Multiparametric Assay To Screen and Dissect the Mode of Action of Anti-Human Immunodeficiency Virus Envelope Drugs

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A flow cytometry-based assay was used to simultaneously quantify X4 and R5 human immunodeficiency virus (HIV) envelope-mediated cell-to-cell viral transfer, cell death, and cell-to-cell fusion. In this assay, different anti-HIV envelope drugs showed characteristic inhibitory profiles for each measured parameter, allowing for the rapid identification of the mode of action of active compounds.

Human immunodeficiency virus type 1 (HIV-1) envelope (gp120/gp41) drives membrane fusion (20). This process involves the interaction of external subunit (gp120) with the receptor CD4 (23) and one of the coreceptors CXCR4 or CCR5 (5) that activates the insertion of the fusion peptide of gp41 into the membrane of the target cell (12). Then, the processes of mixing of external membrane leaflets (hemifusion), pore opening, and irreversible fusion take place (18). Every step in HIV envelope function is susceptible to becoming a drug target (2, 7, 10, 15, 22, 24). To date, one gp41 inhibitor (6, 14) has been approved for the treatment of HIV infection and others are in advanced clinical trials (17).

The growing interest in fusion as a drug target has led to the development of drug-screening assays (11) based on the coculture of envelope-expressing cells and CD4⁺ cell lines that results in the formation of multinucleated giant cells (syncytia) (16, 19, 21). Generally, the readout of these assays requires complete cell-to-cell fusion, making difficult the identification of the mechanism of action of active compounds (9, 16, 19, 21). New assays with increased resolution in the prefusion steps of HIV envelope function may help to discriminate the mechanism of action of novel fusion inhibitors.

We have developed an assay in which we coculture in 96-well plates 2.5×10^5 primary CD4 T cells (purified from peripheral blood mononuclear cells by negative selection; StemCell, Vancouver, Canada) with 2.5×10^5 MOLT/CCR5 cells chronically infected with an X4 isolate, NL4-3 or CI-1-SI, or the R5 isolate BaL (6, 8). After 24 h of coculture, we measured two previously characterized HIV envelope-mediated events occurring during cell-to-cell contacts (viral synapses) (13) but prior to irreversible cell-to-cell fusion (6, 8). Synapses led to a coreceptor-independent transfer of HIV that was revealed by intracellular p24 HIV antigen staining as described previously (8). This passive transfer completely depends on gp120-CD4 interaction and could therefore be used as a surrogate marker of this interaction. Synaptic contacts may also lead to hemifusion

events (7) that are dependent on the coreceptor and may result in a T-20-sensitive single cell death (SCD) (3). SCD may be quantified after staining with the mitochondrial probe DIOC₆ (3,3'-dihexyloxycarbocyanine iodide) (6) and could be used as a surrogate marker of gp41-mediated hemifusion.

The simultaneous study of prefusion (transfer and death) and postfusion (syncytium formation) events should increase the resolution of HIV envelope function. The use of X4 and R5 envelopes gives information on the interaction of gp120 with the coreceptor. Since p24 and DIOC₆ staining are incompatibles due to the fixation/permeabilization procedure (6), we have evaluated cell death by morphological criteria as previously described (3, 4) and cell-to-cell fusion by counting absolute numbers of living and dead target cells. This was achieved by adding 5×10^4 fluorescent beads (Perfect-Count Microspheres; Cytognos, Salamanca, Spain) to each sample before starting the staining procedure. Cells and beads were analyzed in a FACScalibur flow cytometer (BD) (Fig. 1).

Morphological parameters easily identified effector (Fig. 1, R3) and target cells, which appeared as a double population representing living (L) and dead (D) cells (Fig. 1, R1 and R2). Morphological detection of cell death (percentage of death = $D/[L + D] \times 100$) in unfixed cells was compared to the percentage of DIOC₆^{low} (dead) cells, and these parameters showed a strong correlation ($r = 0.995$, $P < 0.001$) (Fig. 2A). Fixation/permeabilization did not modify cell morphology (data not shown); therefore, similar percentages of dead cells in untreated or fixed/permeabilized CD4 T cells cocultured with different infected cells were observed (Fig. 2B).

A last concern was the quantification of the absolute numbers of living and dead cells as a measure of syncytium formation. Since disintegration of dead cells may also contribute to the loss of cells, we measured the effect of puromycin on the absolute number of total (L + D) CD4 T cells. Despite strong apoptosis induction, the absolute cell count in unfixed or in fixed/permeabilized cells was not affected by puromycin after 24 h (Fig. 2C). The absolute number of lost cells was therefore calculated as the difference between total CD4 T cells in HIV-infected and uninfected cocultures (lost cells = $[L + D]_{\text{uninfected}} - [L + D]_{\text{HIV}}$). The utility of this approach was confirmed by the correlation observed between the number of lost cells and the

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FIG. 1. Quantifications of transfer, death, and fusion. Flow cytometry analysis of cocultures of CD4 T cells with uninfected (UNINF; left) or CI-1-SI infected (right) MOLT/CCR5 cells. Forward (FSC)-by-side scatter (SSC) plots (upper panels) allowed the identification of different cell populations. R1 and R2 regions were defined as the living and dead target cell populations, respectively (detail is shown in the middle panels). Morphology of target cells was used as a marker of cell death. R3 was defined as the effector (uninfected or HIV-infected) cell population. Absolute cell counts were obtained by adding a constant number of fluorescent beads to cultures. Beads were identified by morphology (R4) and fluorescence intensity (not shown). P24 antigen staining allowed for the evaluation of HIV particles transferred to the living (R1) target cell population (low panels). Parameters were set up by using uninfected cocultures as a control. Lymph., lymphocytes.

syncytium formation measured in micrographs of cocultures (Fig. 2D).

Our assay was validated by assessing the anti-HIV activity of drugs targeting different steps of HIV envelope function (Fig. 3). The anti-CD4 monoclonal antibody Leu3a (BD, Madrid, Spain) targeting the interaction of gp120 with CD4 blocked HIV transfer, SCD, and cell-to-cell fusion induced by both NL4-3 and BaL envelopes. In contrast, CXCR4 and CCR5 antagonists (AMD3100 and TAK779) failed to block cell-to-cell virus transfer but efficiently inhibited cell-to-cell fusion and SCD induced by X4 and R5 envelopes, respectively. The gp41 inhibitor C34 blocked fusion and death induced by both X4 and R5 envelopes without an inhibitory effect in the transfer of HIV antigen. Rather, coreceptor antagonists and gp41 fusion inhibitors increased the transfer of HIV antigen, which is the unique outcome of synapses when cell death and syncytium

FIG. 2. Validation of the absolute cell count. (A) Correlation of the assessment of cell death by morphological or mitochondrial parameters. A total of 159 samples from CD4 T cells cultured in different conditions with infected MOLT cells were analyzed for mitochondrial membrane potential after DIOC staining (6) and cell morphology (as shown in Fig. 1). (B) The effect of fixation (FIX) and permeabilization (PERM) on absolute CD4 T-cell count was assessed for unfixed (left) and fixed/permeabilized CD4 T cells cocultured for 24 h with NL4-3-, BaL-, or CI-1-SI-infected MOLT/CCR5 cells. The percentage of dead cells in each case is shown. Data are means $\pm$ standard deviations of three experiments. UNINF, uninfected. (C) CD4 T cells were cultured in the absence or the presence of the indicated concentrations of puromycin for 24 h, and the absolute numbers of morphologically living and dead cells were assessed. The numbers of living cells (black columns) and dead cells (white columns) are shown. Data are means $\pm$ standard deviations of triplicate samples. Ctrl, control; FIX, fixated; PERM, permeabilized. (D) Correlation between calculated CD4 T-cell loss induced by NL4-3-infected MOLT/CCR5 cells and the surface of syncytia evaluated in micrographs of the same cocultures by using NIH Image 1.63 software. Correlations in panels A and D were examined with the Pearson correlation coefficient.

formation are completely blocked. This effect was more clearly observed in X4 envelopes due to the higher expression of CXCR4 in target cells (6).

Every drug tested showed comparable 50% inhibitory con-

FIG. 3. Evaluation of the inhibition of HIV envelope functions by several drugs. The effects of Leu3a (filled squares), AMD3100 (open squares), TAK779 (filled triangles), C34 (open triangles), and AZT (circles) on HIV transfer (left), cell death (middle), and fusion (right) were determined in cocultures of CD4 T cells with NL4-3 (upper graphs), CI-1-SI (middle graphs), or BaL (lower graphs) infected MOLT/CCR5 cells, in the presence of increasing concentration of each inhibitor. Dotted lines represent background levels found in uninfected control cocultures. Data are representative of one experiment of three performed. Dose-response inhibition curves were fitted by nonlinear regression using the GraphPad Prism 4 software.

centrations for each parameter evaluated in our assay. However, values were higher than those observed in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay (Table 1) performed on MT-4 cells as described previously (1). This may be due to the higher multiplicity of infection associated with our assay (0.4 pg p24/target cell compared to 0.004 pg p24/target cell in the MT-4/MTT assay) or to mechanistic differences between cell-to-cell and virus-to-cell fusions. Interestingly, a comparison of discordant data from both experimental settings may permit the understanding of such differences. Drugs targeting late steps of HIV replication, such as zidovudine (AZT), did not modify any of the measured parameters but showed potent antiviral activity in MT-4 cells (Fig. 3 and Table 1).

TABLE 1. Anti-HIV activity of HIV envelope inhibitors calculated from the multiparametric and the MT-4/MTT viability assays

Inhibitor	IC ₅₀ (μg/ml) ^a			MT-4 assay ^b
	Multiparametric assay			
	HIV transfer	Cell death	Fusion	
Leu3a	0.7 ± 0.4	0.2 ± 0.1	0.2 ± 0.1	
AMD3100	>1	0.07 ± 0.04	0.3 ± 0.2	0.0026 ± 0.0004
C34	>10	0.2 ± 0.1	0.3 ± 0.2	0.022 ± 0.0013
AZT	>1	>1	>1	0.0006 ± 0.0001

^a IC₅₀, 50% effective concentration, or concentration needed to inhibit 50% HIV-induced transfer, single cell death, or fusion in primary cells cultured with NL4-3-infected MOLT/CCR5 cells or HIV-induced cytopathicity in MT-4 cells (measured by the MTT method). Dose-response inhibition curves were fitted by nonlinear regression using the GraphPad Prism 4 software. Values are means ± standard deviations of three independent experiments.

^b Leu3a was toxic to MT-4 cells after 5 days in culture, with a concentration needed to kill 50% of cells of 0.02 ± 0.01 μg/ml.

The measure of HIV transfer, SCD, and the disappearance of CD4 T cells as surrogates of gp120 binding to CD4, hemifusion, and overt membrane fusion, respectively, combined with the use of X4 and R5 envelopes (providing data on the gp120/coreceptor interaction), served to simultaneously monitor different envelope functions. Assuming that the inhibition of one step of HIV envelope function has no inhibitory effect on earlier steps, some general rules can be drawn: (i) inhibitors of gp120/CD4 interaction block all measured parameters, (ii) coreceptor inhibitors selectively inhibit X4 or R5 envelope-mediated death and fusion in the absence of inhibition of HIV transfer, and (iii) inhibitors of gp41 (and those blocking both coreceptors) inhibit death and fusion induced by X4 and R5 envelopes but not HIV transfer.

Our assay may be improved by using cell lines instead of primary cells as target cells. Although primary cells did not show strong differences among donors, their use makes difficult the full standardization of the assay and required checking of CCR5 expression to avoid donors with too low or null CCR5 expression. Primary cells were preferred because of their high sensitivity to hemifusion-mediated SCD; in contrast, cell lines tested are prone to fuse and show lower levels of SCD (7). The ability to indirectly measure hemifusion makes our assay particularly suitable for testing gp41 inhibitors, since it could differentiate hemifusion and fusion inhibitors, albeit all inhibitors tested to date, either directed against gp41 (C34) or directed against cellular targets (cytocalasin) (8), blocked both events with equal efficiency.

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