Relation of Impaired Lymphocyte Proliferative Function to Other Major Human Immunodeficiency Virus Type 1-Induced Immunological Changes

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Human immunodeficiency virus (HIV) type 1 (HIV-1) induces impairment of immune function reflected in reduced lymphocyte proliferative responses. Many other immune changes are induced by HIV-1, but their relationship to lymphocyte functional defects is not known. The present study was designed to correlate functional defects with other HIV disease parameters. Cryopreserved samples from 118 HIV-1-positive subjects and 40 seronegative individuals were examined. The main findings were that impaired proliferative responses to mitogens correlated with (i) decreased cell surface expression of the interleukin-2 receptor (CD25), (ii) increased expression of HLA-DR antigens on CD4 cells, (iii) reduced CD4 and increased CD8 cell numbers, and (iv) increased levels of serum immune complex dissociated p24 antigen. However, impaired function was not associated with increased serum neopterin, β 2-microglobulin, or soluble interleukin-2 receptor or with CD38 antigen expression on lymphocytes. In summary, proliferative functional impairment correlated with some, but not all, immunological changes associated with HIV-1 infection. Most of the phenotypic markers that correlated with altered function are cell surface molecules with significant roles in lymphocyte proliferation and were associated primarily with CD4 cells, compatible with the view that dysregulation of CD4 cells is responsible for impaired function.

Human immunodeficiency virus (HIV) type 1 (HIV-1) causes a progressive breakdown of immunity that can be quantified by several different immunological assays. The selective depletion of CD4 lymphocytes has been well described (7, 23). The number of peripheral blood CD4 T cells is commonly used to determine the stage of disease and to predict disease progression (19, 24). Impairment of T-cell function is a separate measurement and occurs early in HIV-1 infection, when CD4 T-cell numbers are still in the normal range. T-cell function decreases further with disease progression (5, 20). In addition to the response to phytohemagglutinin (PHA) (12, 13), prognostic assessments have indicated that the proliferative response to pokeweed mitogen (PWM) is a sensitive prognostic marker for AIDS occurrence.

Extensive activation of the immune system is another major effect of HIV-1 infection. The activation occurs early and increases throughout the course of the infection. HIV-1-induced activation results in the production of serum immune activation products, such as neopterin, β 2-microglobulin (β 2M), soluble interleukin-2 (IL-2) receptor (sIL-2R), and soluble CD8 (8, 14, 22, 27). Changes in these markers have also been shown to predict HIV-1 disease progression and to be largely independent of CD4 T-cell numbers (25). The increase in serum immune activation markers may reflect specific cytokine-mediated processes. For example, serum neopterin reflects primarily an increased production of gamma interferon, whereas β 2M reflects increases in other cytokines (1, 15). sIL-2R does not correlate closely with the changes of serum neopterin and

β 2M (16) and presumably reflects a different aspect of HIV-1 immunopathogenesis.

Changed expression of lymphocyte surface antigens also reflects the dynamic interaction between the human immune system and HIV-1. HLA-DR (major histocompatibility complex class II molecule) CD38 (a cell immaturity and activation marker), and CD71 (transferrin receptor) are significantly increased while CD25 (IL-2 receptor alpha chain) is significantly decreased in HIV-infected subjects (2, 10, 11, 16, 21). Multiparameter phenotypic analyses offer opportunities to examine changes within specific lymphocyte subpopulations, e.g., CD4 T cells, CD8 T cells, or B cells. Because CD4 T cells are central to proliferative function and are a major target for HIV-1 infection, the phenotypic changes of these cells are of special interest. Finally, the virologic marker of serum p24 antigen, both free and complexed with antibody, also is known to relate to disease prognosis (26). This marker reflects active, ongoing viral replication.

Thus, studies of AIDS pathogenesis have identified numerous potential surrogate markers for prognosis and therapy in HIV-1 infection. With many different measurements emerging, little work has been done to relate these markers to each other. A goal of the current study was to determine whether the functional changes in lymphocytes indicated by decreased proliferative responses to mitogens (PHA and/or PWM) correlated with other HIV-1-associated immune changes. These parameters include (i) the levels of four major lymphoid cell subsets (CD4, CD8, B, and NK cells), (ii) expression of four lymphoid activation markers (HLA-DR, CD38, CD71, and CD25) in the total lymphocyte population as well as in the major lymphocyte subsets, (iii) levels of free and total serum p24 antigen, and (iv) three soluble immune activation markers

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(serum neopterin, $\beta 2M$, and sIL-2R). A total of 118 HIV-1-seropositive individuals were compared to 40 seronegative homosexual men. The data indicate that reduced proliferative function correlated with several, but not all, immune alterations in HIV-1 infection.

MATERIALS AND METHODS

Subjects. Cryopreserved peripheral blood mononuclear cells (PBMC) from 118 mildly symptomatic and asymptomatic HIV-1-seropositive homosexual and 40 HIV-1-seronegative homosexual individuals were selected from the Multi-center AIDS Cohort Study repository at Los Angeles, Calif. All subjects had been monitored approximately every 6 months since 1985 for presence of HIV-1 antibody, major lymphocyte subsets, antiretroviral therapy, and health status. Details of the recruitment procedures and the characteristics of the cohort at baseline have been reported previously (6, 18). Samples obtained in 1988 were obtained from the frozen repository. Of 118 HIV-1-seropositive subjects without any antiviral treatment, 60 had a baseline CD4 number of $<500/\text{mm}^3$, with a mean of $364/\text{mm}^3$. The remaining 58 subjects had a baseline CD4 number of $>500/\text{mm}^3$, with a mean of $728/\text{mm}^3$.

Preparation of PBMC. PBMC were originally isolated from fresh peripheral blood by centrifugation on a Histopaque density gradient (Sigma) and suspended to $10 \times 10^6/\text{ml}$ in complete RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 20% human serum and 20% dimethyl sulfoxide (Sigma). One-milliliter cell aliquots were dispensed into 2-ml cryovials (Nalgene) for programmed-rate freezing (Cryomed, New Baltimore, Mich.) to -80°C and stored in liquid nitrogen. The vials of cryopreserved cells were placed in a 37°C water bath until just thawed, and 5 ml of RPMI 1640 medium with 10% human serum was added dropwise. The cells were washed twice, and viability was determined by exclusion of 0.2% trypan blue dye. The viability of all thawed samples was over 90%.

Proliferative assay. One-hundred-microliter aliquots of PBMC (2.5×10^5 lymphocytes/ml) in RPMI 1640 medium containing 10% human AB serum were pipetted into triplicate wells of a 96-well round-bottom microtiter plate. Mitogens at a concentration of $360 \mu\text{g}/\text{ml}$ for PHA (Wellcome Diagnostics, Research Triangle Park, N.C.) or a dilution of 1:250 for PWM (GIBCO) were added at $100 \mu\text{l}$ per well. These concentrations had been previously determined as optimal by titration using cells from at least three healthy subjects. The microtiter plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 72 h, the PHA-stimulated plates were pulsed with $10 \mu\text{l}$ of [^3H]thymidine prepared as a fresh stock at $200 \mu\text{Ci}/\text{ml}$. After 5 days, the PWM-stimulated plates were pulsed with [^3H]thymidine. The plates were incubated further for 18 h, and then cells were harvested with an automated cell harvester (PHD, Cambridge, Mass.). The incorporated radioactivity was measured with a liquid scintillation counter (Beckman LS-1800). The results (in counts per minute) were given as medians of triplicate determinations.

Measurement of serum immune activation markers. Serum was stored at -20°C prior to testing. Serum neopterin levels were quantitated by a commercial radioimmunoassay (Neopterin, RIAcid; Henning, Berlin, Germany). Serum $\beta 2M$ levels were measured by automated microparticle enzyme immunoassay (IMX; Abbott Diagnostics, Abbott Park, Ill.). The details of the methods have been described elsewhere (14, 22). Serum sIL-2R was assayed by using an enzyme-linked immunosorbent assay kit from T Cell Diagnostics (Cambridge, Mass.) according to the manufacturer's instructions.

Lymphocyte immunophenotype analysis. PBMC were distributed among 12-by-75-mm plastic tubes (5×10^5 cells per tube) containing the following monoclonal antibody combinations: CD3-CD56+16-CD8, HLA-DR-CD38-CD8, CD71-CD25-CD8, CD57-CD56-CD8, HLA-DR-CD38-CD4, CD71-CD25-CD4, and CD71-CD25-CD19 (all from Becton Dickinson Immunocytometry Systems, San Jose, Calif.). In each case, the first monoclonal antibody was labeled with fluorescein isothiocyanate, the second was labeled with phycoerythrin, and the third was labeled with peridinin chlorophyll protein (PerCP). The tubes were incubated for 15 min at room temperature, washed, and resuspended in 0.25 ml of fixation buffer (1% paraformaldehyde in $1\times$ phosphate-buffered saline). Cells were analyzed by using a FACScan flow cytometer (Becton Dickinson). Lymphocytes were identified by gating on forward (low-angle) and 90°C (wide-angle) light scatter parameters, and anti-CD45 (pan-leukocyte) and anti-CD14 (monocyte) served as markers for validating lymphocyte scatter gating. The fluorescence negatives were defined by using isotype control antibodies, and compensations were adjusted by using the CD3-CD56+16-CD8 tube. List mode data were collected for 10,000 events in the total gate. The absolute number of cells within a subset was determined by multiplying the absolute lymphocyte count by the percent cells positive for that marker(s). The absolute lymphocyte count was obtained from the leukocyte count and differential, which were determined for fresh whole blood prior to cell separation and cryopreservation.

Free and total serum p24 antigen measurement. Free serum p24 antigen (not bound into immune complexes) was measured by an enzyme immunoassay using the Coulter (Hialeah, Fla.) HIV p24 antigen assay kit according to the manufacturer's procedure (26). Total serum p24 antigen (free plus immune complexes dissociated) was measured after acid pretreatment (26). A $100\text{-}\mu\text{l}$ volume of serum from study subjects or standards were mixed with $50 \mu\text{l}$ of 0.5 N HCl, incubated for 1 h at 37°C , and then neutralized with approximately $50 \mu\text{l}$ of 0.5

FIG. 1. Proliferative responses of PBMC to PHA and PWM. Means and standard deviations are shown. HIV(-), HIV negative; HIV(+), HIV positive. CD4 counts per microliter are indicated.

N NaOH to pH 6.5 to 7.5. Thereafter, the assay was performed as for free p24 antigen, with the exception of an overnight incubation at 4°C instead of 1 h at 37°C as the first step.

Statistical analysis. Correlations were calculated by using Spearman's rank correlation coefficient. *P* values are two-sided alternatives, representing the probability of a value as large as (or larger than) that observed if there were no association between the variable measures. For group comparisons and the stratified analysis, comparisons were made with the Wilcoxon rank sum test. In the stratified analysis, groups were obtained from the HIV-seropositive subjects on the basis of absolute CD4 count (above or below $500/\text{mm}^3$).

RESULTS

Lymphocyte proliferative responses to mitogens. Results of proliferative responses to PHA and to PWM in 40 HIV-1-seronegative controls and 118 seropositive individuals are shown in Fig. 1. Seropositive individuals were divided into those with CD4 T-cell levels of greater or less than $500/\text{mm}^3$. There was no difference in the response to PHA between HIV-1-seronegative and -seropositive subjects with CD4 numbers of $>500/\text{mm}^3$. A significant difference, however, was found between the responses of the seronegative control subjects and HIV-1-seropositive subjects with CD4 counts of $<500/\text{mm}^3$. PWM responses, on the other hand, were significantly different between seronegative controls and both groups of seropositive subjects (Fig. 1). Furthermore, the seropositive group with low numbers of CD4 cells had a significantly poorer response to PWM ($P = 0.0015$) than did the seropositive group with higher CD4 cell levels.

Similar results were obtained when proliferative responses

TABLE 1. Summary of lymphocyte subset and phenotypic changes in HIV-1 infection

Subset or marker	Mean value for group $\pm$ SD ^a		Change ^b	P
	HIV negative (n = 40)	HIV positive (n = 118)		
Lymphoid subsets				
CD4	978 $\pm$ 376	543 $\pm$ 239	↓	0.0001
CD8	677 $\pm$ 510	1,051 $\pm$ 428	↑	0.0001
CD56+16 (NK cells)	355 $\pm$ 154	302 $\pm$ 173	↓	0.0123
CD19 (B cells)	295 $\pm$ 148	207 $\pm$ 92	↓	0.0011
Phenotypic markers				
HLA-DR	269 $\pm$ 312	591 $\pm$ 330	↑	0.0001
CD38	879 $\pm$ 393	1,141 $\pm$ 445	↑	0.0001
CD71 (transferrin receptor)	53 $\pm$ 26	64 $\pm$ 36	↑	NS ^c
CD25 (IL-2 receptor)	576 $\pm$ 313	226 $\pm$ 144	↓	0.0013

^a Mean number of lymphocytes per cubic millimeter (for lymphoid subsets) or mean number of lymphocytes expressing the phenotypic antigen (for phenotypic markers).

^b Direction of change in HIV-1-seropositive subjects compared to seronegative controls.

^c NS, not significant ($P > 0.05$).

of the HIV-infected subjects were calculated as a percentage of the mean value of the seronegative controls measured in the same assay or when the proliferation results were expressed as stimulation indices (data not shown). There was a good correlation ($P < 0.0001$) between the proliferative responses to PHA and PWM in seropositive individuals.

Lymphocyte phenotype changes and correlation with proliferative response. The mean levels of the major lymphocyte subsets and cells expressing activation antigens in the seropositive men were compared with those of HIV-1-seronegative controls measured concurrently (Table 1). The absolute numbers of CD4 T cells, NK, B cells, and cells expressing CD25 were significantly lower in HIV-1 infection. In contrast, the numbers of CD8 T cells and cells expressing HLA-DR and CD38 were significantly higher.

In the HIV-1-seropositive group, impaired lymphocyte proliferative responses showed a clear positive correlation with lowered numbers of CD4 cells as well as with decreased total lymphocyte expression of CD25 (Fig. 2). Decreased CD25 expression on CD4 and on B-lymphoid subpopulations also correlated with the proliferation impairment. Increased expression of HLA-DR or CD71 on CD4 cells correlated inversely with proliferative responses (Table 2). There was no significant correlation between lymphocyte proliferative responses and phenotypic markers in the HIV-1-seronegative group (data not shown).

Correlation of proliferative responses and serum p24 levels in HIV-1 infection. Total serum p24 antigen was detected in 62 subjects, and the levels correlated inversely with reduced proliferative response ($P = 0.0273$) (Table 3). Only 8 of the 118 seropositive subjects had detectable levels of free p24 (immune complex dissociated) antigen at the time of the study, and the levels inversely correlated with impaired proliferative response ($P = 0.0165$).

Lack of correlation of proliferative response with phenotypic marker changes on CD8 T cells or with serum immune activation markers. CD8 cell expression of CD38 and HLA-DR was significantly increased and expression of CD25 was decreased (data not shown). However, these changes did not correlate with the proliferative response impairment. Two serum immune activation markers, neopterin and β 2M, were significantly increased in seropositive subjects ($P < 0.0001$). In comparison with a mean $\pm$ standard deviation of 6.6 ± 1.9 nmol/liter for 40 HIV-1-seronegative individuals, the mean neopterin level for seropositive subjects with CD4 counts of

$>500/\text{mm}^3$ was 11.9 ± 3.7 nmol/liter. This further increased in subjects with CD4 counts of $\leq 500/\text{mm}^3$ (14.25 ± 3.5 nmol/liter). Similarly, the mean value ($\pm$ standard deviation) for β 2M in the seronegative group was 1.3 ± 0.3 mg/liter, whereas the levels increased to 2.2 ± 0.6 mg/liter in seropositive subjects with CD4 counts of $>500/\text{mm}^3$ and 2.6 ± 0.6 mg/liter in the seropositive group with CD4 counts of $>500/\text{mm}^3$. sIL-2R was tested only in the HIV-1-seropositive subjects with CD4 $<500/\text{mm}^3$ and was also significantly increased. However, these three serum immune activation parameters appeared to be independent of cell proliferation and did not correlate with either PHA or PWM proliferative responses (Table 3). The correlations of proliferative functional impairment with other immunological changes in HIV infection are also summarized in Table 3.

DISCUSSION

Clinical parameters, such as the onset of opportunistic infection, certain neoplasms, or death, according to the clinical definition of AIDS, are long-term endpoints for evaluation of HIV-1 infection or its treatment. Thus, effective and reliable surrogate markers are needed for more-expedient evaluation of disease progression and therapeutic efficacy. Whereas many immune changes during HIV-1 infection have been identified, few studies have examined their interrelationships. In a previous study, several serum immune activation and lymphoid phenotypic markers were assessed, and it was shown that some serum markers (neopterin, β 2M, and soluble CD8) and lymphoid phenotypic markers (CD38, CD45RA, and 1-selectin) reflect related aspects of immune dysregulation associated with HIV-1 infection (3). Prince et al. reported similar results (28). The relationship of serum and lymphoid phenotypic markers to lymphocyte function, however, has not been determined previously.

The impairment of proliferative function in HIV-1 infection is particularly interesting because this change can be seen before CD4 lymphocytes are substantially reduced in number (5, 20) and because such changes are reported to be related to disease progression (13). Furthermore, proliferative responses return toward normal levels transiently after initiation of zidovudine therapy (29), indicating that this change is induced by HIV-1 infection. Proliferative evaluations, however, are rarely used in HIV-1 disease stage determination and therapeutic evaluation, in part because the assay is more laborious

FIG. 2. Correlations between impaired proliferative responses to PHA (A and B) and PWM (C and D) and the levels of CD25 expression (reductions) on lymphocytes and the levels of HLA-DR expression on CD4 T cells. Correlation coefficients and *P* values are indicated for each correlation.

than serologic or flow cytometric assays and usually must be done with freshly obtained blood. Cryopreserved cells were used in the current study because batch testing allowed for better standardization. We included cryopreserved cells from the same healthy subject as an interexperimental control for

each batch, and the tests were performed in a limited period by the same technician using a single lot of reagents. The coefficients of variation for the interexperimental control were 32% for PHA and 24% for PWM.

Previously, PWM was found to be a more sensitive stimulus

TABLE 2. Significant correlations of proliferative responses with changes in lymphocyte phenotype

Parameter	Change ^a	Proliferative response ^b	
		PHA	PWM
% of total lymphoid cells expressing phenotypic marker			
CD4 cells	↓	0.22 (0.0157)	0.38 (0.0001)
CD8 cells	↑	-0.20 (0.0284)	-0.35 (0.0001)
CD71 cells	↑	-0.20 (0.0375)	NS ^c
CD25 cells	↓	0.26 (0.0061)	0.25 (0.0089)
% of lymphoid subset expressing phenotypic marker			
CD4 cells expressing:			
HLA-DR	↑	-0.28 (0.0026)	-0.24 (0.00109)
CD71	↑	-0.35 (0.0001)	NS
CD25	↓	0.19 (0.0436)	0.18 (0.0489)
CD19 B cells expressing CD25	↓	0.29 (0.0016)	0.20 (0.0384)

^a See Table 1, footnote *b*.

^b Spearman's rank correlation coefficients are given, with *P* values in parentheses.

^c NS, not significant.

TABLE 3. Correlations of proliferative capacity with other immunological changes in HIV-1 infection^a

Marker tested	Correlation ^b
Serum p24 antigen levels	
Free.....	+
Total.....	+
% of CD8 cells expressing:	
HLA-DR.....	NS
CD38.....	NS
CD71.....	NS
CD25.....	NS
Serum activation marker levels	
Neopterin.....	NS
β 2M.....	NS
sIL-2R.....	NS

^a Correlation results were similar for PHA and PWM stimulations for all HIV-positive subjects.

^b +, $P < 0.05$; NS, $P > 0.05$ (not significant).

than PHA for detecting the impairment of proliferative response associated with HIV-1 infection (13). Responses to PHA were reduced only in more-advanced HIV-1-seropositive subjects, while the response to PWM was reduced in most HIV-1-seropositive asymptomatic men (17). Our results also showed that PWM responses can distinguish between seropositive subject groups with CD4 numbers of greater or less than 500/mm³. This may be explained, at least partially, because PHA acts via both the CD2-dependent and the T3/Ti pathway while PWM involves primarily the T3/Ti-induced responses. The proliferative defect associated with HIV-1 infection alters principally the T3/Ti-induced responses (17).

Previously, it was reported that IL-2 receptor expression (CD25) on the major lymphoid subsets is significantly reduced in HIV-1 infection (16). Since IL-2 production and IL-2 receptor expression are essential steps in lymphocyte proliferation, it was expected that reduced CD25 expression would correlate with the functional defect in HIV-1 infection. This was confirmed for CD4 T cells in the present study. CD4 lymphocytes are known to be central to the proliferative responses and are responsible for producing IL-2 and other cytokines necessary for cell proliferation. However, it has been shown that IL-2 cytokine mRNA is not increased in CD4 cells in HIV-1 infection (9), and IL-2 is known to be a stimulus for IL-2 receptor (CD25) expression. Thus, impaired IL-2 receptor expression and IL-2 gene expression may both contribute to the proliferation impairment in HIV infection.

Interestingly, CD4 T cells have been shown to have increased expression of gamma interferon mRNA in HIV infection (9). This may relate to the changes of other cytokines which contribute to HIV-associated increases in HLA-DR and CD71 on CD4 T cells (Table 3). Altered proliferative function may reflect aspects of HIV-1 pathogenesis distinct from those reflected by other immune changes. This possibility is supported by the fact that the kinetics of zidovudine-induced changes in immune activation markers, lymphoid phenotypes, and proliferative response differ markedly. In HIV-1-infected subjects treated with zidovudine, elevated levels of serum activation markers and lymphoid phenotypic markers are most reduced (closest to normal) by 1 to 4 weeks of therapy (2, 4). In contrast, the recovery of proliferative response and increase of CD4 T cells after initiation of zidovudine treatment peak later at about 2 to 3 months (29). Whether zidovudine-induced reduction in viral activity results in functionally competent CD4 T cells or whether other factors are responsible for this transient functional improvement remains to be determined.

Unfortunately, wide variability in proliferative response to mitogens is a major problem inherent in the test system. A standard deviation of 50% is a common finding with this type of assay. This introduces uncertainties in establishing correlations between impairment of proliferation with other measurements of immunological change in HIV-1 infection. Although we batch-tested samples to minimize variation, the possibility of missing some existing correlation between proliferative responses and other measurements cannot be completely ruled out.

The complexity of the immune changes induced by HIV-1 infection is emphasized by our findings. The results show that measurements of lymphocyte functional change reflected by proliferative impairment provide additional information not obtained by measuring the changes in serum immune activation markers or several lymphoid phenotypic markers on CD8 T cells.

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