Antifusion Activity in Sera from Persons Infected with Human Immunodeficiency Virus Type 1

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Cell-to-cell fusion plays an important role in the pathogenesis of human immunodeficiency virus type 1 (HIV-1) infections. An assay to measure the antifusion activity of serum has been developed by using the fusion event that occurs between H9 cells chronically infected with HIV-1 (H9_{IIIB}) and fusion-susceptible MT-2 cells. The endpoint is determined by measuring neutral red uptake in cells after syncytium formation is allowed to occur in the presence of various serum dilutions. The assessment of antifusion activity in serum by neutral red uptake has been shown to correlate with syncytium reduction as determined by direct counting. The optimal number and ratio of cells in the suspension for efficiency and speed of the assay have been determined. With this assay it was shown that 50% of 36 serum specimens capable of neutralizing cell-free virions failed to inhibit syncytium formation. The assay can thus measure a distinct activity in HIV-1-immune human sera which is a subset of neutralization activity. Because of the potential role of this activity in the rate of disease progression and protective immune responses, the antifusion assay will be an important tool for the investigation of disease pathogenesis and for acquired immunodeficiency syndrome vaccine development. The assay can also be applied to the investigation of the pathogenesis of the fusion event at the cellular level. The ability to use absorbance measurements rather than syncytium counts as the endpoint facilitates direct computer-assisted data analysis, which expedites the performance of the assay.

Syncytium formation is a major cause of cell killing induced by human immunodeficiency virus type 1 (HIV-1) in vitro (7, 10) and may contribute to CD4 depletion and cell-to-cell spread of the virus in the absence of cell-free virus in vivo (1, 6, 13). Syncytium formation occurs when the viral surface glycoprotein, gp120, expressed on the surfaces of HIV-1-infected cells binds to the viral receptor, CD4, on the surfaces of uninfected target cells (7, 14). The viral transmembrane glycoprotein, gp41 (5), and additional cellular surface molecules, particularly the leukocyte adhesion receptor LFA-1 (4), may then become involved in membrane fusion events in which one infected cell can fuse with multiple surrounding uninfected cells. The end result of this fusion process is the formation of multinucleated giant cells called syncytia, and this process is a cardinal element in the pathogenesis of acquired immunodeficiency syndrome. We have developed an assay to measure the antifusion activity of serum which is based on neutral red uptake and correlated to syncytium reduction in cell cultures. The assay is reproducible and is amenable to computer-assisted analysis because absorbance measurements are used as the endpoint.

MATERIALS AND METHODS

Sera. Sera were obtained from persons who were confirmed to be HIV-1 seropositive by an enzyme-linked immunosorbent assay (Abbott Diagnostics) on at least two separate occasions and by Western blot (immunoblot) (DuPont) on at least one occasion.

Cells. MT-2 is a fusion-susceptible cell line (3) used as the target cell line for the assay. It is a CD4⁺ T-lymphoblastoid cell line derived from human T-cell lymphotropic virus type I-transformed cord blood lymphocytes (9). The subclone of MT-2 cells used here was a gift from Doug Richman, San

Diego Veterans Administration Hospital. H9 cells chronically infected with HIV-1 (H9_{IIIB}) and uninfected H9 parent cells were gifts from Robert Gallo, National Cancer Institute (12). All cell lines were maintained in RPMI 1640 containing 12% fetal bovine serum and 50 µg of gentamicin per ml.

Antifusion assay. The assay was performed in 96-well microdilution plates. All sera were heat inactivated at 56°C for 60 min prior to use. Serum from an HIV-1-seronegative person and a standard serum from an HIV-1-seropositive person were used as controls in each assay. Fifty microliters of growth medium was added to all wells, except for one row used as a blank control. Test sera (50 µl) were added to wells 3 to 11 in the bottom row and serially diluted 1:2 in triplicate. H9_{IIIB} cells were dispersed, washed once with growth medium, and resuspended to yield 6×10^5 cells per ml. The H9_{IIIB} cell suspension (100 µl) was added to all wells, except for the cell control column, in which an equal number of H9 cells was placed. Plates were incubated at 37°C under 5% CO₂ for 1 h. MT-2 cells were dispersed and adjusted to 3×10^6 cells per ml, and 100 µl was added to all wells, including the cell control column, resulting in an MT-2/H9_{IIIB} ratio of 5:1. The final dilution range for the test sera was 1:5 to 1:640.

Plates were incubated for 16 to 24 h in modular incubator chambers (Billips-Rothenberg, Del Mar, Calif.) flushed with 5% CO₂. The chambers were used for biosafety purposes, since 96-well microdilution plates are open vessels. Plates were processed when the majority of cells in the full cytopathic effect control wells (MT-2 plus H9_{IIIB} cells and no test serum) had undergone syncytium formation as observed by light microscopy. Cells were suspended by micropipette action, and 100 µl was transferred to corresponding wells of poly-L-lysine-coated plates containing 100 µl of Finter's neutral red (2) in growth medium per well. Plates were incubated for 1 h at 37°C to achieve optimal adherence and vital dye uptake in viable cells and syncytia (11). The medium was removed, and all wells were rinsed twice with 150 µl of phosphate-buffered saline. The dye was extracted

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with 100 μ l of 50% ethanol in 1% acetic acid. The extracted dye was colorimetrically quantitated at 540 nm with a Flow Titertek colorimeter. Antifusion activity (percent protection) is defined as the difference in the absorbance between the averages of test wells (MT-2 plus H9_{IIIB} cells and test serum) and full cytopathic effect control wells divided by the difference in the absorbance between the averages of noncytopathic effect control wells (MT-2 plus H9 cells and no test serum) and full cytopathic effect control wells: $[A_{540}(\text{test}) - A_{540}(\text{positive control})]/[A_{540}(\text{negative control}) - A_{540}(\text{positive control})]$. For noncytopathic and full cytopathic effect control wells, $n = 8$. For test wells, $n = 3$. Three serum specimens were tested per plate.

Data analysis was performed with Lotus 1-2-3 spreadsheet software installed on an IBM PC-AT interfaced to a Titertek type 3100 microdensitometer by a type 312B module (Flow Laboratories). The serum dilution resulting in 50% protection (or preservation of neutral red uptake) as determined by linear regression analysis was recorded as the antifusion titer.

Neutralization assay. The neutralization assay was similar to the antifusion assay, except for the following differences. Cell-free HIV-1 (2×10^6 to 4×10^6 infectious particles per ml), instead of H9_{IIIB} cells, was incubated with sera for 1 h prior to mixing with MT-2 cells. The number of MT-2 cells was reduced to 3×10^4 per well. Final dilutions of test sera ranged from 1:4 to 1:512. The assay required 72 to 96 h of incubation, and the results were analyzed by the neutral red uptake method (11).

RESULTS

Absence of serum toxicity. Wells containing serum from the lower dilutions of each serum sample were inspected for evidence of cytotoxicity by light microscopy. Occasionally, sera are toxic for MT-2 target cells and result in cell killing, which can result in reduced neutral red uptake despite the lack of syncytium formation. None of the sera used for this study were found to exhibit cytotoxic effects by light microscopy. The effect of the test sera on neutral red uptake and cell growth was evaluated with dilutions of HIV-1-immune and nonimmune sera in a mock antifusion assay with H9 instead of H9_{IIIB} cells and with harvesting of plates at 0 and 24 h for neutral red uptake measurements and counting. The sera neither conferred a growth advantage on cells nor altered the capacity of cells to take up neutral red. A doubling of cell numbers per well was noted after 24 h of incubation.

Correlation of neutral red uptake with syncytium formation. To determine the correlation between percent protection measured in the antifusion assay and syncytium reduction, we performed the assay in duplicate without sera and with nonimmune control sera, sera with neutralization but not antifusion activity, and sera with both neutralization and antifusion activities. Neutral red uptake was determined by the method described above, and duplicate wells were fixed with 10% Formalin and stained with Wright-Giemsa stain. The numbers of syncytia, determined by direct counting with an inverted microscope, could be estimated by a logarithmic function of the A_{540} with a correlation coefficient of 0.8. The correlation between syncytium counts and the absorbance values for sera with antifusion activity showed that 40% protection (preservation of neutral red uptake) corresponded to 90% syncytium reduction.

Ratio of target cells to effector cells and effect on the efficiency and timing of the assay. The ratio of effector cells to

target cells was relevant to the length of the assay and to the efficiency of fusion events. Under the conditions described in Materials and Methods (target/effector ratio of 5:1), the assay is ready to harvest at 16 to 24 h and there is one fusion event for every 250 to 400 H9_{IIIB} cells. If the number of MT-2 cells is kept constant and the target/effector ratio is increased beyond 50:1, the efficiency of the fusion event improves so that one fusion event occurs for every 100 to 125 H9_{IIIB} cells, but the length of the assay is extended to >36 h.

Differential neutral red uptake by cell type. The differential contributions of the three cell lines, H9, H9_{IIIB}, and MT-2, to total neutral red uptake were investigated. A total of 3×10^5 MT-2 cells per well or 6×10^4 H9 or H9_{IIIB} cells per well (the same number as that used in assay wells) were individually incubated in growth media (total volume, 250 μ l) for 30 h and transferred to poly-L-lysine-coated plates for dye extraction. The mean and standard deviation A_{540} s were 0.501 ± 0.035 for MT-2, 0.027 ± 0.007 for H9, and 0.014 ± 0.005 for H9_{IIIB}. A series of control wells with MT-2 plus H9 or MT-2 plus H9_{IIIB} had A_{540} s of 0.437 ± 0.028 and 0.058 ± 0.010 , respectively. Thus, MT-2 cells make the dominant contribution to neutral red uptake, with either H9 or H9_{IIIB} cells accounting for 5% or less of the absorbance measurement. The direct effect that HIV-1-immune sera may have on H9_{IIIB} cell function thereby does not affect the assay endpoint measurement.

Timing of test serum additions to assays. Test sera were added to wells prior to the addition of cells for efficiency and for the convenience of making serial dilutions in test wells. Because the assay is intended to be a measure of antifusion activity and not solely a measure of the inhibition of attachment, the timing of serum additions was investigated. Test serum dilutions were added to the assay system prior to the addition of cells and from 15 min to 6 h after MT-2 cells were mixed with H9 or H9_{IIIB} cells. Equivalent antifusion activity was detected when serum was added up to 2 h after cells were mixed. Serum added after 2 h had a diminished effect on syncytium inhibition (i.e., the preservation of neutral red uptake). These results suggest, but do not prove, that the antifusion activity measured by this assay is a postattachment phenomenon.

Comparison of neutralization and antifusion activities in HIV-1-immune sera. Neutralization and antifusion assays were performed on serum specimens from 36 HIV-1-infected individuals. Sera were number coded, and clinical information was not available. Neutralization titers of >1:10 were detected in 34 of 36 serum specimens, but only 17 of 36 serum specimens (47%) had antifusion titers of >1:10 (Fig. 1). When the $\log_2$ reciprocal serum dilution giving neutralization versus antifusion activity was plotted (Fig. 2), it was found that there was no antifusion activity in the sera without neutralization activity and that many sera had substantial neutralization titers but no detectable antifusion activity.

Reproducibility of the antifusion assay. Intraassay variability was assessed by determining standard deviations of the mean A_{540} in the triplicate experimental wells and in control wells. Assays in which the standard deviations were greater than 10% were repeated. Standard deviations usually fell in the range of 2 to 7%. Interassay variability was monitored by using a standard serum from an HIV-1-seropositive individual. In eight consecutive assays with this serum, the geometric mean titer of the $\log_2$ reciprocal serum dilution that gave 40% inhibition of fusion activity was 5.7, with a range of 5.2 to 6.2 and a standard deviation of 0.42.

FIG. 1. Neutralization (NT) and antifusion activities in individual sera, recorded as the log₂ reciprocal serum dilution yielding 40% syncytium reduction in the assays. For activity that was undetectable (<1:10), the value is represented by an open circle on the x axis.

DISCUSSION

The process of cell fusion is important in the pathogenesis of HIV-1 infection (7, 10, 14) and other viral infections (8). Inhibition of the fusion process in particular has been thought to be a critical determinant for immunity and virulence in paramyxovirus infections.

Cell-to-cell fusion should be considered separately from the fusion event that occurs between a virion envelope and a cell membrane, even though the mechanisms may have similarities. The property of cell-to-cell fusion inhibition should also be regarded as distinct from virus neutralization. Neutralization can be defined as activity that diminishes the infectivity of cell-free virions. For purposes of this study, we have defined antifusion activity as the inhibition of the fusion process that leads to syncytium formation between HIV-1-infected cells and CD4⁺ cells. To measure this event, we have used neutral red uptake, which has been inversely correlated with the number of syncytia. Cells involved in syncytia have a markedly diminished ability to take up neutral red, so the measurement reflects the number of target cells not involved in syncytium formation.

Several steps in addition to the correlation with syncytium formation were taken to ensure that the neutral red uptake assay was a valid measure of cell-to-cell fusion and did not represent an artifact of differential cell growth or a direct effect of serum on individual cell neutral red uptake efficiency. It was determined that neither HIV-1-immune nor nonimmune sera exhibited a toxic effect or conferred a growth advantage on cells in the assay which may have affected the efficiency of neutral red uptake. Furthermore, it was shown that neither H9 nor H9_{IIB} cells contributed

FIG. 2. Log₂ reciprocal neutralization (NT) and antifusion titers in the same serum sample plotted against each other to demonstrate the high NT titers in many of the sera with undetectable antifusion activity.

significantly to the total neutral red uptake in the system, because of the high target/effector ratio and because the capacity of an individual MT-2 cell for neutral red uptake was fivefold greater than that of an individual H9 or H9_{IIB} cell. Therefore, greater than 95% of the neutral red uptake was a function of the MT-2 target cell involvement in syncytium formation.

The conditions for the assay were adjusted for specificity and efficiency. The development of syncytia in MT-2 cells as caused by cell-free HIV-1 can be first noted between 72 and 96 h. Neutralization assays therefore must be incubated for 4 days before endpoint analysis. With a target/effector cell ratio of 5:1, the antifusion assay is ready to harvest and analyze at about 20 h, clearly distinguishing the activity being measured from neutralization activity. While the efficiency of fusion events per H9_{IIB} cell improves when the target/effector cell ratio is increased, the length of incubation required for the assay is also increased and becomes less distinct from that required for the neutralization assay. A target/effector cell ratio of 5:1 was chosen for its specificity and for the convenience of overnight incubation.

The data from clinical samples also suggest that the measurement of antifusion activity by this assay is distinct from the measurement of neutralization activity. There are several samples with high neutralization activity and no antifusion activity. On the other hand, there is no sample with measurable antifusion activity and no neutralization activity, suggesting that antifusion activity is a subset of neutralization activity. The explanation for this is not known. It is possible that a different quantity of antibody molecules is needed to prevent the more diffuse interaction that occurs between two cells than between a cell-free virus and a susceptible cell. Another explanation may be that all epitopes mediating fusion are also neutralization epitopes,

but not vice versa. Individuals with antibodies able to recognize only neutralization epitopes not involved in fusion would not develop antifusion activity. A third possibility is that a different set of protein interactions is involved in cell-to-cell fusion than in cell-free virus-target cell attachment and entry. While the gp160-CD4 interaction is certainly involved in the cell-to-cell fusion event, accessory protein interactions may also be necessary (4). Persons failing to evolve an antibody response to epitopes involved in accessory ligand formation may also have diminished antifusion activity.

In summary, we have developed an antifusion assay that is applicable on a large scale because the endpoint measurement is much easier to obtain than is direct counting of syncytia in standard plaque reduction assays. We believe that the assay specifically measures the inhibition of cell-to-cell fusion events, an activity distinct from neutralization activity. The assay will be used to investigate the pathogenesis of fusion, the clinical importance of antifusion activity, and the ability of candidate HIV-1 vaccines to elicit antifusion activity.

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