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Discriminating in vitro cell fusion from cell aggregation by flow cytometry combined with fluorescence resonance energy transfer

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Abstract

Expression of fusion proteins in the plasma membrane enables cells to bind and fuse with surrounding cells to form syncytia. Cell fusion can have important functional outcomes for the interacting cells, as syncytia formation does in AIDS pathogenesis. Studies on cell fusion would be facilitated by a quantitative method able to discriminate between cellular aggregates and bona fide fused cells in a cell population. Flow cytometry with fluorescence resonance energy transfer is applied here for analyzing fusion of HIV-1 envelope-expressing cells with $CD4^+$ Jurkat cells. Fusion partners were labeled with the vital lipophilic fluorescent probes DiO (green) and DiI (red) and FRET is manifested by an enhancement of the DiI red fluorescence intensity in double fluorescent cells, thus allowing discrimination between fused and aggregated cells. The inhibitory effect of anti-CD4 monoclonal antibodies and the inhibitory peptide T-20 upon cell fusion were readily quantified by this technique. This method allows the distinction of fused and aggregated cells even when they are at low frequencies.

Keywords: Cell fusion; Flow cytometry; FRET; Syncytia; T-20; Membrane fusion

1. Introduction

Cells infected with the human immunodeficiency virus (HIV) express the virus envelope fusion protein (Env) on their plasma membrane, a process that enables infected cells to bind and fuse with neighbor cells to form syncytia (Lifson et al., 1986; Sodroski et al., 1986). Although the relevance of cell-cell fusion for the pathogenesis of AIDS is unknown, evidences indicate that syncytia formation may be a mechanism for $CD4^+$ T cell depletion (Andreau et al., 2004; Furrer et al., 1998; Miedema et al., 1994). Additionally, syncytia could constitute important sites for virus replication in brain and lymphoid tissues (Frankel et al., 1997; Raghavan et al., 1999). Other processes where cell fusion is important include the formation of placenta and muscle, malignant transformation and stem cell-induced tissue repair (Alvarez-Dolado et al., 2003; Ogle et al., 2005). It is possible that distinct physiological or pathological outcomes of cell fusion could originate from differences in the relative numbers of fused cells, their types and physiological states, the nature and degree of alteration of the individual cell functions after fusion and from the stimuli from the microenvironment, all issues largely unattended.

Cell fusion has been studied using a number of assays, including reporter genes (Rucker et al., 1997) and spectrofluorometric measurement of fluorescent probe redistribution, such as fluorescence dequenching, photosensitized labeling, and fluorescence resonance energy transfer (FRET) (Blumenthal et al., 2002; Struck et al., 1981). Several of these methods provide a global estimation of fusion and others have been useful to monitor detailed kinetic changes in the lipid and cytosolic compartments allowing the study of fusion mechanism (Melikyan et al., 2005). However, studies on the cell population dynamics during cell fusion, investigation of their biological features, and the effect of fusion on surrounding cells, cannot be accomplished by these methods. Flow cytometry provides a quantitative approach which, combined with the analysis of functional markers, can be applied to study cell population fusion dynamics, determinants and consequences of cell fusion. However, the validity of this method can be compromised by mistaking cellular aggregates for fused cells (Gabrijel et al., 2004). In order to identify and quantify fused cells, a FACS-based method was developed using Jurkat lymphoid cells expressing HIV-1 envelope proteins,

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which fuse with neighbor $CD4^+$ target cells by a mechanism analogous to the virus-cell fusion process. In this assay, fusion partners are stained with the lipophilic fluorescent dyes DiI (red) and DiO (green), and fused cells are detected as double fluorescent particles (Huerta et al., 2002). Since the emission and absorption spectra of these dyes overlap, FRET assays would allow the distinction between fused and aggregated cells. Here it is reported that fused cells consistently exhibit FRET while non-fused, aggregated cells do not, thus allowing the discrimination of cellular aggregates from bona fide fused cells by flow cytometry.

2. Material and methods

2.1. Cells

Jurkat cell lines HXBc2(4) and 522F/Y containing an inducible tetracycline-dependent transactivator and transfected with the *env* and *rev* genes from the HIV-1 HXBc2 strain coupled to a cytomegalovirus promoter and to tetracycline operator sequences. HXBc2(4) cell line expresses a functional gp120/gp41 glycoprotein, while the 522F/Y cell line contains a F/Y substitution at position 522 in gp41 that prevents fusion (Cao et al., 1996). Both transfected cell lines and Jurkat clone E6-1 (E6 cells), were obtained through the AIDS Research and Reference Reagent Program.

2.2. Cell culture

Transfected cells were grown in RPMI medium (Gibco BRL, Rockville, MD) containing 10% fetal bovine serum (Gibco BRL) (RPMI-10), 200 μg/ml of G418, 200 μg/ml of hygromycin, and 1 μg/ml of tetracycline. Env expression was induced by removal of tetracycline by washing cells with PBS, and culturing for 3 days in medium without tetracycline before the fusion experiments (Cao et al., 1996). Jurkat E6-1 cells were maintained in RPMI-10.

2.3. Fluorescent dyes

Red fluorescent DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and green fluorescent DiO (3,3′-dioctadecyloxacarbocyanine perchlorate) were obtained from Molecular Probes (Eugene, OR). Maximal excitation and emission energies are 549/565 and 484/501 nm, respectively. Stock solutions of dyes (3.5 mg/ml) were prepared in dimethylsulfoxide (DMSO) as described (Huerta et al., 2002), and stored at room temperature protected from light. DiI stock can be used for 8 months. DiO stock should be prepared fresh monthly.

2.4. Cell labeling, fusion assay, nuclei counting and cell sorting

Cell labeling was performed the day before the fusion experiments (Huerta et al., 2002). To label cells with DiI, 8 μl of a 1/10 dilution of the DiI stock solution were added to $5–15 \times 10^6$ cells suspended in 1 ml of RPMI-10. After incubation for 15 min at room temperature in the dark, cells were washed twice with 10 volumes of RPMI-10, resuspended in the same medium (adding hygromycin and G418 to transfected lines) and cultured overnight at 37 °C, with 5% CO_2. To label cells with different concentrations of DiO (from 3.9 to 132 μM), appropriate aliquots of the stock solution (from 1 to 34 μl) were added to $5–15 \times 10^6$ cells suspended in 1 ml of RPMI-10, and the same procedure described for DiI labeling was performed. Fusion experiments were performed by coculturing 2×10^5 cells from each fusion partner in serum-free medium (AIM-V medium, Gibco BRL). Cocultures were incubated for 5 h at 37 °C, in 5% CO_2. Wells containing anti-CD4 mAb (Pharmingen) and a coculture of labeled non-fusogenic 522F/Y and E6 cells were always included as controls for fusion inhibition and cellular aggregation, respectively. When anti-CD4 mAb was used, it was added to E6 cells before the addition of HXBc2(4) cells. After coculture, cells were collected from plates, washed once with 2 ml of PBS, and analysed immediately by FACS. Adjustment for autofluorescence was carried out using non-labeled cells, whereas single-labeled cells that had not been cocultured were used for compensation of red and green signals. Loosely aggregated cells were dissociated by gently pippeting immediately before analysis of 10,000 events in a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using the CellQuest software (Becton Dickinson). Fusion and cell aggregation were determined after exclusion of cell debris. Results are shown as averages of duplicates.

For nuclei counting, cells were collected from plates after coculture, washed and incubated for 15 min at 37 °C with 1.5 μM of Hoechst 33342 dye (Molecular Probes). Nuclei were counted in triple-fluorescent cells.

Sorting of FRET-positive and FRET-negative double fluorescent cells was performed in a FACSAria flow cytometer (Becton Dickinson) using the FACSDiva software (Becton Dickinson). Cells were collected in Dulbecco's phosphate buffered saline containing 10% FBS and photographed immediately.

2.5. Fusion inhibitors

T-20 peptide (Fusion inhibitor from Roche) was obtained through the NIH AIDS Research and Reference Reagent Program. Preparations of peptide solutions and concentration determination were carried out as indicated by the provider. Anti-CD4 mAb (RPA-T4 clone) was purchased from BD Pharmingen (San Diego, CA).

3. Results

Coculture of HIV-1 Env-expressing Jurkat HXBc2 cells with Jurkat E6 cells (denoted here as Env^+ and $CD4^+$ cells, respectively) leads to cell fusion and syncytia formation, manifested by the formation of multinucleated giant cells containing different numbers of nuclei (Fig. 1). Quantification of syncytia was accomplished by labeling the fusion partners with the lipophilic probes DiO (green) and DiI (red), followed by flow cytometry analysis once fusion takes place (Huerta et al., 2002). An excellent agreement was found between the numbers of

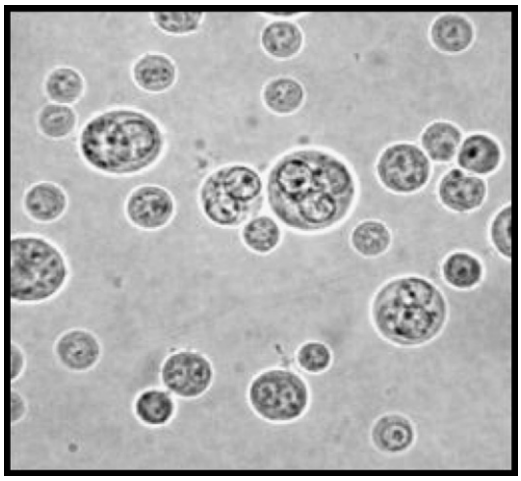

Fig. 1. Diversity of syncytia generated in cocultures of Jurkat CD4$^+$ and Env$^+$ cells. Magnification: 400×.

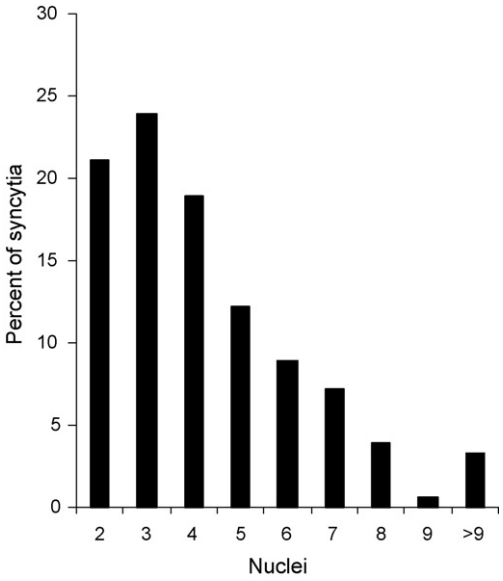

Fig. 2. Number of nuclei in syncytia formed in cocultures of DiI-CD4$^+$ and DiO-Env$^+$ cells. Nuclei were stained with the Hoechst 33342 dye and counted in 180 triple-fluorescent cells.

Table 1
Percentage of syncytia and single cells obtained by visual counting and flow cytometry

	Microscopy counting[a]	Flow cytometry[b]
Single red (CD4$^+$)	45.1	41.3
Single green (Env$^+$)	34	36.4
Syncytia	21	20.8

[a] Syncytia and single cells were identified as double and single fluorescent cells, respectively, in cocultures of DiI-CD4$^+$ and DiO-Env$^+$ cells. A total of 863 cells were analyzed in 58 microscope fields.
[b] FACS quantitation was carried out on 10,000 events.

syncytia and unfused cells determined by microscopic counting and by flow cytometry (Table 1). Small syncytia, containing two to four nuclei, accounted for 64% of the total syncytia population. There was, however, a small population of large syncytia with nine or more nuclei (Fig. 2).

When analyzed by flow cytometry, double fluorescent cells consistently exhibited an increased red DiI fluorescence intensity relative to that of their non-fused counterparts. The enhancement of red fluorescence in syncytia was observed regardless which fusion partner was DiI-stained. Thus, in cocultures of DiI-CD4$^+$ with DiO-Env$^+$, and of DiO-CD4$^+$ with DiI-Env$^+$ cells, the double fluorescent particles showed an approximately three-fold increase in the average red fluorescence intensity over their red-only counterparts (Fig. 3). In both cases, the percentage of double fluorescent particles was quite similar (18.6 and 17.7%, respectively), and monoclonal antibody against CD4 diminished fusion to 3 and 2.7%, respectively (data not shown). This indicates that enhancement of red fluorescence in syncytia occurs regardless of the kind of the red-labeled cell and that fluorescent dyes do not influence the specificity of the cell fusion.

The enhancement of red fluorescence in fusion products could originate from incorporation of multiple red cells into syncytia relative to green cells, or to an energy transfer from DiO to DiI, or both. The emission spectrum of DiO and the absorption spectrum of DiI overlap extensively: DiO emission energy ranges between 480 and 650 nm, while excitation energy of DiI ranges between 450 and 585 nm (Molecular Probes, http://www.probes.com). Thus, fluorescence resonance energy transfer (FRET) could take place when dyes are located close

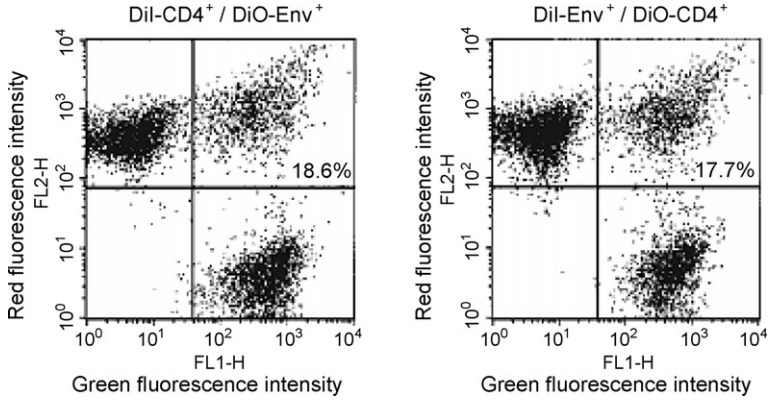

Fig. 3. FACS analysis of fusion between Env$^+$ and CD4$^+$ Jurkat cells labeled either with DiI (red) or DiO (green), as indicated. Note that red fluorescence in fused cells is enhanced with respect to that of the non-fused red cells regardless of the type of red-labeled cell.

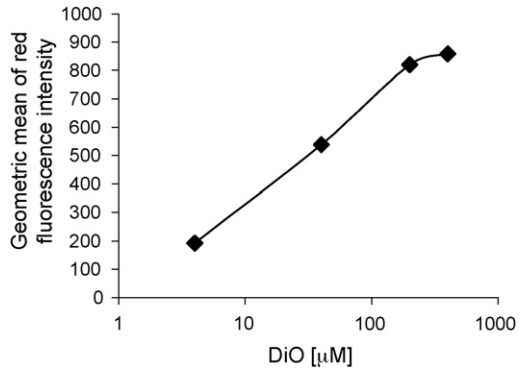

Fig. 4. Red fluorescence intensity of CD4+ cells labeled with 4 μM of DiI and with increasing concentrations of DiO, as indicated.

together in the same membrane compartment, increasing the red emission of DiI. To test this, DiI-labeled CD4+ cells were further stained with increasing concentrations of DiO and analyzed by FACS. The red DiI fluorescence intensities of double-stained cells were compared to that of DiI-CD4+ red-only cells. As can be seen in Fig. 4, the intensity of red fluorescence increased along with the amount of DiO incorporated, indicating FRET from DiO to DiI.

Labeling Env+ cells with a concentration of DiO higher than that used in experiments shown in Fig. 3 could potentiate FRET, providing a clearer distinction between fused and cellular aggregates. As shown in Figs. 5A and 6, two populations of double fluorescent cells were actually observed when DiI-labeled CD4+ cells were cocultured with Env+ cells labeled with high concentrations of DiO, ranging from 12 to 120 μM: one showing a red DiI-fluorescence enhancement, namely FRET-positive, and another with the same fluorescence intensity as non-fused cells, here denoted as FRET-negative. Incubation in the presence of an anti-CD4 mAb reduced the percentage of the FRET-positive population from 14 to 1.6% (90% inhibition) while the percentage of FRET-negative cells was not affected (Fig. 5B). When DiI-labeled CD4+ cells were cocultured with non-fusogenic DiO-labeled 522F/Y cells, only a small percentage of FRET-positive cells (0.4%) was detected. In this coculture, the percentage of FRET-negative particles remained 1.7% (Fig. 5C). FRET-positive and FRET-negative cells from a coculture similar to that in Fig. 5A were separated by cell sorting and examined by fluorescence microscopy; the double fluorescent FRET-positive cells consisted of syncytia, whereas, the FRET-negative population was composed of single-colored individual cells (Fig. 7). Thus, presence of FRET allows differentiation

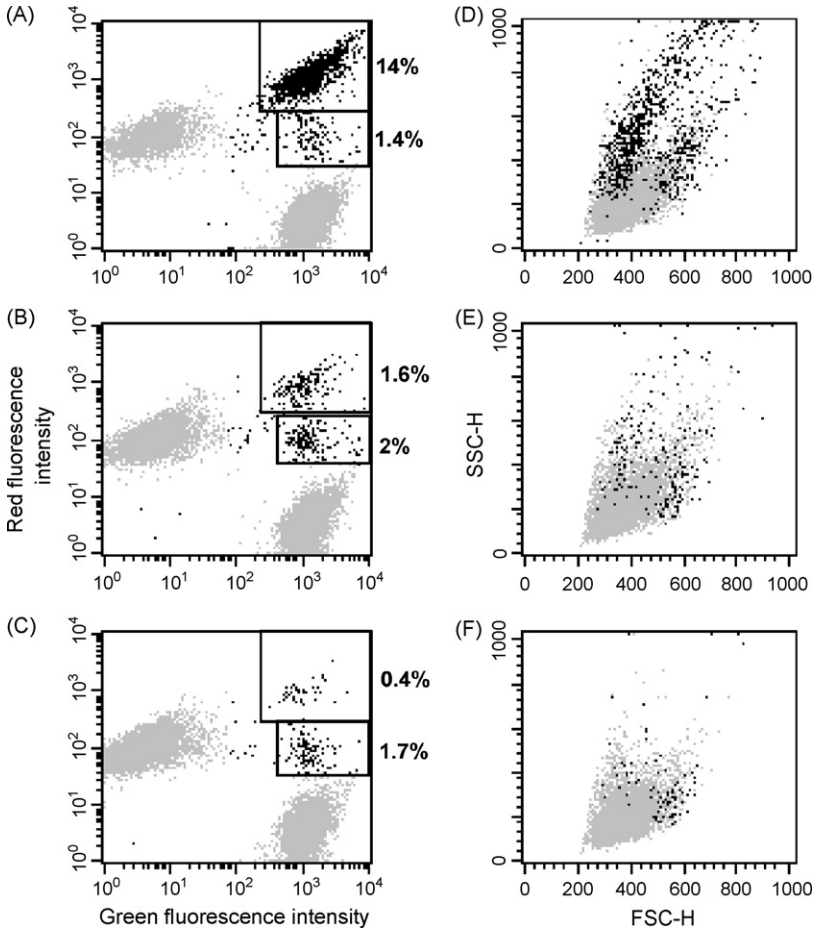

Fig. 5. Fluorescence and side vs. forward scatter profiles of FRET-positive (fused cells, upper region) and FRET-negative (cellular aggregates, lower region) in cocultures of DiI-CD4+ cells with: DiO-Env+ cells (A and D); DiO-Env+ cells plus 30 μg/ml anti-CD4 mAb, (B and E); and DiO-522F/Y cells (Jurkat cell carrying a non-fusogenic mutation in gp41) (C and F).

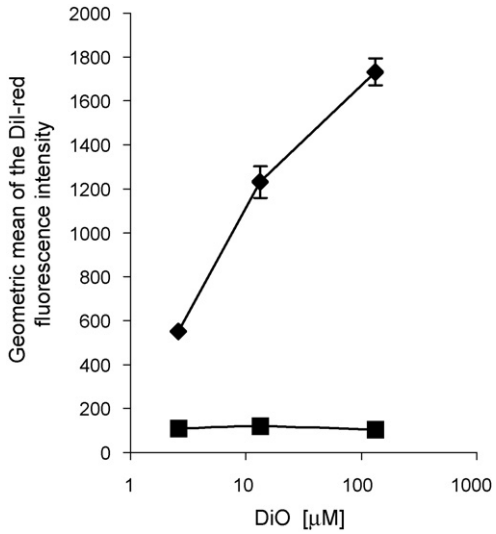

Fig. 6. Red fluorescence intensity of FRET-positive (fused cells) and FRET-negative (cellular aggregates) of double fluorescent particles generated in cocultures of DiI-CD4$^+$ cells with Env$^+$ cells labeled with increasing concentrations of DiO.

between fused cells and transiently aggregated cells in the flow cytometric assay. Syncytia showed an enhancement of red fluorescence intensity which positively correlated with the level of DiO incorporated in the Env$^+$ cells, while red fluorescence intensity of cell aggregates was not modified (Fig. 6). The labeling of Env$^+$ cells with high concentrations of DiO did not modify the percentages of fusion and aggregated cells nor the extent of inhibition by the anti-CD4 mAb (data not shown).

FRET-positive cells showed a wide distribution of size and granularity whereas the FRET-negative population only had greater size than single fluorescent cells (Fig. 5D–F). These plots also illustrate that a clear distinction between syncytia and unfused cells cannot be made on the basis of their size and granularity profiles because of the extensive overlap between fused and single cell populations caused by the high proportion of small syncytia.

Whether multiplicity of red cells in syncytia contributes to the increase in red fluorescence intensity observed was determined by fusion experiments performed with cells labeled with the red and green cytoplasmic dyes CMTMR and CMFDA. These dyes do not give rise to FRET, since double labeling of single cells with CMTMR and increasing concentrations of CMFDA does not produce an increase of the CMTMR red fluorescence (data not shown). Simultaneous experiments performed with both cytoplasmic and lipophilic dyes, showed that the red intensity of fused cells relative to that of the respective red unfused cells is considerably higher when lipophilic DiI and DiO dyes are used (1.3 and 8.9 for cytoplasmic and lipophilic dyes, respectively). Thus, a large number of red cells in syncytia does not account for the observed increase in DiI fluorescence.

The FRET-based flow cytometry method was used to determine the sensitivity of the HIV-envelope dependent cell fusion assay to anti-CD4 mAbs and the fusion inhibitor T-20 peptide (Wild et al., 1994). T-20 strongly inhibited cell fusion with an IC_{50} value of 1.7 nM (IC_{90} = 9 nM). This was nearly 45 times lower than the molar concentration of the anti-CD4 mAb required to get the same level of inhibition (IC_{50} = 75 nM, IC_{90} > 200 nM). The percentage of cellular aggregates was not significantly affected by these compounds (Fig. 8).

4. Discussion

In FRET, the excited state energy from a donor is transferred to an acceptor fluorochrome. The increase in fluorescence intensity of the acceptor is the best indicator of FRET. The rate of energy transfer depends upon the extent of overlap of their respective emission and absorption spectra, the relative orientation of the donor and acceptor transition dipoles, and the distance between these molecules (Wu and Brand, 1994). Here it was shown that cell fusion and syncytia formation can be quantitated by flow cytometry and that the appearance of FRET is a useful criterion to distinguish true fusion from cellular aggregation. Double-labeling of a cell line with the two fluorescent lipophilic dyes DiI and DiO produced an enhancement of the DiI-red emission equal to that observed in fusion experiments, demonstrating that incorporation of DiO and DiI in the membrane of the same cell produces an energy transfer that can be detected by flow cytometry. Increasing the amount of DiO incorporated into the cell membrane produced a proportional enhancement of the DiI fluorescence in both double-labeled

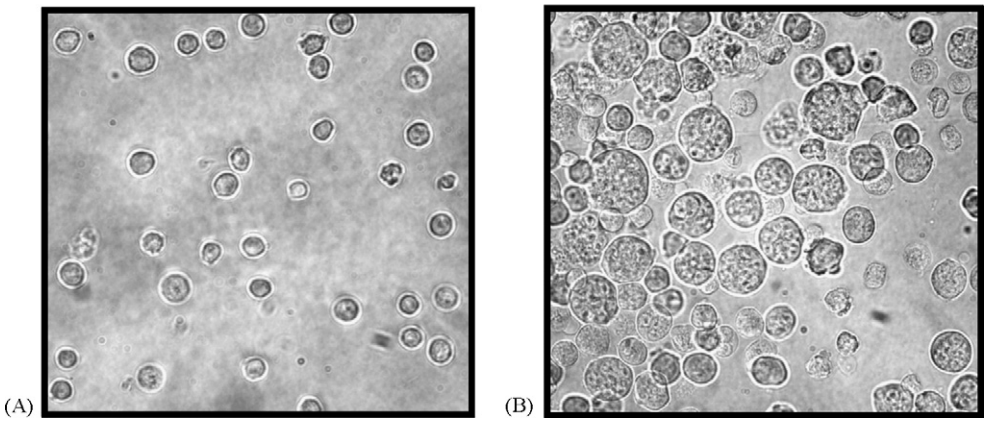

Fig. 7. Appearance of double fluorescent cells after sorting of (A) FRET-negative and (B) FRET-positive particles. Magnification: 400×.

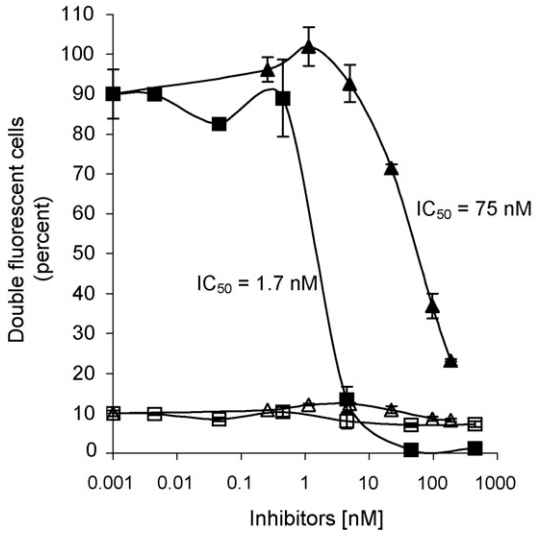

Fig. 8. Effect of the fusion inhibitor peptide T-20 (squares) and anti-CD4 monoclonal antibody (triangles) on cell fusion (solid symbols) and cellular aggregation (open symbols), as analyzed by the FRET-based flow cytometry method. Fused cells are sensitive to both inhibitors at different degrees, while aggregated cells are not appreciably affected.

single and fused cells, consistent with an increased excitation of the DiI molecule. FRET may be favored by the partition of DiI and DiO to detergent resistant microdomains in the cell membrane due to their two 18-carbon saturated fatty acid chains (Klausner and Wolf, 1980; Mukherjee et al., 1999).

The cell labeling procedure does not interfere with fusion specificity, as indicated by the fact that neither total fusion nor sensitivity to inhibition by the anti-CD4 mAb were altered by the labeling of cells with several fold increments of DiO. The small percentage of FRET-positive double fluorescent cells observed in cocultures of $CD4^+$ with non-fusogenic 522F/Y cells, may be due to spontaneous fusion, a phenomenon that has been reported to occur at low levels in tumor cell lines (Duelli and Lazebnik, 2003).

The FRET-based flow cytometry method was useful to determine the inhibitory activities of an anti-CD4 mAb, which blocks the CD4 gp120-binding site (Esser et al., 2000), and the HIV gp41-binding peptide T-20, that hinders the gp41 transition to the fusogenic stage (Furuta et al., 1998). Both molecules blocked fusion in a dose dependent manner, with potencies differing by 45-fold. The IC_{50} obtained here for the anti-CD4 mAb (RPA-T4 clone) was intermediate compared to other reported values using HeLa and CHO cells for Env-expression and reporter genes for fusion quantitation (Hong et al., 1999; Sakamoto et al., 2003). Additionally, the concentration of T-20 peptide required for cell fusion inhibition was quite similar to that required to inhibit cell fusion induced by a panel of prototypic HIV strains, as determined by counting of multinucleated giant cells (Wild et al., 1994) and monitoring of cell-to-cell transfer of a cytoplasmic dye by fluorescence microscopy (Liu et al., 2005). Thus, combination of flow cytometry and FRET provides a method with a similar degree of sensitivity compared to other current methodologies, allowing us to distinguish inhibition due to different mechanisms but with the advantages of technical simplicity and non-ambiguous numerical quantification. Fusion efficiency can be determined (i.e., the actual percentage of syncytia in the cell population) and the study of cell population dynamics during cell fusion can be accomplished by tracking of the number of syncytia and unfused cells in a series of coculture wells analyzed successively. Furthermore, studies on the phenotypic features of both fused and unfused cells may be performed using antibodies coupled to third party fluorescent molecules. However, it is clear that homologous fusion (red–red or green–green cell fusion) cannot been detected simultaneously with heterologous fusion in this system, although it may be evaluated by coculture and FACS-analyses of cells of the same type that have been labeled with DiI and DiO.

FRET combined with flow cytometry has been applied in cellular biology to study nonrandom co-distribution of membrane proteins (Batard et al., 2002; Szöllösi and Damjanovich, 1994). In the present study, it is shown that FRET combined with flow cytometry can be used to discriminate between cell fusion and cellular aggregation.

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