Volume 239, number 1, 88–92 FEB 06423 October 1988

Quantitative measurement of fusion between human immunodeficiency virus and cultured cells using membrane fluorescence dequenching

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Received 19 July 1988; revised version received 31 August 1988

Human immunodeficiency virus (HIV) was purified by sucrose gradient centrifugation and labeled with octadecylrhodamine B-chloride (R-18) under conditions resulting in 90% quenching of the fluorescence label. Incubation of R-18-labeled HIV (R-18/HIV) with CD4-positive CEM and HUT-102 cells, but not with CD4-negative MLA-144 cells, resulted in fluorescence dequenching (DQ, increase in fluorescence) of 20–25%. Similar level of DQ was observed upon incubation of CEM cells with R-18-labeled Sendai virus. DQ was observed when R-18/HIV was incubated with $CD4^+$ cells at 37°C, but not at 4°C. Most of the increase in fluorescence occurred within 5 min of incubation at 37°C and was independent of medium pH over the range of pH 5–8. Preincubation of cells with the lysosomotropic agent NH_4Cl had no inhibitory effect on DQ. Complete inhibition was observed when target cells were fixed with glutaraldehyde prior to R-18/HIV addition. Our results demonstrate application of membrane fluorescence dequenching method to a quantitative measurement of fusion between HIV and target cell membranes. As determined by DQ, HIV penetrates into target cells by a rapid, pH-independent, receptor-mediated and specific process of fusion between viral envelope and cell plasma membrane, quite similar to that observed with Sendai virus.

HIV; Fluorescence dequenching; Membrane fusion; Target cell membrane

1. INTRODUCTION

Human immunodeficiency virus (HIV), the primary etiological agent of the acquired immunodeficiency syndrome (AIDS) [1–3], is characterized by a remarkable potential to induce fusion and lysis of CD4-positive human T lymphocytes [1–4]. The cytopathic effects of HIV are considered to be the main cause of the immune defects leading to AIDS [5]. According to recent reports, HIV enters CD4-positive target cells via fusion between viral envelope and target cell membranes [6–8]. Binding and penetration of infectious virus requires the presence of functional envelope glycoproteins, gp41 and gp120 [9,10], and neutralization or mutational inactivation of any of these proteins also abolishes cell-to-cell fusion [11,12]. The presence of surface CD4 receptors is necessary for HIV infection and for HIV-mediated cell fusion and lysis [4,13]. Hence, the interaction between fusogenic HIV envelope components and target membrane (via specific membrane receptors), and the consequent cell fusion and lysis, may play a crucial role in determining the cytopathogenic outcome of HIV infection.

The exact parameters of the HIV-membrane fusion process have been difficult to establish

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Abbreviations: HIV, human immunodeficiency virus; SV, Sendai virus; AIDS, acquired immunodeficiency syndrome; DQ, membrane fluorescence dequenching; R-18, octadecylrhodamine-B chloride

Preliminary results of this study were presented at the IVth International AIDS Conference, Stockholm, June 12–16, 1988

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because of the lack of a reliable quantitative assay. Confusing results have been published, with some groups claiming that HIV penetrates by pH-dependent endocytosis [6], and others suggesting a pH-independent HIV-plasma membrane fusion [7,8]. Recently, fluorescently labeled virions and fluorescence dequenching methods have been applied to a quantitative analysis of membrane fusion occurring during penetration of various enveloped viruses, such as Sendai virus [14], influenza [15] or vesicular stomatitis virus [16]. The assay involves incorporation of a self-quenching dye, such as the octadecylrhodamine B-chloride (R-18), into intact virus particles. Intermixing (fusion) of R-18-labeled viral envelopes with target membranes results in relief of self-quenching measured as an increase in fluorescence (or dequenching, DQ) (reviewed in [17]). In the present report, we describe the application of the membrane fluorescence dequenching method to the study of HIV-membrane fusion. The measurements of the kinetics, extent, temperature, pH dependence and host cell dependence of the HIV fusion reaction demonstrates that HIV, similar to paramyxoviruses, penetrates target cells by a receptor-mediated, rapid and pH-independent process of envelope-cell membrane fusion.

2. MATERIALS AND METHODS

2.1. *Materials*

Octadecylrhodamine B-chloride (R-18) was obtained from Molecular Probes (Oregon, USA); all other chemicals were of analytical grade.

2.2. *Cells*

The $CD4^+$ cell line of human T cell leukemia origin, CEM [18], was obtained from L. Montagnier. The $CD4^+$ T lymphoblastoid cell line carrying HTLV-I virus, HUT 102, subclone B2 [19] was obtained from W. Greene. MLA 144 cells, a CD4-negative T cell of gibbon lymphoma origin [20], were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown as stationary cultures in RPMI 1640, supplemented with 5% fetal bovine serum and antibiotics.

2.3. *Propagation of HIV and SV, and preparation of fluorescently labeled virions*

HIV (N1T isolate) was propagated in CR10 cells as described previously [21]. Cell-free supernatants from virus-producer cultures were centrifuged at 17500 × *g* for 2 h at 4°C in a Beckman JA-10 rotor, and virus sediment was resuspended in 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4, and purified by centrifugation through a 10–60% sucrose gradient (18 h at 160000 × *g* at 4°C) as described previously [22]. Purified HIV was resuspended at 2 mg viral protein/ml and frozen in aliquots. SV was isolated from the allantoic fluid of fertilized chicken eggs as described [14] and kept stored at −80°C. Intact virions were labeled with R-18 essentially as described for Sendai virus [14]. Briefly, 0.6 μl of 1 mg/ml ethanoic solution of R-18 were rapidly added to 35 μl PBS (150 mM NaCl and 10 mM phosphate buffer, pH 7.4) containing 50 μg of HIV or SV. After 15 min incubation in the dark, virus preparations were diluted in PBS to 1.5 ml and separated from free dye by 15 min centrifugation in an Eppendorf centrifuge. Labeled virions (R-18/HIV or R-18/SV) were resuspended in PBS to a final concentration of 0.5 mg/ml viral protein/ml.

2.4. *Fluorescence measurements*

R-18-labeled viruses were adsorbed to target cells (2.5 μg viral protein/1 × 10^6 cells) in 100 μl RPMI 1640 for 30 min at 4°C, followed by washing of nonadsorbed virus and incubation at 37°C for the designated period of time. Cells were then diluted to 3 ml and analyzed for fluorescence in a Perkin-Elmer LS-3B fluorometer (560 nm excitation, 608 nm emission). Cell suspension in a cuvette was then supplemented with 0.1 ml Triton X-100 (5% stock solution) to elute R-18 from membranes as a measure of 100% relaxation. The background fluorescence value was obtained by mixing R-18/HIV and target cells immediately before measurement, so no intermixing of R-18/virus and target cell membranes could occur. Specific DQ was calculated as follows [17]:

% DQ = fluorescence units of:

$$\left[\left(\frac{(\text{system} \times \text{Triton}) - \text{system}}{\text{system} \times \text{Triton}}\right) - \left(\frac{(\text{background} \times \text{Triton}) - \text{background}}{\text{background} \times \text{Triton}}\right)\right] \times 100$$

3. RESULTS

Addition of R-18 to sucrose gradient-purified HIV, under the conditions previously established for other enveloped animal viruses [17], resulted in self-quenching of the dye. Solubilization of R-18-labeled HIV with Triton X-100 led to 10–15-fold increase in fluorescence, suggesting that the dye was incorporated into the viral envelope bilayer membrane. The R-18-labeled HIV remained biologically active, as determined by infectivity tests using CD4-positive CEM cells (not shown).

Incubation of R-18-labeled HIV with CD4-positive human T lymphoid cells CEM and HUT 102 resulted in membrane fluorescence dequenching (i.e., increase in fluorescence signal) of 20–25% (table 1). The extent of the DQ was similar to that observed under the same conditions

Table 1

Interaction of fluorescently-labeled human immunodeficiency virus and Sendai virus with cultured cells

System	Dequenching (DQ) (%)
Expt I	
CEM + R-18/HIV, 37°C	22
CEM + R-18/HIV, 4°C	0
MLA 144 + R-18/HIV, 37°C	0
MLA 144 + R-18/HIV, 4°C	0
HUT102 + R-18/HIV, 37°C	25
Expt II	
CEM + R-18/HIV, 37°C	21
GA/CEM + R-18/HIV, 37°C	3
CEM + NH_4Cl + R-18/HIV, 37°C	15
Expt III	
CEM + R-18/SV, 37°C	33
CEM + R-18/SV, 4°C	1
GA/CEM + R-18/SV, 37°C	15

Experimental conditions were as described in section 2. Crosslinking of CEM cell surface with glutaraldehyde (GA/CEM) was achieved by incubating freshly washed CEM cells (2×10^6/ml) in PBS containing 1% glutaraldehyde for 15 min at 4°C, followed by 3 washes in PBS

during incubation of R-18-labeled Sendai virus with CEM cells (table 1). The DQ most probably resulted from a specific interaction between R-18/HIV and virus receptor-positive CEM or HUT 102 cell membranes, because no increase in fluorescence was observed upon addition of R-18/HIV to gibbon T cells MLA 144 which do not express surface CD4 molecules/HIV receptors (Volsky, D.J., unpublished). MLA 144 were also refractory to infection with R-18/HIV or unlabeled virus (not shown). The specificity of R-18/HIV-membrane interaction was further demonstrated by using glutaraldehyde-fixed CEM cells as targets. As shown in table 1 (expt II), incubation of R-18/HIV to GA/CEM cells resulted in DQ of only 3%, an 85% inhibition vs the DQ value for unfixed CEM cells. DQ of R-18/HIV fluorescence could also be prevented to a large measure by preincubating target cells with OKT4a monoclonal antibody, which specifically blocks the HIV-CD4 receptor binding site ([4], not shown).

The degree of DQ observed following incubation of fluorescently labeled HIV with CEM cells was dependent on the number of cells present in the incubation medium, reaching a peak value at

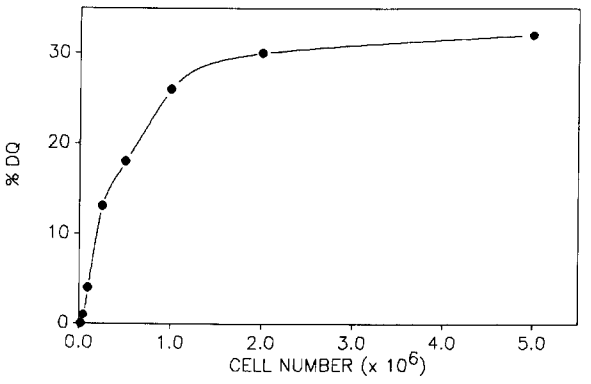

Fig.1. Quantification of HIV-target membrane fusion by membrane fluorescence dequenching (DQ): cell saturation kinetics. Experimental conditions were as described in section 2. Time of incubation was 30 min at 37°C.

2×10^6 cells/1 μg R-18/HIV (fig.1). The peak values differed for different target cells accordingly with the relative expression of surface CD4 receptors (not shown).

Kinetic studies revealed that the interaction between R-18/HIV and target cell membranes, as measured by DQ, was rapid and temperature dependent (fig.2 and table 1). While only negligible DQ was observed during 60 min of incubation of R-18/HIV with CEM cells at 4°C, transfer of the virus-cell mixture to 37°C resulted in a rapid increase in fluorescence, reaching 50% of the maximum DQ value within less than 2 min (fig.2). The process was essentially completed in 10–15 min at

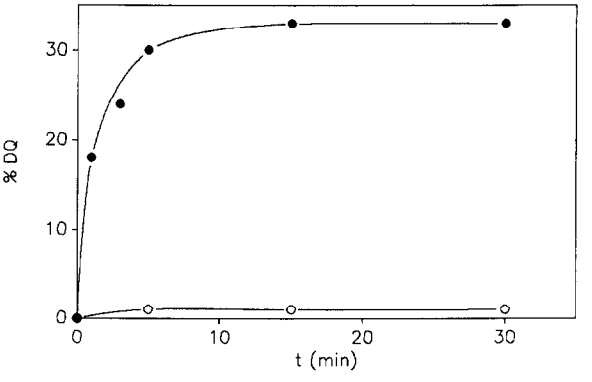

Fig.2. Quantification of HIV-target membrane fusion by membrane fluorescence dequenching (DQ): time kinetics and temperature dependence. Experimental conditions were as described in section 2. Open and shaded symbols designate incubation at 4°C and 37°C, respectively.

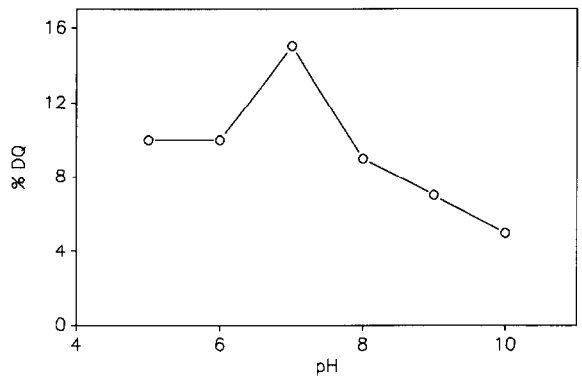

Fig.3. pH-dependence of HIV-target membrane fusion as determined by membrane fluorescence dequenching. Experimental conditions were as described in section 2, except that 0.16 M NaCl solution, buffered to appropriate pH with phosphate buffer was used instead of RPMI. Time of incubation: 30 min at 37°C.

37°C, similar to time kinetics data presented for other enveloped fusogenic viruses [14,15], including Sendai virus used here for comparison (table 1, expt III).

The experiments described above established the basic quantitative parameters for HIV-cell interaction as measured by DQ method (table 1, figs 1 and 2). We then approached the controversial issue [7–9] of the pH dependence of HIV fusion reaction. Our result show (fig.3) that the highest increase in DQ was obtained at the pH range of 5–8 with a peak value at pH 7.0. Preincubation of CEM cells with a lysosomotropic agent ammonium chloride (50 mM) had little effect on DQ (table 1, expt II).

4. DISCUSSION

The results presented here demonstrate the application of membrane fluorescence dequenching technique [17] to a quantitative measurement of the process of HIV penetration into target cells. Purified HIV virions can be labeled with a fluorescent lipotropic dye R-18 under the conditions of 90% fluorescence quenching and without impairment of virus infectivity. Incubation of R-18/HIV with target cells results in 20–25% increase in fluorescence. We suggest that this increase, which represents a relaxation of quenched molecules in the viral envelope [17], reflects the actual process of fusion between HIV envelopes and target membranes. This conclusion is supported by the results showing the temperature and target cell number dependence of DQ, the requirement for the presence of specific HIV receptors, and DQ inhibition by crosslinking of target cell surface proteins.

Use of DQ technique allowed us to elucidate several characteristics of HIV-membrane fusion that could not be determined by previously used semiquantitative methods [6–8]. One such feature is the kinetics of HIV-membrane fusion at 37°C which, at 2 min for the half-maximal degree of DQ, resembles rapid fusion kinetics of potent fusogenic RNA viruses such as Sendai [4] or influenza [15]. Another feature is the apparently high efficienty of HIV-membrane fusion process as reflected by the high degree of DQ (20–25%). This result indicated that a significant proportion of labeled HIV particles interact with target cell membranes. Similarity in this respect with Sendai virus fusion should be noted again, as is evident from the comparison between the two viruses shown in table 1. Our results also show that HIV, like SV and other enveloped viruses, does not fuse significantly with target membranes at 4°C. This finding is in contrast with the observation of Stein et al. [7] who showed electron micrographs of HIV particles presumably fusing with cells at 4°C. While this discrepancy might be due to differences among the HIV strains used, it is not likely to be so. Generally, fusion between biological membranes is favored by an increased membrane fluidity in temperatures above 4°C [23]. Papahadjopoulos et al. [24] have shown, for example, that fusion between phospholipid vesicles requires the interacting membranes to be in a fluid state, i.e., above the phospholipid-phase transition temparature. Similarly, fusion and infectivity (but not binding) of Sendai, influenza and other enveloped viruses are completely blocked at 4°C [14,15]. Hence, fusion between HIV and plasma membranes at 4°C might be an extremely rare event, which is undetectable by DQ but can be occasionally visualized by EM.

Stein et al. [7] and McClure et al. [8] demonstrated that synthesis of proviral DNA and syncytium formation, following exposure to HIV, was not affected by inhibitors of endocytosis, and suggested that HIV enters target cells by a pH-independent fusion with plasma cell membrane.

Maddon et al. [6] provided evidence to the contrary, favoring endocytosis as a mode of HIV penetration. Interpretation of results presented in these investigations is difficult because neither directly measured HIV penetration when it occurs, namely, within 15 min of HIV addition at 37°C (fig.2). The data shown here clearly demonstrate that HIV entry into CD4-positive cells, as measured by DQ, does not require low pH and occurs most efficiently at pH 7. This evidence favors a pH-independent HIV envelope-plasma membrane fusion as the principal mechanism of HIV penetration. On the other hand, HIV-membrane fusion also clearly occurred at acidic pH (fig.3), suggesting that under specific conditions (or in certain cells) HIV may penetrate cells via the endocytotic pathway. Whether or not this mode of virus entry may lead to a productive infection has not yet been determined.

In conclusion, our data demonstrate that HIV may enter cells by a rapid, pH-independent, receptor-mediated and temperature-dependent process of fusion with plasma membrane. However, the virus can also penetrate into cells at pH 5, compatible with receptor-mediated endocytosis. The parameters of virus-membrane fusion as determined by DQ put HIV in the same category of fusogenic viruses as Sendai virus. Studies are underway to determine functional similarities between HIV envelope glycoproteins and fusion factors of paramyxoviruses.

Acknowledgements: We would like to thank Li Gongrong and Barbara Volsky for help in propagating viruses and cells. Special appreciation is extended to Drs J. Cantor and S. Keller for allowing us to use their equipment during preliminary stages of this work. The secretarial assistance of Marilyn Steed is also gratefully acknowledged. This work was supported in part by grants from the National Institutes of Health (CA 43465 and AI 25902) and by a grant from the American Foundation for AIDS Research (AmFAR).

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