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Slow Human Immunodeficiency Virus (HIV) Infectivity Correlated with Low HIV Coreceptor Levels

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The absolute number of $CD4^+$ lymphocytes in blood is prognostic for disease progression, yet the cell surface density of CD4 receptors or chemokine receptors on a single cell has not previously been found to be predictive of human immunodeficiency virus (HIV) infectivity outcome. It has recently been shown that human leukocyte elastase (HLE) and its ligand α_1 proteinase inhibitor (α_1PI; α_1 antitrypsin) act as HIV fusion cofactors. The present study shows that decreased HIV infectivity is significantly correlated with decreased cell surface density of HLE but not with decreased CD4 nor chemokine receptors. In vitro HIV infectivity outcome in this study was predicted by the surface density of HLE on mononuclear phagocytes but not on lymphocytes. The set point HLE surface density was in part determined by α_1PI. Decreased circulating α_1PI was correlated with increased cell surface HLE and with increased HIV infectivity. The correlation of HIV infectivity outcome with surface HLE and circulating α_1PI supports the utility of these HIV cofactors in diagnostic analysis and therapeutic intervention.

We previously demonstrated that cell surface human leukocyte elastase (HLE) specifically and reversibly binds the HIV fusion domain (Bristow et al. [3]). We have recently found that human immunodeficiency virus (HIV) preferentially binds to copatches of HLE, CD4, and chemokine receptors (C. L. Bristow et al., unpublished data). HIV receptors were found to copatch in response to $\alpha_1$proteinase inhibitor (α_1PI) potentially explaining the requirement for α_1PI during HIV entry. These studies led to the hypothesis that α_1PI might impact HIV disease progression. Indeed, in a study of HIV-seropositive patients, we recently found that decreased circulating α_1PI is significantly correlated with decreased viral load (3a). The prognostic value of measuring α_1PI was found to be comparable to measuring CD4 and considerably better than measuring HIV RNA. During the asymptomatic category of HIV disease, 100% patients were found to manifest deficient levels of active α_1PI. In contrast, circulating levels of the proteinase inhibiting α_2 macroglobulin (α_2M) in this patient population were not significantly different from those of normal controls. Individuals with the inherited form of α_1PI deficiency, especially males, are notably susceptible to respiratory infections, and 80% who survive to adulthood succumb to respiratory failure between the fourth and sixth decades of life (1). This suggests that the α_1PI deficiency acquired during HIV infection could provide a mechanistic explanation for the onset of attendant infections and inflammation which subsequently initiate an increase in α_1PI. Recent evidence has suggested that increased HIV is produced by blood cells from individuals having the inherited form of α_1PI deficiency (6). To further investigate the possibility that variation in circulating α_1PI concentrations might directly impact HIV infectivity outcome of peripheral blood mononuclear cells (PBMC), in vitro infectivity of PBMC from HIV-nonexposed individuals having the inherited form of α_1PI deficiency was compared in the presence and in the absence of exogenous α_1PI in autologous serum.

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MATERIALS AND METHODS

Subjects. Blood was collected from eight different HIV-seronegative healthy volunteers after we obtained informed consent. Subjects were selected to represent a range of α_1PI concentrations in serum. All subjects were normally healthy; however, the female counterpart from a pair of siblings homozygous for the α_1PI-deficient genotype PI_{ZZ} (2) was included for comparison despite her history of systemic lupus erythematosis.

Quantitation of α_2M and α_1PI. Sera were measured at the time of collection for α_2M, as well as for active- and inactive-α_1PI concentrations. Methods for quantitating active and total α_1PI have been described elsewhere (2). Active α_2M and α_1PI concentrations were determined by elastase inhibitory capacity. Total α_1PI was determined by enzyme-linked immunosorbent assay. Inactive α_1PI was expressed as the difference between total α_1PI and active α_1PI.

Active-site standardization of exogenous α_1PI. Active-site standardization was performed as previously described (2). One mole of active-site titrated porcine pancreatic elastase, type 1 (EC 3.4.21.36; Sigma) was found to saturate 3.06 moles α_1PI (Sigma), suggesting that this preparation was 32.7% active.

In vitro HIV infectivity of mononuclear cells. PBMC were isolated by Ficoll-Hypaque gradient centrifugation from whole blood collected in tubes containing ACD (Becton Dickinson, San Jose, Calif.). Three primary non-syncytium-inducing HIV type 1 (HIV-1) clinical isolates were generously provided by the Retrovirology Core Laboratory, UNC–Chapel Hill. In vitro infectivity outcome was determined by quantitating p24 accumulation or reverse transcriptase (RT) activity as previously described (3, 5). Isolated PBMC were resuspended and maintained in the wells of a 96-well tissue culture plate at 2×10^6 cells/ml in RPMI 1640 containing 20% autologous serum and 10% interleukin-2 (Cellular Products, Buffalo, N.Y.). PBMC in 100 μl in autologous medium were stimulated by the addition of 5 μg of phytohemagglutinin (PHA; Sigma) per ml for 3 days at 37°C in humidified 5% CO_2. Cells were washed and resuspended in fresh autologous medium containing various concentrations of exogenous active-site standardized α_1PI (Sigma).

For the determination of RT activity, PHA-stimulated PBMC were incubated with a 8×10^{-8} 50% tissue culture infective dose ($TCID_{50}$) of HIV-1 for 2 h at 37°C and 5% CO_2. Cells were subsequently washed three times, resuspended, and cultured in fresh autologous medium containing matched exogenous α_1PI. Aliquots of 50 μl of culture supernatants were collected and replaced with fresh autologous medium containing matched exogenous α_1PI every other day on days

TABLE 1. Coreceptor levels and circulating α_1PI and α_2M in HIV-seronegative volunteers

Subject	Sex[a]	α_2M concn (μM)[b]	α_1PI concn (μM)[b]	Lymphocyte (MFI)[c]				Mononuclear phagocytes (MFI)[c]			
				CD4$^+$	CCR5$^+$	CXCR4$^+$	HLE$^+$	CD4$^+$	CCR5$^+$	CXCR4$^+$	HLE$^+$
1	M	5.65	1.9	105	31	24	176	16	23	39	257
2	F	5.57	3.0	98	25	101	190	25	32	112	341
3	F	5.12	11.0	109	11	32	165	19	17	42	248
4	M	3.53	34.0	114	17	71	245	14	21	50	167
5	M	3.34	42.4	97	21	109	178	32	43	63	325
6	F	3.26	61.2	121	13	50	178	19	15	19	177
7	F	3.50	13.7	ND	ND	ND	ND	ND	ND	ND	ND
8	M	5.57	39.5	ND	ND	ND	ND	ND	ND	ND	ND

[a] M, male; F, female.
[b] Active concentration determined by elastase inhibition.
[c] Geometric mean fluorescence was detected by flow cytometry. Lymphocytes identified by forward and side scatter were gated to represent the CD4$^+$ population. Mononuclear phagocytes identified by forward and side scatter were gated to represent the CD4$^+$ CD14$^+$ population. CCR5, CXCR4, and HLE are represented as the geometric mean fluorescence intensity (MFI) within gated cells. ND, not determined.

2 through 8. Culture supernatants were stored at −80°C for analysis of the RT activity.

For the determination of the p24 accumulation, PHA-stimulated PBMC were incubated with 10 or 40 $TCID_{50}$ of HIV-1 for 2 h at 37°C and 5% CO_2. Cells were subsequently washed three times and resuspended in fresh autologous medium and cultured at 3×10^6 cells/1.5 ml/well. Aliquots of 225 μl were removed each day for p24 determination (without the replacement of fresh media) on days 2 through 7. Cell counts and viability were determined at the final time point. In the uninfected cell controls, $(1.68 \pm 0.36) \times 10^6$ PBMC were 96% ± 7% viable. In the infected cells, $(2.39 \pm 0.37) \times 10^6$ PBMC were 88% ± 3% viable.

Immunofluorescent staining and flow cytometric analysis. Three-parameter flow cytometric analysis using direct immunofluorescent staining of whole blood was performed on a FACScan flow cytometer (Becton Dickinson) using fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyl protein (PerCP) by using methods recommended by the manufacturer. To detect chemokine receptors, blood was stained simultaneously in a single step with three monoclonal antibodies which included (i) anti-CD14-FITC, anti-CCR5-PE, and anti-CD4-PerCP (PharMingen, San Diego, Calif.); (ii) anti-CD14-FITC, anti-CXCR4-PE, and anti-CD4-PerCP (PharMingen); or (iii) anti-CD14-FITC, immunoglobulin G2a (IgG2a)-PE, and anti-CD4-PerCP (PharMingen). To detect cell surface HLE, blood was stained stepwise with three antibodies, including polyclonal sheep anti-HLE-FITC (Biodesign, Inc., Kennebunkport, Maine), monoclonal anti-CXCR4-PE (PharMingen), and monoclonal anti-CD4-PerCP (Becton Dickinson). The isotype-matched controls were IgG2a-PE, IgG1-PerCP, or nonspecific IgG-FITC. Blood was washed in 2 ml of phosphate-buffered saline between each staining step.

For each analysis, 30,000 events were acquired. List mode multiparameter data files were analyzed using CellQuest Software (Becton Dickinson). Because the fraction of CD4$^+$ lymphocytes and CD4$^+$ mononuclear phagocytes varied considerably between individuals, the fluorescence intensity was normalized to the CD4$^+$ cells in the gate containing either lymphocytes or mononuclear phagocytes. The relative geometric mean fluorescence intensities (RFI) were determined as geometric mean fluorescence intensity (MFI) relative to CD4 = geometric MFI of coreceptor/geometric MFI of CD4.

RESULTS AND DISCUSSION

Influence of α_1PI on HIV-1 produced by PBMC infected in vitro. To determine whether α_1PI might influence HIV infectivity, healthy volunteers were selected to represent a range of circulating α_1PI levels (Table 1). PBMC from these individuals were infected in vitro using three concentrations of a non-syncytium-inducing clinical isolate of HIV-1. PBMC were maintained at all times in autologous serum containing various concentrations of exogenous α_1PI. We have observed that circulating α_1PI significantly impacts the adherence of PBMC to tissue culture tubes ($r^2 = 0.81$, $P < 0.0001$; data not shown). To avoid exclusion of adherent cell populations, isolated PBMC were stimulated, infected, and cultured without removal from their original tissue culture wells. Cultures were performed in duplicate, and supernatants were collected and replaced with fresh medium every other day for the determination of RT activity.

In the absence of exogenous α_1PI, RT activity was negligible regardless of the cell source (Fig. 1) or viral inoculum (data not shown). However, in the presence of exogenous α_1PI, RT

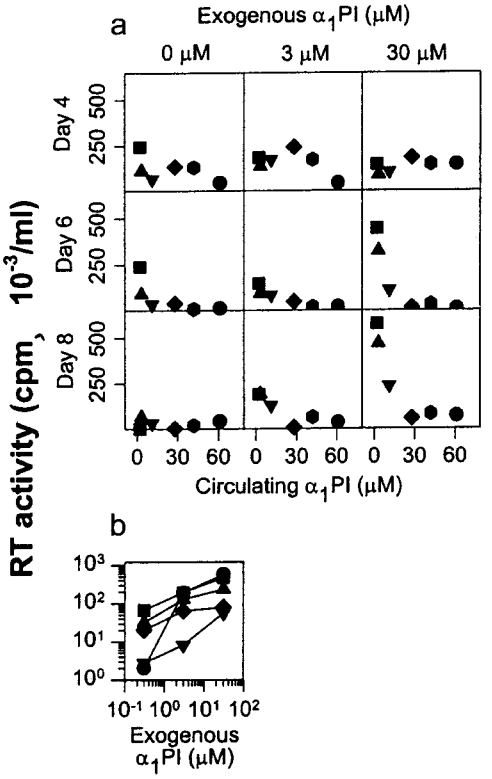

FIG. 1. In vitro HIV infectivity of PBMC in the presence of exogenous α_1PI. (a) Infection outcome using a non-syncytium-inducing clinical isolate of HIV-1 was determined in duplicate by measuring the RT activity produced by PBMC from subjects 1 (■), 2 (▲), 3 (▼), 4 (◆), 5 (⬢), and 6 (●), represented in Table 1 in the absence or presence of 3 or 30 μM exogenous α_1PI in autologous serum. Mean values are depicted. (b) On day 8, the RT activity in the culture supernatants increased as the exogenous α_1PI concentration increased.

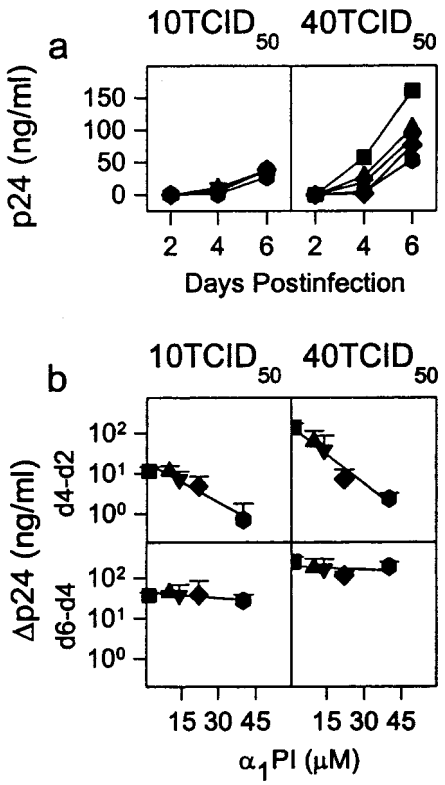

FIG. 2. Correlation between the rate of HIV p24 production and the circulating α_1PI concentration. (a) PBMC were infected with 10 or 40 $TCID_{50}$, and the infectivity outcome was determined by measuring p24 accumulation. PBMC from subjects 1 (■), 3 (▲), 4 (▼), 7 (◆), and 8 (⬢) represented in Table 1 were infected with a non-syncytium-inducing clinical isolate of HIV-1 and cultured in autologous serum. (b) The rate of p24 accumulation was calculated as the difference in p24 between consecutive measurements. The computer-fit linear regression curve for 10 $TCID_{50}$ is $\log [y] = 1.29 - 0.03 \log [x]$ ($r^2 = 0.93$, $P < 0.008$) and for is 40 $TCID_{50}$ is $\log [y] = 1.81 - 0.04 \log [x]$ ($r^2 = 0.95$, $P < 0.005$), where y represents Δp24 and x represents active serum α_1PI.

activity was detected in PBMC from three subjects. These three subjects were found to constitutively manifest deficient circulating α_1PI. New virus increased as circulating α_1PI decreased ($r^2 = 0.95$, $P < 0.001$). In contrast, new virus increased in a manner dependent upon increasing active α_1PI tissue culture concentration. These results suggest the hypothesis that cells conditioned in vivo by increased concentrations of α_1PI are less sensitive and less responsive to α_1PI in tissue culture, perhaps due to downregulation of an HIV coreceptor. Two of the subjects studied are known to be homozygous for the PI_{ZZ} genotype (2). The phenotype of the third subject is not known; however, the α_1PI levels (11 μM) in this subject are inconsistent with PI_{ZZ}. This suggests that α_1PI levels, but not the genotype producing α_1PI deficiency, determine HIV outcome.

To further investigate the influence of circulating α_1PI on HIV infectivity of PBMC, p24 production was determined in cells infected in vitro in autologous serum. Active levels of circulating α_1PI were again found to be related to p24 production; the lower the concentration of α_1PI in serum, the greater the p24 produced (Fig. 2). That p24 produced by cells infected with 40 $TCID_{50}$ was proportionally greater than by cells infected with 10 $TCID_{50}$ suggests that infectious dose was a primary determinant in p24 synthesis. Because p24 accumulates in tissue culture supernatants under these conditions, comparing HIV produced by PBMC from these individuals was facilitated by determining the rate of accumulation. It was found that the levels of α_1PI were significantly correlated with the rate of p24 accumulation prior to day 4 when cells were exposed to either infectious dose. In contrast, the rate of p24 accumulation after day 4 was equivalent regardless of the PBMC source or individual α_1PI concentration in serum. These results are consistent with previous evidence using homogeneous cell populations, suggesting that once an infection is initiated in vitro, subsequent infectious cycles are kinetically indistinguishable (4). That the rate of p24 accumulation prior to day 4 maintained the rank order of all five individuals at either infectious dose suggests that p24 synthesis was not limited by the capacity of the infected cells for new protein synthesis but was limited by the number of initially infected cells in a manner determined by the infectious dose. In vitro HIV infectivity of PBMC is notoriously variable; however, the consistency of results in three independent experiments with eight individuals representing a range of α_1PI concentrations in serum supports the hypothesis that α_1PI is a physiologically relevant cofactor for HIV. One of the individuals in this study has been found routinely to have normal active α_1PI levels (40 μM) but at the time of the current study exhibited an unusually low level (13.7 μM). This further supports the conclusion that it is the α_1PI level, and not the α_1PI phenotype, which influences HIV outcome.

It has previously been reported that α_1PI inhibits HIV infectivity (6). Evidence presented here suggests that α_1PI facilitates HIV infectivity. We have recently found that α_1PI produces a short-lived window for HIV entry (unpublished results). HIV coreceptors are initially disperse, are stimulated to copatch within 15 min of exposure of cells to α_1PI, and then pinch off from the plasma membrane following a period of 30 to 60 min, forming small platelet-like transitory cytoplasmic bodies (SPTBalls). The corresponding disappearance of HIV infectivity and the appearance of SPTBalls suggests the possibility that SPTBalls may serve to uncouple cellular responsiveness including HIV entry.

Influence of circulating α_1PI on HIV coreceptor density. The relationship between infectivity and coreceptor densities was compared using blood collected from the same six volunteers as those for which RT activity was measured. We have observed that cell surface HLE on promonocytic cells appears to be increased when the cells are interacted with antibodies specific for CD4 first and specific for HLE secondarily and then decreased when the order of antibody addition is reversed (C. L. Bristow, unpublished data). When blood was first reacted with anti-HLE and secondarily with anti-CD4 or antibodies specific for CXCR4 or CCR5, HLE density was negligible on $CD4^+$ lymphocytes or $CD4^+$ $CD14^+$ mononuclear phagocytes (Fig. 3). When blood was reacted with antibodies in the reverse order, considerable levels of HLE were detected on both $CD4^+$ lymphocytes and $CD4^+CD14^+$ mononuclear phagocytes. In contrast, the order of coreceptor ligation had no influence on the fluorescence intensities of CD4, CD14, CCR5, or CXCR4 (data not shown). These results suggest a dynamic

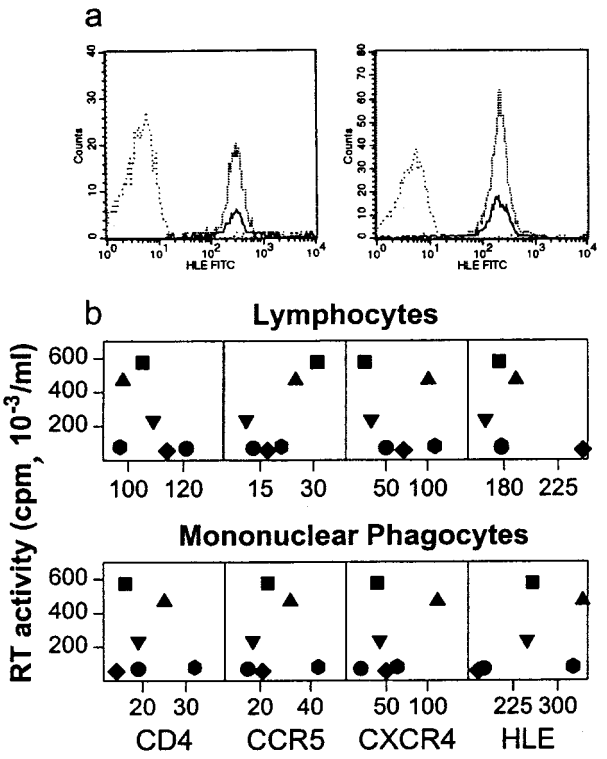

FIG. 3. Relationship between RT activity and HIV coreceptor expression. PBMC from subjects 1 to 6 represented in Table 1 were analyzed for coreceptor levels using three-color flow cytometry. (a) Whole blood first interacted with anti-CD4 and second with anti-HLE (tightly dotted line) exhibited considerable HLE on the cell surface of $CD4^+$ $CD14^+$ mononuclear phagocytes (left panel) and $CD4^+$ lymphocytes (right panel) compared with isotype controls (loosely dotted line). In contrast, blood first interacted with anti-HLE and secondly with anti-CD4 (solid line) exhibited diminished HLE. Two representative subjects are depicted. (b) The RT activity produced by subjects 1 (■), 2 (▲), 3 (▼), 4 (◆), 5 (⬢), and 6 (●) showed no relationship to the cell surface densities of any coreceptors examined on $CD4^+$ lymphocytes or $CD4^+$ $CD14^+$ mononuclear phagocytes.

functional association between membrane-associated HLE and the HIV coreceptors CD4, CXCR4, and CCR5. As would be expected, receptor densities were found to vary in the subjects examined here without any obvious pattern (Table 1). For example, a low receptor density for one receptor was not related to a concordantly low or high receptor density for any other receptor. Nor was RT activity found to be related to the receptor densities of any HIV coreceptors examined (Fig. 3). Since the fractions of $CD4^+$ lymphocytes and $CD4^+$ mononuclear phagocytes varied considerably between individuals, the fluorescence intensity of coreceptors was normalized to $CD4^+$ cells and was expressed as RFI. Increased HLE RFI, but neither the CXCR4 nor the CCR5 RFI value on $CD4^+$ $CD14^+$ mononuclear phagocytes was found to be directly correlated with increased RT activity ($r^2 = 0.81$, $P = 0.01$; Fig. 4). These results suggest increased cell surface HLE molecules associated with each CD4 molecule resulted in increased RT activity. In contrast, RT activity was not related to the RFI for any coreceptors on $CD4^+$ lymphocytes. Nor was RT activity related to RFI when coreceptors were expressed relative to CXCR4 or CCR5 (data not shown). Consistent with these results, when coreceptors were expressed as the fluorescence intensity relative to HLE, increased RT activity was found to be related to CD4 on $CD4^+$ $CD14^+$ mononuclear phagocytes but not to either CXCR4 or CCR5 (data not shown). In this case, increased numbers of CD4 molecules associated with each HLE molecule resulted in decreased RT activity. These results suggest that, although all coreceptors may participate during HIV entry, the ratio of cell surface of HLE and CD4, but neither CXCR4 nor CCR5, is determinant during HIV infectivity outcome.

As was found for RT activity, decreased cell surface HLE relative to CD4 on $CD4^+CD14^+$ mononuclear phagocytes was correlated with increased circulating α_1PI ($r^2 = 0.95$, $P = 0.0008$). Neither circulating α_1PI, nor α_2M was related to CXCR4 or CCR5 relative to CD4 on $CD4^+$ $CD14^+$ mononuclear phagocytes or any coreceptors on $CD4^+$ lymphocytes.

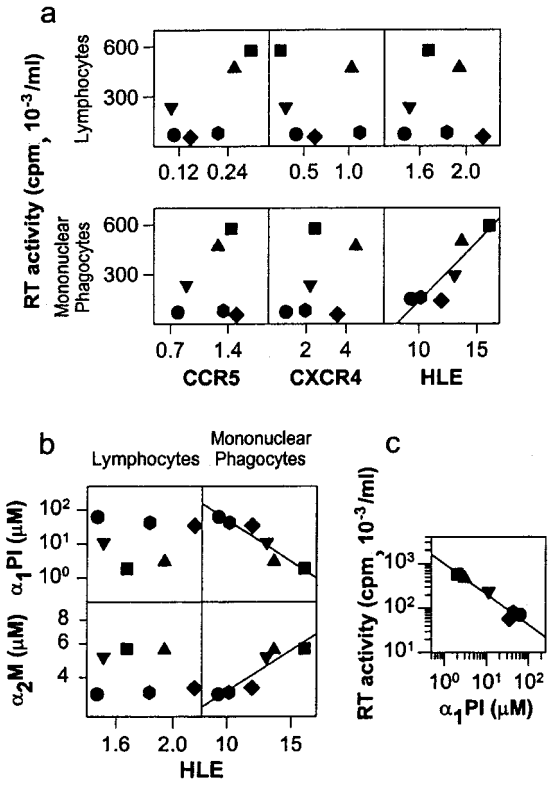

FIG. 4. Relationship between RT activity and HIV coreceptor expression relative to CD4 levels. (a) The RT activity produced by PBMC from subjects 1 (■), 2 (▲), 3 (▼), 4 (◆), 5 (⬢), and 6 (●) represented in Table 1 was correlated with the HLE relative to CD4 (RFI) on $CD4^+$ $CD14^+$ mononuclear phagocytes but not with other coreceptors. The computer-fit linear regression curve predicts $y = -770 + 80x$ ($r^2 = 0.81$, $P = 0.01$), where y represents the RT activity and x represents the HLE RFI. (b) Circulating α_1PI, but not α_2M, was correlated with HLE RFI on $CD4^+$ $CD14^+$ mononuclear phagocytes but not with other coreceptors on these cells. The computer-fit linear regression curve predicts $y = 137 - 9x$, where y represents the α_1PI and x represents the HLE RFI relative to CD4 ($r^2 = 0.88$, $P = 0.006$). (c) The RT activity decreased as the circulating α_1PI concentration increased on day 8 as represented in Fig. 1. Computer-fit linear regression of these data predict that $\log [y] = 3.0 - 0.69 \log [x]$, where x represents the α_1PI concentration in serum and y represents the in vitro HIV RT activity ($r^2 = 0.95$, $P < 0.001$).

These results suggest that circulating α_1PI may modulate cell surface HLE on peripheral blood mononuclear phagocytes, thereby diminishing the number of HIV-responsive cells.

Although tropism was not addressed in the present study, the relationship between RT activity and coreceptors on mononuclear phagocytes suggests that the viral isolate used may have been tropic for these cells. The dynamic relationship between coreceptors on PBMC may not be consonant with their relationship in lymph nodes or in tissue where differentiation pathways are subject to the local environment; however, these results suggest new targets for therapeutic intervention which could potentially prolong or prevent the onset of the symptomatic clinical status and AIDS.

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