which may be a more direct indicator of HIV-1 transcription [22]. In the present study, we measure baseline and inducible levels of cell associated HIV-mRNA in peripheral CD4+ T cells of ES and CPs using a previously validated primer probe set [23]. These results further our understanding of the HIV-1 latent reservoir in ES.

**MATERIALS AND METHODS**

**Study Participants**

All studies were approved by the Johns Hopkins Institutional Review Board. All patients provided written informed consent before participation in this study. CPs are patients with undetectable viral loads on suppressive ART regimens for at least one year. ES have maintained undetectable viral loads by standard commercial assays without ART.

**Isolation of HIV-1 mRNA and DNA**

PBMCs were isolated from fresh blood samples by Ficoll gradient centrifugation. CD4+ T Cells were isolated using Miltenyi CD4+ T Cell Isolation Kit and cultured in RPMI1640 supplemented with 1% Pen/Strep and 10% FBS. 5x10^6 cells in 2 mL of complete media were either cultured in the presence of DMSO (0.2%) or PMA (50 ng/mL) and ionomycin (2 µM), or microbeads coated with antibodies against CD3 and CD28 (Dynal) for 24 hours and HIV-mRNA was isolated and quantified by qPCR. Fold upregulation upon stimulation is plotted. Squares are used to represent subjects where either the DMSO control value and/or the value from stimulated cells were below the limit of detection. In these subjects the limit of detection was arbitrarily used as the DMSO control value. No significance difference in fold upregulation was detected between the two populations after stimulation with PMA and ionomycin (p = 0.27 with all symbols, 0.24 without squares).

**Quantification of HIV-1 mRNA and DNA**

Total intracellular RNA was reverse transcribed using qScript from Quanta Biosciences as per manufacturer’s instructions. Reverse transcribed HIV-1 mRNA was
quantified as described previously [22] using a primer and probe set that anneals to a highly-conserved region of the 3’ end of HIV-1 mRNA [23]. The limit of quantification was set as the dilution point at which the Ct of the plasmid molecular standard replicates had an s.d. > 0.5. We determined that the limit of quantification for all intracellular HIV-mRNA transcripts was 10 copies [22]. HIV-1 proviral DNA levels were quantified by qPCR using ToughMix (Quanta Biosciences). Previously published primers that target a small region of Gag [24] were used to detect HIV-1 proviral DNA, and cellular input was quantified measuring RNaseP levels (ThermoFisher) of a human genome standard (Roche).

RESULTS

Cell associated HIV-1 mRNA from unstimulated CD4+ T cells ex vivo was quantifiable in only 2 of 10 ES (median < 10 copies/5M cells) as opposed to 8 of 10 CPs tested (median = 92 copies/5M cells; p = 0.03) (Figure 1a). Proviral HIV DNA was measured in 10 ES (median of 52 copies per 10⁶ CD4+ T cells) and 10 CPs (median of 309 copies per 10⁶ CD4+ T cells) to verify that our patients represent previously seen ES and CP cohorts (data not shown).

To determine whether transcription was induced with T cell activation, we compared cell associated HIV-1 mRNA at baseline and after maximal T cell activation. Treatment of CD4+ T cells with the T cell activation control of PMA plus ionomycin (PMA/I) increased cell associated HIV-1 mRNA levels in 7 of 9 ES and 4 of 5 CPs (Figure 1b). The measured cell associated HIV-1 mRNA per 10⁶ CD4+ T cells remained statistically higher for CPs (median = 33,676 per 10⁶ cells) than for ES (median = 873 per 10⁶ cells) (p = 0.047). However, there was no statistical difference between fold increase of cell associated HIV-mRNA between ES (13.1-fold, interquartile range = 12.1) and CPs (19.2-fold, interquartile range = 13.4, p = 0.22) per 10⁶ stimulated cells. Interestingly, cell associated HIV-1 mRNA levels remained below the limit of detection in 2 ES after stimulation with PMA and ionomycin stimulation. In order to determine whether more physiological T cell stimulation would result in similar upregulation of transcription, CD4+ cells from a subset of the patients were activated with microbeads that were coated with antibodies to CD3 and CD28. There was a robust increase in intracellular mRNA in 2 of 3 ES whereas mRNA was undetectable at baseline and after T cell activation in the third subject. The level of mRNA upregulation seen in the 2 ES was comparable to the level seen in 2 CPs (Figure 1C).

We also assessed the effect of T cell activation on viral release into culture supernatant. Upon stimulation of replicates of 5x10⁶ CD4+ T cells from ES with PMA and ionomycin, HIV-1 mRNA was detected in the culture supernatant from 5 of 8 ES (Figure 2). Three ES (ES6, ES9, and ES42) had undetectable HIV-1 mRNA measurements in culture supernatant even when a total of 10-20x10⁶ CD4+ T cells were stimulated. In contrast, in ES22, ES23, and ES24 we detected HIV-1 mRNA in the culture supernatant from the majority of stimulated CD4+ T cell replicates. Interestingly in ES36 and ES43,
HIV-1 mRNA was detected in the culture supernatant in only 1 of 5 stimulated replicates, with 3,012 and 5,116 copies of HIV-1 mRNA present in the positive wells. The frequency of detected released HIV-1 RNA suggests an 89 percent probability by Poisson distribution that the signal observed reflects a single cell releasing virus, and our HIV-1 mRNA measurements fit with our recent estimates of the burst size of an infected CD4+ T cell in vitro [25]. Thus, of the 2.5x10^6 cells stimulated, it appears that only one cell harbored an inducible virus in both of these patients. Interestingly, ES36 and ES43 also had undetectable cell associated HIV-1 mRNA in 5x10^6 CD4+ T cells after stimulation with PMA and ionomycin. Taken together, these data suggest that there is significant heterogeneity in the inducible HIV-1 reservoir seen in ES which is consistent with results obtained from the viral outgrowth assay [1,2].

DISCUSSION

Control of HIV-1 without ART by ES can be considered a model of a functional cure of infection. We have previously shown that these patients harbor much lower frequencies of latently infected CD4+ T cells [1] than CPs on suppressive ART regimens [26], and in this study we further analyze the inducible reservoir in ES. We demonstrate that cell associated HIV-1 mRNA levels observed in ES CD4+ T cells at baseline were significantly lower than those observed in CPs. These results further characterize the nature the latent reservoir in ES patients. Recent studies have found that ES had low levels of HIV-1 RNA in peripheral blood and in the GALT [11,21]. However, despite the low baseline levels of HIV-1 mRNA, there was no significant difference in the fold increase of cell associated HIV-1 mRNA levels upon PMA and ionomycin stimulation in CD4+ T cells isolated from ES and CPs, suggesting a similar induction of transcription in response to PMA and ionomycin treatment in both patient populations. This is consistent with results from a recent study that found a correlation between copies of proviral DNA and the inducibility of the HIV reservoir in ES and CPs [27]. Another recent study has shown that some defective proviruses present in CP CD4+ T cells can be transcribed [28]. It is not known whether this phenomenon also occurs in ES and so while we cannot determine whether the observed increase in HIV-1 mRNA was due to transcription of defective or intact proviral sequences, our data are consistent with our ability to culture replication-competent virus from some ES.

There was significant heterogeneity in the amount of cell associated and released virus seen in CD4+ T cells from different ES. Interestingly, ES23 and ES24 had detectable HIV-1 mRNA at baseline and both patients had a relatively high frequency of CD4+ T cells that released virus into culture supernatant following T cell stimulation. In contrast, the frequency of CD4+ T cells that released virus into culture supernatant was much lower in other ES. However, we were able to culture and characterize replication-competent virus from two such patients, ES9 [1] and ES36 [12] in prior studies. Further studies will be needed to determine whether virus that is released into culture supernatant following CD4+ T cell activation is in fact replication-competent.

Studies have shown that virus in the plasma of ES evolves over time suggesting that there is ongoing viral replication [12-16]. In spite of that we still found very low levels of HIV-1 mRNA in peripheral CD4+ T cells in some of these patients. It is possible that the robust CTL (cytotoxic T lymphocytes) response in these patients [29] is able to eliminate transcriptionally active CD4+ T cells in peripheral blood that may be producing viral proteins, while CD4+ T cells in anatomical compartments that exclude CTL are the true source of ongoing viral replication. Lymph nodes represent one such compartment [30,31] and results from a recent study that found that CD8+ T cells are excluded from B cell follicles in ES monkeys may be consistent with this hypothesis [32]. Furthermore, sequence analysis from controllers with low level viremia suggest that there is ongoing viral replication in lymphoid tissue [33]. It will be interesting to look at mRNA expression in ES CD4+ T cells in B cell follicles and in other potential reservoirs such as myeloid cells. It will also be interesting to determine whether there is a correlation between the HIV-1 mRNA levels present in CD4+ T cells and the immune phenotype of ES and CPs.

In summary, we characterize baseline CD4+ T cell associated HIV-1 mRNA and show a robust upregulation following T cell stimulation in some ES. The results will have implications for patients who are subjected to curative procedures but still have residual low level HIV-1 reservoirs.

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