Short Communication

Activating Stimuli Enhance Immunotoxin-Mediated Killing of HIV-Infected Macrophages

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Abstract

Strategies for purging persistent reservoirs in human immunodeficiency virus (HIV)-infected individuals may be enhanced by including agents that specifically kill virus-expressing cells. Anti-HIV envelope immunotoxins (ITs) represent one class of candidate molecules that could fulfill this function. We have previously utilized an anti-gp120 IT in conjunction with various stimulants to kill latently infected T cells *ex vivo*. Here we show that primary macrophages expressing HIV Env are relatively refractory to killing by IT when used alone. However, including stimulants such as prostratin or granulocyte-macrophage colony-stimulating factor to increase HIV gene expression in infected macrophages enhanced IT-mediated killing. Therefore, “activation-elimination” strategies similar to those proposed for purging the latent HIV reservoir may prove useful in clearing chronically infected macrophages *in vivo*.

WITH THE USE OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART), plasma viral loads in human immunodeficiency virus (HIV)-infected patients can be maintained for years at levels that are undetectable using standard clinical assays.^{1–3} Yet during this time, HIV persists in various cellular reservoirs, and if therapy is ceased then viral loads rapidly rebound causing renewed disease progression toward AIDS.^{4–6} One confirmed reservoir of replication competent HIV is within latently infected CD4⁺ T cells.^{7–10} Various methods have been proposed to clear this reservoir, including the combinatorial use of molecules that induce latent virus expression with compounds that specifically kill HIV-infected cells.

A wide range of cytokines, antibodies, and other molecules is capable of stimulating HIV from latency,^{11–15} and it has been suggested that use of anti-HIV envelope (Env) immunotoxins (ITs) may accelerate the elimination of infected cells activated by such agents.¹⁶ ITs are hybrid molecules consisting of a targeting domain derived from a monoclonal antibody linked to a toxic moiety.^{17,18} Certain ITs and related targeted toxins have proven clinically useful as anticancer therapies,^{19–21} and in the context of HIV, various ITs have

been developed that are effective against HIV-infected T cells and macrophages.^{22–24} We have previously shown that one such anti-gp120 IT can be used to kill latently infected T cells after stimulation *ex vivo*.²⁵ However, the effect that such “activation-elimination” strategies would have on other persistent viral reservoirs is uncertain.

In addition to latently infected CD4⁺ T cells, macrophages may also play a role in HIV persistence during therapy. Notably, in contrast to activated CD4⁺ T cells,^{26–28} macrophages are relatively resistant to the cytopathic effects of HIV, and can continue producing virus for weeks or months after infection in culture.^{29–31} While the frequency of persistently infected macrophages in patients undergoing HAART is not clear, this capacity for long-term chronic infection potentially allows infected macrophages *in vivo* to produce virus for months after initiation of HAART. Similarly, any temporal or spatial reductions in drug concentrations during HAART may lead to a “topping up” of the macrophage reservoir that could take months to reverse. Productively infected monocytes have been isolated from patients on HAART with sustained viral loads below 50 copies/ml,^{32–34} and although this observation requires further investigation, it does lend

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weight to the notion that monocyte/macrophage cells may represent an independent reservoir of replication-competent HIV in some patients. Macrophages are also the principal target cell type in potential anatomical reservoirs such as the central nervous system,^{35–37} where suboptimal drug concentrations may allow continued low-level virus replication with the potential to reseed the lymphoid system upon cessation of therapy.

The purpose of the current study was to determine what effect regimens suggested for use in purging the latent reservoir in CD4⁺ T cells would have upon chronically infected macrophages. Infected macrophages in HAART-treated patients are relatively rare *in vivo*, and it is difficult to obtain viable tissue samples from HIV-infected patients to directly test these cells for susceptibility to stimulants/immunotoxin. This sampling issue is particularly problematic in central nervous system tissues, where a large number of macrophages are present. Because of limited accessibility to these *in vivo* cells, we have used peripheral blood mononuclear cell (PBMC)-derived macrophages in this exploratory study. However, the majority of previously published macrophage studies suggest that infected macrophages act as chronic producers of virus, rather than truly latent reservoirs (where

virus is not produced until the cell is stimulated). There are few data to suggest that these chronically infected macrophages differ in their response to cytokines or ITs from macrophages that have been infected with HIV for several days. For this reason we utilized peripheral cells infected for 2 days before exposure to stimulants and ITs as surrogates for assessing the effect of these factors on chronically infected macrophages.

A new HIV-based reporter virus was constructed for this study (Fig. 1A). This virus was generated by first replacing the 1978-bp *SpeI*-*AgeI* region of NL4-3³⁸ with an EGFP_{Luc} reporter gene, which encodes a fusion protein comprising an N-terminal enhanced green fluorescent protein (EGFP)^{39,40} and C-terminal firefly luciferase (Luc).⁴¹ The EGFP_{Luc} coding sequence was amplified from plasmid pEGFP_{Luc} (Clontech) by using a PCR with the sense primer 5'-ATTGAC-TAGTAAGGCCGCGCTACCAGTCGCCACCATG-3' and antisense primer 5'-GTCCGTACCGGTTTACACGGCGAT-CTTCCGCCCTT-3' (underscored bases represent relevant restriction sites). The *AatII*-*AgeI* (upstream) fragment of this vector was then combined with the downstream *AgeI*-*AatII* fragment of NFN-SX⁴² to create a chemokine receptor 5 (CCR5)-tropic HIV proviral clone with the same genetic

FIG. 1. Testing of reporter viruses in 293FT cells. (A) Schematic representation of R5-EGFP_{Luc}, a new HIV-based reporter virus. R5-EGFP_{Luc} was constructed using the recombinant R5-tropic provirus NFN-SX,⁴² which is composed of NL4-3³⁸ with the indicated region replaced with that of JR-FL.⁴² The proviral sequence of NFN-SX was modified by exchanging a portion of gag-pol with a reporter gene encoding the enhanced green fluorescent protein fused to firefly luciferase (EGFP_{Luc}). (B) 293FT cells were mock infected or inoculated with either R5-EGFP_{Luc} or denv (Wt) virus. GFP expression was present in the infected cultures but absent from mock-infected cells. Pictures were taken at 3 days postinfection with an original magnification of 20×. (C) 293FT cells infected as in (B) were lysed at 3 days postinfection and assayed for Luc activity (Luciferase Assay System, Invitrogen). Infected cells expressed levels of Luc >10000-fold above the background levels of mock-infected cells. (D) 293FT cells were infected with each virus, then different concentrations of the anti-gp120 IT HY-PE were added to infected wells at 1 day postinfection. Cells were lysed and assayed for Luc activity at 3 days postinfection. Error bars are present at all data points and represent mean values ±SD of quadruplicate infections.

FIG. 2. Testing of reporter viruses in primary macrophages. **(A)** Macrophages from healthy donors were differentiated for 2 weeks then infected with reporter viruses. Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 15% human AB serum, 5% fetal bovine serum, 100 U of penicillin/ml, and 100 μ g of streptomycin/ml. At 5 days postinfection, cells were harvested and the cell lysates assayed for Luc activity. Mean values $\pm$ SD for quadruplicate wells are shown. **(B)** GFP expression at 5 days postinfection. GFP⁺ macrophages were present in infected cultures but absent from mock-infected cultures. Original magnification $\times 40$.

structure as NFN-SX, except for the portion of gag-pol that was replaced with EGFP-Luc. For producing virus, this plasmid was cotransfected into 293FT cells together with pCMVR8.2,⁴³ which expresses the HIV structural genes, and (for enhancing viral infectivity) a third vector that encodes the vesicular stomatitis virus G (VSV) envelope protein.⁴⁴

Cells infected with the resultant virus express EGFP-Luc as a late gene product and also express HIV Env and all viral accessory/auxiliary proteins. This expression is driven from the native HIV long terminal repeat (LTR) promoter. In some experiments a control virus that does not express Env but does express EGFP-Luc was required. For this purpose VSV-

FIG. 3. Effect of different molecules upon postintegration gene expression in HIV-infected macrophages. **(A)** Overview of the assay system. Monocytes were isolated from PBMC then differentiated into macrophages for 2 weeks before infection with the R5-EGFP-Luc reporter virus. At 2 days postinfection (after viral integration) different concentrations of various molecules were introduced into the cultures. Cells were lysed and assayed for levels of HIV expression (Luc activity) at 5 days postinfection. **(B)** Assay results. Values are expressed as a percentage of the Luc activity observed in untreated infected cultures. GM-CSF and prostratin treatment produced significant increases in HIV expression within the cultures. Treatment with IT alone did not significantly reduce HIV gene expression in this system.

pseudotyped *denv*(Wt)⁴⁵ was utilized, which encodes EGF-PLuc in place of NL4-3 Env. Viral titers were established by infection of 293FT target cells, and all infections were performed at 37°C for 2 h in the presence of 10 μ g/ml polybrene.

The new R5-EGFP_{Luc} virus was initially tested in parallel with *denv*(Wt) by infecting 10⁵ 293FT cells at a multiplicity of infection (MOI) of approximately 0.1. At 3 days postinfection, GFP⁺ cells were visible in the infected cultures (Fig. 1B) and abundant Luc activity was present in infected cell lysates (Fig. 1C). To verify that an anti-HIV gp120 IT was active against cells infected with the R5-EGFP_{Luc} but not against those challenged with the *denv* (Wt) virus, 293FT cells were infected as in Fig. 1C, then at 1 day postinfection, different concentrations of the IT 3B3:N31H/Q100eY[dsFv]-PE (HY-PE)^{23,25,46,47} were added to each culture. At 3 days postinfection the cells were harvested and assayed for luciferase activity. As expected, cells infected with R5-EGFP_{Luc} were susceptible to IT-mediated killing, while those infected with *denv*(Wt) were not (Fig. 1D).

To test infection of mature macrophages with R5-EGFP_{Luc}, monocyte cells were isolated from PBMCs obtained from HIV-seronegative donors, essentially as previously described.⁴² At 2 weeks postisolation, cells were scraped off the tissue culture flask and infected in suspension at an MOI of approximately 0.1. After infection, cells were washed and seeded at 12,500 cells per well in a flat-bottomed 96-well plate. Some wells were assessed for Luc activity and GFP expression at day 5 postinfection. Both Luc activity (Fig. 2A) and GFP expression (Fig. 2B) were present in all infected cultures, but absent from mock-infected cultures. Other wells of infected macrophages were maintained for up to 1 month in culture, and GFP⁺ cells were visible throughout this time (not shown), indicating that chronically infected macrophages can be created using this approach.

Cytokines and other potentially stimulatory molecules can have inconsistent or even opposing effects upon different stages of the HIV life cycle, and these effects can vary depending upon the specifics of the experiment and culture system.⁴⁸ Much of the prior work relating to the effect of stimulants upon HIV replication in primary macrophages has focused upon virus spread in culture, rather than the specific collection of events between integration and virus production. Since the latter situation is likely more important when developing strategies for purging chronically infected macrophages *in vivo* (which would be expected to already harbor an integrated provirus), we elected to focus exclusively on this phase of macrophage infection while developing our single-round infection assay. Integration takes approximately 2 days to complete in HIV-infected macrophages,⁴⁹ and GFP⁺ cells were present in the infected cultures at this time after infection with the R5-EGFP_{Luc} virus (not shown). Hence, for testing the effect of stimulants and/or IT upon infected macrophages, these molecules were added at 2 days postinfection; cells were then lysed and assayed for Luc activity (HIV expression) at 5 days postinfection (Fig. 3A).

We tested different concentrations of several molecules in this manner, including interleukin (IL)-2, IL-7, and prostratin, each of which has been suggested for use as a component of purging strategies intended to eliminate the latent HIV reservoir within CD4⁺ T cells.^{13,15,50} We also tested

macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage-stimulating factor (GM-CSF), and IL-4, which can significantly alter the efficiency of HIV spread in macrophages.⁴⁸ Using the current assay system, certain concentrations of both GM-CSF and prostratin resulted in up-regulation of HIV gene expression in the macrophage cultures (Fig. 3B), while the addition of IL-4, IL-7, or M-CSF did not significantly alter HIV expression levels. GM-CSF is FDA approved for treatment of certain neutropenias,⁵¹ and the possibility of using prostratin as a therapeutic to aid in elimination of latent HIV is under evaluation in preclinical trials in nonhuman primates.

Notably, treatment with up to 1 μ g/ml of HY-PE also failed to alter HIV gene expression (Fig. 3B), indicating that the IT alone has minimal effect on HIV-infected macrophages in this single-round infection system. One potential explanation for this result is that the majority of HIV budding in macrophages occur intracellularly, rather than directly at the cell surface plasma membrane.^{29,30,52,53} This may impair the function of an anti-Env IT by reducing the concentration of Env at the cell surface that is available for recognition by the

FIG. 4. Treatment with a stimulant in conjunction with IT enhances killing of infected macrophages. (A) Macrophage cells were infected and assayed as described in Fig. 3A, but rather than adding single compounds, 20 ng/ml of GM-CSF and 1 μ g/ml of IT were added in combination to the cultures. Values are presented in pairs, with the "no IT" value set to 100% and the corresponding +IT value expressed as a percentage thereof. (B) The experiment was performed as in (A), but with 0.1 μ g/ml prostratin added in conjunction with IT. All statistical comparisons were performed using a two-tailed, two-sample (equal variance) Student's *t*-test. **p* < 0.002; ***p* < 0.001.

IT. It was thus possible that upregulating HIV expression could enhance IT-mediated killing by increasing Env expression at the cell surface. Consequently, assays were set up where stimulants and IT were added to infected macrophages, either alone or in combination. Combinatorial use of GM-CSF + IT (Fig. 4A) and prostratin + IT (Fig. 4B) each resulted in a significant reduction in luciferase activity (HIV expression) in cultures infected with R5-EGFP_{Luc}, but not in those infected with denv(Wt). This reduction was less than that observed in either 293FT cells (Fig. 1D) or latently infected CD4⁺ T cells²⁵ treated with this same IT, which may reflect the different distribution of Env within these various cell types.

Using stimulants such as prostratin in conjunction with anti-Env ITs represents one approach for purging the latent reservoir of HIV in patients undergoing treatment with HAART. It is likely that multiple ITs with different specificity would be needed to effectively target antigenically diverse clades of HIV. Yet the data described here indicate that suboptimal IT-mediated killing of HIV-infected macrophages can also be improved by stimulation of the host cell. In principle, this type of approach could be used in the future to help compensate for the low affinity that anti-HIV envelope agents, such as anti-gp120 ITs, may have for some of the diverse global isolates of HIV. In summary, activation-elimination strategies designed to eliminate latently infected CD4⁺ T cells may be adapted to simultaneously target chronically infected macrophages, potentially leading to a broader impact and improved efficacy against the various persistent reservoirs of HIV in HAART-treated patients.

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Disclosure Statement

No competing financial interests exist.

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