Infection by human immunodeficiency virus type 1 (HIV-1) has been associated with increased cell death of both infected and bystander cells. The envelope glycoprotein complex appears to play an active role in HIV-induced death of bystander cells. We quantified cell-to-cell fusion, single cell death and membrane lipid mixing in cocultures of effector, HIV-1 envelope-expressing cells with peripheral blood mononuclear cells or purified CD4 T lymphocytes from HIV-negative donors, in the presence or the absence of the fusion inhibitor enfuvirtide (T-20, pentafuside, Fuzeon™). T-20, which blocks gp41-dependent virus–cell fusion, showed a complete and dose-dependent inhibition of syncytium formation in cocultures of envelope-expressing cells with uninfected cells. Similarly, T-20 totally abrogated death of single bystander CD4 T cells with an IC₅₀ of 0.04 µg/ml. Membrane lipid mixing, as a measure of interaction between envelope-expressing cells and CD4 cells, was also dose-dependently inhibited by T-20. Moreover, effector cells chronically infected with a T-20-resistant virus recovered the ability to induce bystander cell death in the presence of the drug, supporting the role of gp41 in single cell death. In conclusion, T-20 is able to protect CD4 T cells from envelope presentation with a dual effect: inhibition of virus replication and blockade of HIV-1 envelope-induced cell death of bystander CD4 T cells. Protection of cells prior to infection from HIV envelope-dependent bystander effect could lead to a better immune restoration of HIV-1-infected patients that are treated with T-20.

Introduction

HIV entry is a sequential process that begins with attachment of the virus to the cell surface followed by binding of the gp120 glycoprotein to CD4 and the interaction with the appropriate chemokine receptor, mainly CXCR4 and CCR5 [1]. In response to such coreceptor binding, there are conformational changes that allow gp41-mediated membrane fusion [2]. Compounds that mimic the C peptide region of gp41 bind to this glycoprotein after interaction of the envelope complex with cellular receptors and block fusion [2,3]. Enfuvirtide (T-20, pentafuside, Fuzeon™). Is a synthetic 36-amino acid peptide that binds to gp41 and blocks HIV infection of CD4 T cells and cell-to-cell virus transmission by preventing the conformational change required for membrane fusion [2,4]. T-20 was the first entry inhibitor demonstrated to be effective in suppression of HIV-1 replication in humans [4] and is currently undergoing Phase III clinical trials, with promising results.

HIV infection has been associated with cell death affecting both infected CD4 cells (by direct infection or killing by cytotoxic cells) and bystander CD4-positive and CD4-negative cells (by chronic immune activation or toxic effects of viral proteins) [5]. HIV-induced cell death has been associated with different viral proteins, including the envelope glycoprotein complex gp120/gp41 (Env) [6–8].

Since fusion inhibitors interfere with envelope function, we evaluated whether these compounds, which are now relevant as therapeutic agents against HIV/AIDS, had an effect on envelope-induced bystander cell death. Here we show that the fusion inhibitor T-20 blocked bystander CD4 T cell death induced by HIV Env-expressing cells.

Material and methods

Cells and reagents

H9/IIIB [9] and HeLa-Env [10] cells were obtained through the Medical Research Council, from RC Gallo and R Ruprecht, respectively. MOLT-4/CCR5 (CCR5+, CXCR4+) and HeLa cells were obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH from M Baba, H Miyake, Y Iizawa [11] and R Axel, respectively. Chronically infected MOLT-4/CCR5 cells were generated in the laboratory after infection of MOLT-4/CCR5 cells with the T cell-adapted strain NL4-3 (MOLT-NL4-3) and a T-20-resistant virus, NT38 (MOLT-NT38). Generation of resistance was done as previously described [12–14],...
by sequential passages of the NL4-3 strain in vitro in MT-4 cells in the presence of increasing concentrations of T-20. The virus generated was genotypically and phenotypically similar to those reported before [14]. Envelope cell surface expression of chronically infected cells was assessed by labelling with a pool of serums from HIV-infected patients and a FITC-conjugated anti-human IgG (SIGMA, St Louis, Mo., USA).

Figure 1. T-20 inhibition of single cell death induced by chronically infected cells

(A) Target CD4 cells can be easily identified by flow cytometry as cells with low forward (FSC) and side scatter (SSC) pattern (R2) unlike effector H9/IIIB cells (R4). Dead cells may be identified by reduced FSC and increased SSC (R3). (B) Magnification of R1 and R3 regions. T-20 (10 µg/ml) blocked single cell death occurring in cocultures of isolated CD4 T cells with chronically infected H9/IIIB cells (ratio 1:1), assessed by changes in cell morphology (percentages of dead cells are indicated) and (C) Annexin-V and propidium iodide labelling in the absence or presence of T-20. Results shown (A, B, C) are representative of at least three separate experiments. (D) Primary CD4 T cells were cultured with increasing concentrations of H9/IIIB cells for 24 h and morphological changes in target cells were analysed. Values represent the mean ±standard deviation of two separate experiments.
Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by Ficoll-Hypaque sedimentation. CD4 T cells were purified from PBMC by immunomagnetic-negative selection (StemCell Technologies, Vancouver, Canada) as indicated by the manufacturer and were used without any stimulation. The fusion inhibitor T-20 was kindly provided by Roche/Trimeris, Inc. (Durham, NC, USA). C-34, another peptidic fusion inhibitor covering the sequence 628–661 in the second extracellular helical region of gp41 [15,16] was kindly provided by P Kim (MIT, Cambridge, Mass., USA). Azidothymidine (AZT) was purchased from SIGMA (St Louis, Mo., USA).

Cocultures and measurement of envelope-induced cell death
Chronically HIV-1-infected H9/IIIB, MOLT-NL4-3, MOLT-NT38 or HeLa-Env cells (effector cells) were cocultured for 24 h with PBMC from uninfected donors or isolated CD4 primary T cells (target cells) in 96-well plates by seeding 2×10^5 target and 2×10^5 effector cells except for cocultures with HeLa-Env cells that were performed in 24-well plates. Single cell death was quantified by flow cytometry in a forward versus side scatter plot. Dead cells show increased side and reduced forward scatter values compared to living cells [17] or effector cells (effector chronically infected cells or detached HeLa cells). To calculate the 50% inhibitory concentration (IC50) values, target and effector cells were cocultured with serial dilutions of drugs as described before [6–8]. Alternatively, cell death was evaluated by flow cytometry analysis of phosphatidyl serine expression in the cell membrane as measured by Annexin-V labelling and propidium iodide exclusion as described before [8,18].

Inhibition of virus replication was evaluated by quantification of MT-4 cell viability by a colorimetric assay as described before [12].

Lipid mixing assays
HeLa or HeLa-Env cells were incubated with the fluorescent membrane probe 3,3′-dioctadecyloxacarbocyanine perchlorate, DiO (Molecular Probes) in a 1:0.6 RPMI:Diluent C (Sigma) mixture (34 μM, 15 min, 37°C). After extensive washes, labelled cells were cocultured with primary CD4 T cells for 24 h. At this time, HeLa and HeLa-Env cells were mostly attached to the plastic, whereas target CD4 T cells remained bound or fused to the cell monolayer. Fusion inhibition was evaluated by counting the number of syncytia in each well. Unfused cells were recovered, and cell death and the transfer of the lipidic fluorescent probe from effector HeLa-Env cells to unfused CD4 T cells were assessed by flow cytometry analysis (FACScalibur, BD). Lymphocytes were gated as living or dead in a forward versus side scatter plot. The extent of lipid mixing was represented as the mean fluorescence intensity (MFI) of the total lymphocyte population.

Results
T-20 protects CD4 T cells from HIV envelope-induced cell death
Distinct lymphoid cells may be identified by flow cytometry by analysis of their forward scatter (FSC) versus side scatter (SSC) pattern. HIV-1 IIIB persistently infected H9 lymphoid cells have increased FSC and SSC compared with primary CD4 cells (Figure 1A). Twenty-four-hour cocultures of effector envelope-expressing cells (H9/IIIB) with purified primary CD4 T cells resulted in the appearance of a subset of dead target cells, which could be morphologically identified in FSC vs SSC dot plot because of their reduced forward and increased side compared to living CD4 cells (Figure 1B). HIV envelope-induced cell death could also be quantified by phosphatidyl serine exposure and cell membrane integrity by labelling with Annexin-V and propidium iodide (PI), respectively (Figure 1C), confirming our previous results on the effect of HIV envelope in bystander T cell death [7,8].

As shown (Figure 1), T-20 (10 μg/ml) was able to inhibit the death of CD4 cells as measured by FSC vs SSC pattern, or Annexin-V and PI staining. This inhibitory effect was not observed in cocultures in the presence of the reverse transcriptase inhibitor AZT (0.2 μg/ml) (data not shown), suggesting that the effect observed is not dependent on virus production or viral replication in the CD4 cell population.

Different ratios of effector (H9/IIIB cells) to target (CD4 cells) yielded a dose-dependent induction of target cell death. Nevertheless, the effect of 10 μg/ml
of T-20 on CD4 cell death could be maintained if the ratio of effector to target (CD4 cells) was modified (Figure 1D).

In cocultures of uninfected HeLa cells that stably express the envelope glycoproteins of HIV-1-IIIB (HeLa-Env) with primary CD4 T cells, T-20 protected target cells from death in a dose-dependent manner (Figure 2, IC\textsubscript{50} 0.04 µg/ml), in parallel to inhibition of cell-to-cell fusion (IC\textsubscript{50} 0.07 µg/ml), and with similar efficacy to its anti-HIV activity (IC\textsubscript{50} 0.04 µg/ml), suggesting that the effect observed on bystander cells is dependent on the expression of HIV envelope in effector cells. Cocultures treated with T-20 at high drug concentration (10 µg/ml) showed identical levels of single cell death to negative controls (cocultures with HeLa cells not expressing HIV envelope proteins). Conversely, AZT did not inhibit single cell death at any of the concentrations tested (data not shown).

Protection of CD4 T cells from envelope-induced cell death is due to its specific effect on gp41 function. To confirm that the effect of T-20 was due to inhibition of envelope function, we evaluated the effect of T-20 on a defined gp41 function: the lipid mixing observed in the intermediate state that takes places before complete cell to cell fusion [19], measured by transference of a lipid probe (DiO) from labelled effector cells to target cells. To assess unspecific lipid mixing we used cocultures of CD4 T cells with labelled HeLa cells not expressing the HIV-1 envelope glycoproteins. In these cocultures there were no variations in CD4 T cell viability and no transfer of lipids from labelled HeLa cells to CD4 T cells. In cocultures with HeLa-Env cells there was a 10-fold increase in fluorescence in the target CD4 dead cells. This effect was inhibited by T-20 (10 µg/ml) or C34 (1 µg/ml) (Figure 3). Conversely, AZT (0.2 µg/ml) did not block lipid mixing between effector (HeLa-Env) cells and CD4 target cells (data not shown).

T-20-resistant envelope is able to induce single cell death

To confirm the role of gp41 in bystander cell death, we evaluated the effect of cells expressing T20-resistant (MOLT-NT38) envelope glycoproteins with CD4 cells. The NT38 virus was generated in the laboratory by sequential passage of HIV-1 NL4-3 wild-type virus in MT-4 cells in the presence of increasing concentrations of T-20 (EC\textsubscript{50} 75.4 µg/ml or a 251-fold increase in EC\textsubscript{50} as compared to the wild-type strain). Cells expressing wild-type (NL4-3) HIV envelope and MOLT-NT38 cells showed similar expression of HIV envelope in their cell surface (Figure 4). Unlike uninfected effector cells (MOLT-4-CCR5), both MOLT-NL4-3 and MOLT-NT38 cells induced single CD4 T cell death. T-20 completely blocked CD4 cell death induced by MOLT-NL4-3 cells. Conversely, cell death induced by MOLT-NT38 could not be blocked by T-20 (10 µg/ml) (Figure 4). AZT did not inhibit single cell death induced by MOLT-NL4-3 or MOLT-NT38 cells.

These results suggest that the HIV envelope glycoproteins expressed on the cell surface of effector cells are sufficient to induce cell death. HIV resistance to T-20 abrogates the inhibitory effect of this compound on cell death suggesting that bystander cell death occurs through a process that is mediated by gp41.

**Discussion**

We have used a well-characterized model of coculture (coculture of envelope-expressing cells, the chronically infected cell lines H9/IIIB, MOLT-NL4-3, MOLT-NT38 or HeLa cells transfected with Env, with PBMCs or primary CD4 cells) [7,8,20–22] to assess the effect of fusion inhibition in HIV envelope-dependent bystander cell death. In our study, HIV envelope-induced cell death was inhibited by the fusion inhibitor T-20 as measured by morphological cell changes that correspond with cell death or by double staining with Annexin-V and PI that are common markers of cell death. We show that death of single CD4 cells was dependent on the expression of HIV-1 Env, could be blocked by T-20 in a dose-dependent manner but was independent of HIV-1 replication. Lipid mixing due to close contact of membranes, which takes place at an early step of the membrane fusion process (intermediary state often called hemifusion), was also inhibited by T-20, suggesting that the inhibitory effect observed on cell death was concomitant to inhibition of gp41 function.
In addition, single cell death was dependent on the sensitivity to T-20 of the envelope expressed by effector cells. Cells that were chronically infected with a T-20-resistant virus recovered the ability to induce cell death in the presence of T-20.

Since the role of gp41 in the death of single CD4 T cells by envelope-expressing cells may have important implications for AIDS pathogenesis [23] it seemed attractive to evaluate the possibility that T-20 could block such bystander effect in addition to its potent inhibitory effect on viral replication. T-20 and the second-generation fusion inhibitor T-1249 are potent in vitro and in vivo inhibitors of both wild-type HIV-1 strains and those that are resistant to other antiretroviral drugs currently used in clinical practice [24,25] that work inside the cell at a later stage of infection and do not have an effect on the initial interactions of HIV with target cells. In addition, fusion inhibitors have displayed an in vitro activity that is additive or synergistic with other anti-HIV compounds, including other entry inhibitors [26,27]. Here, we propose a novel mechanism for anti-HIV activity of T-20, that is, inhibition of bystander cell death induced by HIV envelope glycoproteins.

It has been suggested that direct HIV infection may not account for the CD4 T cell depletion observed in patients [28]. We hypothesize that a component that contributes to this bystander effect, together with chronic immune activation, is envelope-induced cell death. While this effect is unlikely in peripheral blood lymphocytes in vivo, it is most probable to occur at sites of active viral replication such as lymph nodes where cell-to-cell contacts between infected and uninfected cells are likely to occur. It has been shown that HIV-infected lymphoid tissues show increased apoptosis of uninfected cells and this may weaken the immune response against HIV [28]. The inhibition of cell death in HIV infection has been thought to be counterproductive. Survival of HIV-infected cells would increase virus production and favour the spread of the virus because of the increase in the number of target cells [29]. Cell apoptosis is, in fact, not a virus function but an immune defence mechanism against invading/unwanted stimuli. Nevertheless, fusion inhibitors, that work at the first steps in the viral life cycle, should protect uninfected cells because of their dual activity: inhibition of viral infection and protection of CD4 cells from envelope-dependent cell death.

Figure 4. Induction of single cell death by cells that were chronically infected with a T-20-resistant virus

Chronically infected cells expressing the wild-type virus NL4-3 and the T-20-resistant virus NT38 were cocultured with CD4 T cells. (A) Flow cytometry histograms of HIV envelope expression in MOLT-4/CCR5-uninfected cells and in chronically infected MOLT-NL43 and MOLT-NT38 cells (thick lines) compared to cells that were labelled with isotype mAb (thin lines) (B) Cell death evaluated by measuring changes in morphology of gated CD4 T cells in cocultures with uninfected or chronically infected cells in the absence or presence of the corresponding drug (T-20 10 µg/ml, AZT 0.2 µg/ml). Data shown are the mean of three independent experiments. White bars, grey bars and black bars represent cocultures of CD4 T cells with uninfected, NL4-3- or NT38-infected cells, respectively.
Recent findings have emphasized that increased CD4 T cell death rather than decreased production [30,31] is the major cause of T cell depletion in HIV infection. With its dual effect, fusion inhibitors would help to increase specific anti-HIV CD4 T cell responses, since, as it has been recently shown, HIV-specific memory CD4 T cells are preferentially infected and destroyed by HIV [32]. The hypothesis that we have demonstrated in vitro is being tested in vivo in the context of a currently undergoing clinical trial with patients receiving T-20 in combination with optimized antiretroviral therapy. This trial will also help to evaluate the extent of envelope-induced bystander effect on uninfected cells as a component of HIV pathogenesis in vivo.

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