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Differential Susceptibilities of U-937 Cell Clones to Infection by Human Immunodeficiency Virus Type 1

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Single-cell clones derived from the U-937 monocytic cell line were studied for susceptibility to infection by human immunodeficiency virus type 1 (HIV-1). Of four such clones, we found that three (UC12, UC14, and UC18) supported replication of HIV-1 more efficiently than parental U-937 cells, as measured by reverse transcriptase activity and p24 core antigen production. In contrast, another clone (UC11) showed only baseline infection throughout an 8-week culture period, before finally becoming positive for expression of viral antigen. This differential susceptibility to infection directly correlated with accumulation of intracellular viral DNA. Furthermore, the UC11 clone expressed lower levels of Sendai virus-inducible tumor necrosis factor alpha mRNA than did the UC12 or UC18 clones. Susceptibility to infection did not correlate with expression of cell surface CD4, since all clones expressed similar levels of CD4 mRNA and surface membrane CD4 protein. Prior exposure of both susceptible UC18 and resistant UC11 clones to Leu3a antibody completely blocked infection by HIV-1, suggesting that no other independent receptors were recognized by the virus.

Cells of monocytic lineage may be infected with high frequency by human immunodeficiency virus type 1 (HIV-1). We and others have used the U-937 promonocytic cell line to examine the biological and molecular consequences of such infection (3, 6, 11, 15, 16).

Susceptibility to infection by HIV-1 is mediated initially by the cell surface CD4 receptor and subsequently by intracellular factors that mediate viral replication and gene expression. Monoclonal antibodies to CD4 can block infection of T lymphocytes and monocytes/macrophages, as well as interfere with HIV-1-mediated syncytium formation (9). Transfection of $CD4^-$ human cells with CD4 DNA can confer HIV-1 susceptibility to cells otherwise refractory to infection (12). Other workers have argued that the CD4 receptor may not be the only route for viral entry and that alternate receptors might be present on certain types of human cells (1, 4). The possibility that intracellular factors, acting after viral entry, govern the likelihood of successful infection is supported by the observations that neither $CD4^+$ human-mouse T-cell hybrids nor CD4-transfected mouse cells are susceptible to HIV-1 (12, 19). Furthermore, productive infection is dependent on cellular factors associated with proliferation of the infected cell (7, 8, 13).

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Previous work by our group pointed to the heterogeneity of HIV-1-infected U-937 cells; certain clones were shown to produce high levels of infectious progeny virus, while others could only generate particles that were defective in processing of either *gag* or *env* gene products (3). In this study, we derived cell clones from uninfected U-937 cells and showed that the lines thus generated displayed distinct differences in their susceptibility to productive infection by HIV-1, despite high levels of CD4 mRNA and cell surface CD4 protein on all clonal types. These data are consistent with the notion that CD4 receptors are necessary for successful HIV-1 infection but that intracellular events affecting viral replication are also important determinants of infectivity.

The U-937 monocytoid cell line (18) was obtained from the American Type Culture Collection and was maintained as a cell suspension (2×10^5 to 1×10^6 cells per ml) in RPMI 1640 medium (GIBCO Products Ltd., Toronto, Canada), supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 250 U of penicillin per ml, and 250 μg of streptomycin per ml. The IIIB strain of HIV-1 (kindly supplied by R. C. Gallo, National Institutes of Health, Bethesda, Md.) was propagated in U-937 cells as described previously (3) and was concentrated from clarified culture fluids by ultracentrifugation. Pelleted virus was resuspended in small volumes and frozen at −70°C until use.

U-937 cells were serially diluted in 96-well microtiter plates to obtain between 0.15 and 0.5 cell per well. The plates were incubated at 37°C under 5% CO_2 and observed daily under an inverted microscope for the presence of colonies derived from single cells. After 2 weeks, well-defined colonies (maximum of one to three per well) consisting of 10 to 50 cells were subcloned in the same way. Colonies thus derived were harvested by micromanipulation under an inverted microscope, using extra-small-bore Pasteur pipettes, seeded individually into another 96-well plate, and expanded into cell clones which were designated UC (for U-937 clones). Cells were seeded onto wells of multitest slides (Flow Laboratories, Inc., McLean, Va.), dried, and fixed in citrate buffer (0.383 M/liter pH 5.4), containing 10% (vol/vol) methanol and 54% (vol/vol) acetone. They were stained for nonspecific esterases by using an α-naphthyl acetate esterase staining kit (Sigma Diagnostics, St. Louis, Mo.).

A panel of 36 single-cell clones of U-937 cells (UC) was thus derived, of which four (UC11, -12, -14, and -18), as well

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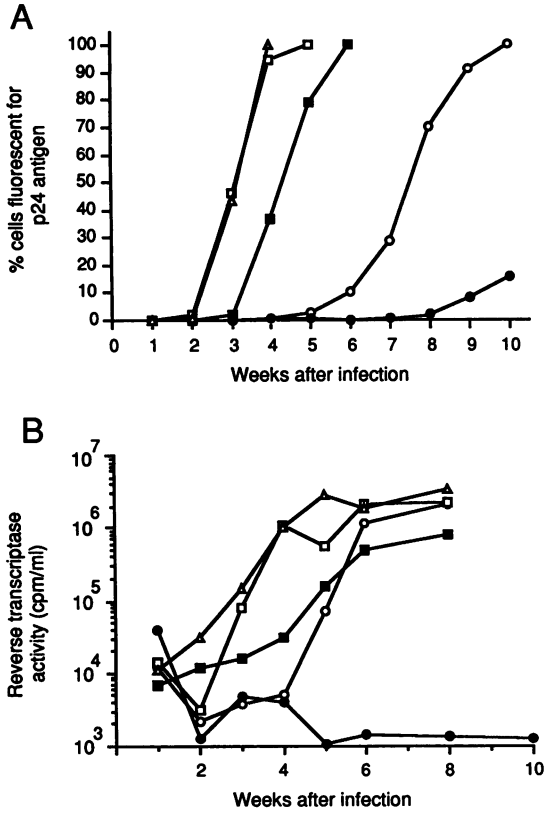

FIG. 1. Course of HIV-1 infection of UC clones. (A) Cells were infected at an MOI of 3 50% tissue culture infective doses per cell and screened for p24 immunofluorescence at regular intervals. (B) Reverse transcriptase activity in culture fluids from UC clones after infection by HIV-1. ○, U-937; ●, UC11; □, UC12; ■, UC14; ▲, UC18.

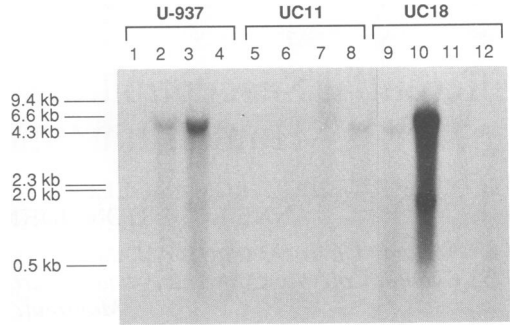

FIG. 2. Southern blot analysis of genomic DNA from infected U-937, UC11, and UC18 cells. For each cell type, infection with HIV-1 was allowed to proceed for 1 (lanes 1, 5, and 9), 2 (lanes 2, 6, and 10), 3 (lanes 3, 7, and 11), or 4 (lanes 4, 8, and 12) weeks prior to extraction of DNA, which was cleaved with *Xba*I and hybridized with a ^{32}P-labeled, nick-translated pBH-10 proviral DNA probe.

as the parental U-937 cell line, were studied for susceptibility to infection by HIV-1. Under light microscopy, all cells were round and polygonal. Their mean cell diameter did not generally differ significantly from that of the parental cell line (13.75 ± 1.57 μm). Only clone UC12 showed a significantly larger diameter (18.63 ± 2.82 μm). Cytochemical staining for α-naphthyl esterase was positive for all clones, although some variations in staining intensity were observed. No significant differences were observed among clones UC11, -12, -14, and -18 in terms of cell growth, with the exception of UC12, which reached a growth plateau after 4 days. The four clones all behaved similarly in response to differentiating agents such as phorbol myristate acetate (5 ng/ml), which caused over 95% of the cells in each case to become adherent within 48 h and to display morphological characteristics of mature macrophages (not shown).

To examine whether all four clones were equally susceptible to infection by HIV-1, we inoculated each cell type with HIV-IIIB at a multiplicity of infection (MOI) of 3 50% tissue culture infective doses per cell. On the basis of an immunofluorescence assay (3), UC12, UC14, and UC18 each showed a rapid increase in numbers of p24 antigen-positive cells, with 100% of cells becoming positive by 3 to 6 weeks after inoculation (Fig. 1A). Parental U-937 cells were 100% p24 positive after 10 weeks. In contrast, clone UC11 was negative for expression of p24 antigen after 8 weeks and only 12% positive after 10 weeks. Ultimately, clone UC11 became 100% positive for p24 antigen expression after 16 weeks (data not shown). Similar results, in terms of cell susceptibility, were obtained with MOIs between 0.15 and 3.0. These data were confirmed by measurements of viral reverse transcriptase activity on the respective culture supernatant fluids (Fig. 1B).

The ability of the U-937-derived clones to become infected by HIV-1 correlated directly with the appearance of intracellular viral DNA (Fig. 2). U-937, UC11, and UC18 were infected with HIV-IIIB at an MOI of 10 to allow synchronous infection of the cells; high-molecular-weight DNA was extracted at 7-day intervals for 28 days to assess the accumulation of HIV-1 viral DNA as described previously (3). In parental U-937 cells, viral DNA was detected at 2 and 3 weeks after infection (Fig. 2, lanes 2 and 3) at a time when the majority of the cells were p24 positive. In UC18, viral DNA was detectable 1 week after infection (lane 9) and increased in amount thereafter (lane 10). In contrast, UC11 cells contained little viral DNA after 2 weeks of infection (Fig. 2, lane 6) but did have detectable viral DNA at weeks 3 and 4 (lanes 7 and 8). Thus, the appearance of intracellular viral DNA reflected the susceptibility of the different clones to infection by HIV-1.

Previous observations have documented the stimulatory effect of tumor necrosis factor alpha (TNF-α) on HIV-1 expression and demonstrated that cytokines such as TNF-α and interleukin 1 may serve as physiological activators of HIV-1 expression. The inducibility of TNF-α RNA in response to Sendai virus infection was measured by polymerase chain reaction-mediated RNA amplification in clones UC11, UC12, and UC18 as described previously (6). The UC11 clone produced four to five times less TNF-specific RNA after induction by Sendai virus (Fig. 3, lane 2) than did either of the highly susceptible clones UC12 and UC18 (lanes 4 and 6). Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were equivalent in all three cell types before and after Sendai virus infection. These findings were confirmed by data showing that UC11 cells secreted lower levels of radioimmunoassay-detectable TNF-α after infection than did the other cell types (ICN Chemicals, Montreal, Canada) (data not shown). This suggests that the differences in susceptibility to HIV-1 infection are related mechanistically to the ability of cytokine genes such as TNF to be induced. We were further interested in learning whether exposure of our various clones to TNF-α (Genzyme, Bos-

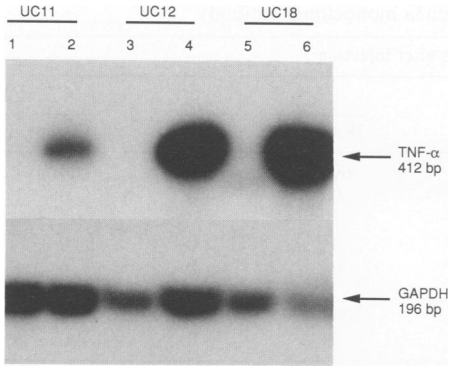

FIG. 3. Expression of TNF-α mRNA in UC11, UC12, and UC18 cells. Total cellular RNA was analyzed by polymerase chain reaction-mediated amplification after 8 h of Sendai virus induction (lanes 2, 4, and 6). Lanes 1, 3, and 5 represent cells not induced by Sendai virus. mRNA expression was studied as a control.

ton, Mass.) might alter their permissiveness to infection, as has been reported by others (17). We found that TNF-α concentrations of 0.1 and 1 ng/ml caused both U-937 and UC14 cells to become 90% positive for expression of p24 antigen about 1 week earlier than usual (data not shown). In contrast, no such effect was seen with UC11 cells. The nonpermissiveness of UC11 to infection, even in the presence of exogenous TNF-α, may thus not be related to the inability to produce this cytokine.

To determine whether relative susceptibility of our clones to infection by HIV-1 could be correlated with levels of CD4, we analyzed each cell type for CD4 surface representation by flow cytometry using anti-Leu3a monoclonal antibodies (Becton Dickinson and Co., Mountain View, Calif.) as described previously (3). The results of Fig. 4A show that epitopes reactive with Leu3a were expressed at high levels in all the clones tested (>99%). CD4 mRNA was also quantified by dot-blot hybridization, using β-actin RNA as a control (Fig. 4B). All four clones, including UC11, which was found to be refractory to active replication of HIV-1, expressed high levels of CD4 mRNA.

We then asked whether differences might exist among our clones in terms of ability to bind ^{125}I-labeled gp120 during a 2-h incubation. As controls, we employed CD4-negative A2.01 cells and CD4-positive MT-4 cells. Toward this end, cells (3.5×10^6) were washed twice in binding medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 0.1% bovine serum albumin (Sig-

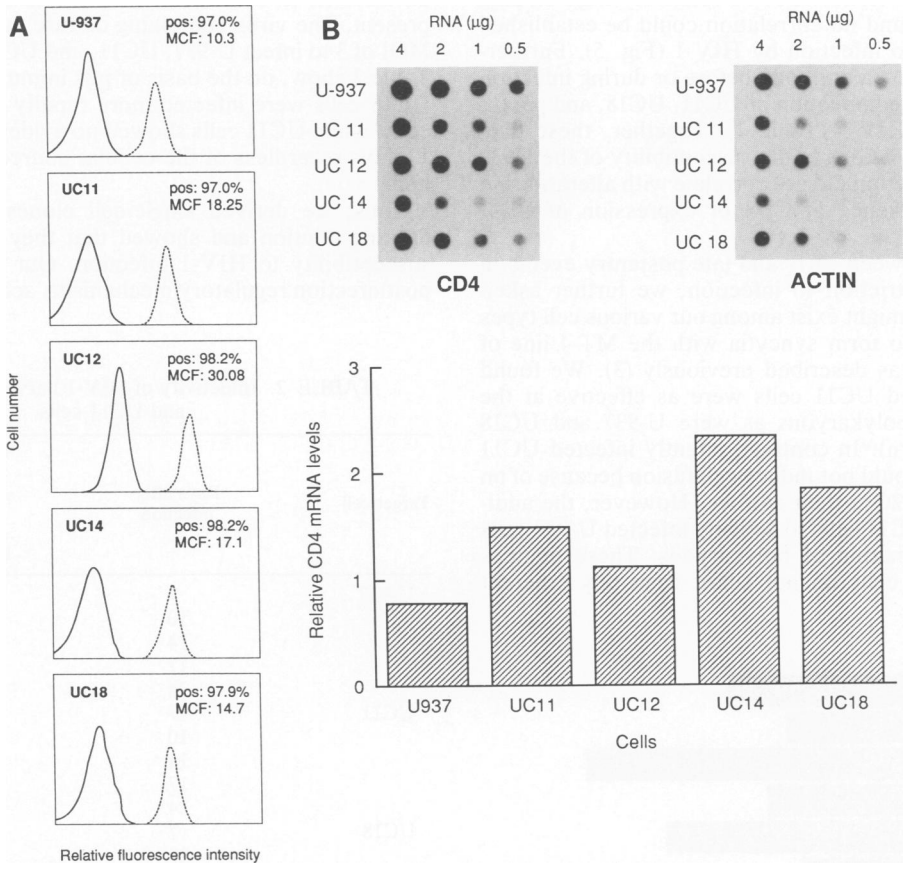

FIG. 4. (A) Flow cytometry analysis of UC clones. Cells were sequentially labeled with anti-Leu3a monoclonal antibody and fluorescein-conjugated goat anti-mouse immunoglobulin. The percentage of cells positive for CD4 and the mean channel fluorescence (MCF) are indicated. (B) Quantitative analysis of CD4 mRNA levels in UC clones. RNA dot blots show CD4 and actin mRNA levels in UC clones and parental U-937 cells. The blots were sequentially hybridized with pT4B, a CD4 probe, and then pβ-actin, which contains mouse actin sequences. Serial dilutions of total RNA were performed, and the amount of RNA in each blot is indicated above each column. The graph shows the relative amount of CD4 mRNA to β-actin mRNA in each clone. RNA levels were quantitated by scanning the autoradiograms with an LKB densitometer.

TABLE 1. Blocking of HIV-IIIB infection by anti-Leu3a monoclonal antibody

Cell type	Anti-Leu3a (μg/ml)	Wks after infection							
		3		4		5		6	
		IFA[a]	p24[b]	IFA	p24	IFA	p24	IFA	p24
U-937	0	3	188	22	151	69	>200	100	>200
	0.02	0	0	0	0	0	0	0	0
UC11	0	0	0	0	0	0	0	0	0
	0.02	0	0	0	0	0	0	0	0
UC18	0	7	163	100	194	100	>200	100	>200
	0.02	0	0	0	0	0	0	0	0

[a] Immunofluorescence (IFA) results are expressed as percentage of cells positive for p24 antigen. A minimum of 2,000 cells were examined in each instance.
[b] HIV p24 antigen levels (picograms per milliliter) were measured in culture fluids by a solid-phase enzyme-linked immunoassay (Abbott Laboratories).

ma). Cells were incubated on ice in 50 μl of binding medium for 1 h, washed in ice-cold binding medium to remove excess antibody, and incubated on ice for 2 h in the presence of 3.5 × 10^6 cpm of ^{125}I-gp120 (gift from R. Sekaly, Institut de Recherche Clinique de Montréal, Montreal, Canada) with frequent agitation. The cells were washed thoroughly four times in cold binding medium and resuspended in 20 μl of medium, and bound radioactivity was counted in a gamma counter. All the UC clones showed relatively high binding capacity for gp120, and no correlation could be established with susceptibility to infection by HIV-1 (Fig. 5). Furthermore, addition of Leu3a antibody before or during infection blocked CD4-mediated infection of UC11, UC18, and parental U-937 cells by HIV-1 (Table 1). Together, these data demonstrate that variations in the susceptibility of the U937 clones to HIV-1 infection did not correlate with alterations in the cell surface presence of CD4 or expression of CD4-specific mRNA.

To distinguish between early and late postentry events in determining the restriction to infection, we further asked whether differences might exist among our various cell types in terms of ability to form syncytia with the MT-4 line of $CD4^+$ lymphocytes as described previously (3). We found that actively infected UC11 cells were as effective at the formation of such polykaryons as were U-937 and UC18 cells (data not shown). In contrast, latently infected UC11 cells, as expected, could not induce cell fusion because of an absence of viral gp120 at their surface. However, the addition of uninfected UC11 cells to actively infected U-937 cells did lead to the formation of polykaryons. These findings indicate that UC11 cells do not suffer from any inherent inability to participate in cell fusion reactions, suggesting that the observed restriction in HIV replication in UC11 cells occurs subsequent to both viral binding and penetration.

Finally, we sought to determine whether the HIV strain that was ultimately recovered from UC11 had decreased infectivity compared with virus from UC18 and U-937 cells. Culture fluids from each cell type were serially diluted in medium and used to infect MT-4 target lymphocytes; based on titration on MT-4, similar titers of infectious virus were present. The virus-containing culture fluids were used at an MOI of 3 to infect U-937, UC11, and UC18 cells. The data of Table 2 show, on the basis of p24 immunofluorescence, that UC18 cells were infected more rapidly than parental U-937 cells, while UC11 cells showed no evidence of infection over 17 days regardless of the cellular source of the virus inoculum.

Thus, we derived single-cell clones of U-937 cells by limiting dilution and showed that they possess differential susceptibility to HIV-1 infection. Our results suggest that postinfection regulatory mechanisms account for the various

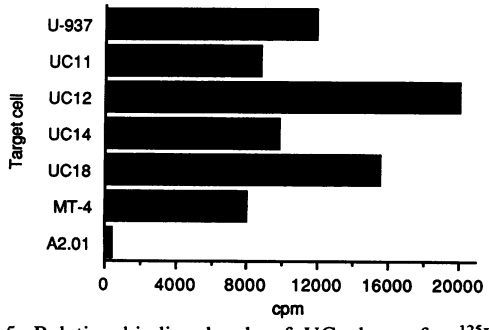

FIG. 5. Relative binding levels of UC clones for ^{125}I-labeled gp120.

TABLE 2. Infectivity of HIV-1 harvested from U-937 and UC11 cells

Target cell	Days after infection	% of cells positive for p24 antigen after infection with culture fluids derived from[a]:	
		U-937	UC11
U-937	7	0	0
	10	0	0
	14	3	0
	17	16	12
	24	100	100
UC11	7	0	0
	10	0	0
	14	0	0
	17	0	0
	24	0	0
UC18	7	3	3
	10	14	13
	14	46	65
	17	100	94
	24	100	100

[a] Culture fluids from chronically infected U-937 and UC11 cells were adjusted to 3 50% tissue culture infective doses per ml and used to infect 5 × 10^5 uninfected U-937, UC11, or UC18 cells. All values were calculated as the means of experiments performed in duplicate.

periods of latency observed. The possibility that differences in CD4 expression contributed to the observed patterns of susceptibility was ruled out by measurement of CD4 levels in the derived clones. Also, attenuation of infectivity of HIV-1 derived from the UC11 clone was ruled out as a mechanism in studies which used U-937 and MT-4 cells as susceptible targets.

Among the factors that may influence the outcome of HIV-1 infection is the cellular protein, NF-κB, which binds to the long terminal repeat region of HIV-1 to increase expression of HIV-1 mRNA (10, 13). This regulatory protein was originally described in B and T cells but is also present in monocytes and may be present at higher levels in UC12, -14, and -18 cells than in either U-937 or UC11 cells. The secretion of cytokines, including interleukin-1 and TNF-α, may also play a role in the different susceptibilities to infection. Both of these molecules may act to increase HIV-1 expression in U-937 cells via posttranslational activation of NF-κB (6, 14, 17). In addition, granulocyte macrophage colony-stimulating factor and alpha interferon have been shown to stimulate and inhibit HIV-1 multiplication, respectively (7, 11). Previous studies have shown that addition of anti-interferon antibody to HIV-1-infected cultures resulted in more rapid virus multiplication (11), while conversely, cells which expressed alpha interferon subtype 2 under the control of the HIV long terminal repeat were unable to replicate HIV-1 (2). Because TNF-α has been reported to stimulate HIV-1 replication in U-937 cells (17), we investigated whether differential levels of Sendai virus-inducible TNF-α mRNA expression might help to explain our results and found that the highly susceptible clones UC12 and UC18 synthesized four to five times more TNF mRNA after such induction than did UC11. In contrast, treatment of HIV-inoculated UC11 cells with exogenous TNF-α did not enhance the rapidity of infection by HIV-1. Thus, our data do not support the conclusion that levels of TNF-α may themselves determine HIV-1 permissiveness, but rather that the ability of cells to be stimulated to produce TNF-α mRNA may be an important consideration. The possibility that TNF-α-mediated enhancement of NF-κB activity is an important factor is now under investigation. All the clones produced similar levels of alpha and beta interferon as determined by radioimmunoassay (data not shown).

The duration of latency among our various clones could not be correlated with the expression of cell surface CD4 as (i) all cell types showed similar CD4 profiles when screened for reactivity with anti-Leu3a monoclonal antibodies; (ii) relative expression of CD4 mRNA in each case was comparable to that of parental U-937 cells; (iii) levels of binding of radiolabeled gp120 did not correlate with susceptibility to infection. We also showed that infection of the clones could be prevented by Leu3a antibody, suggesting that other independently functioning receptors for HIV-1 were absent in these cells.

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