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Effect of Shipment, Storage, Anticoagulant, and Cell Separation on Lymphocyte Proliferation Assays for Human Immunodeficiency Virus-Infected Patients

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Lymphocyte proliferation assays (LPA), which can provide important information regarding the immune reconstitution of human immunodeficiency virus (HIV)-infected patients on highly active antiretroviral therapy, frequently involve shipment of specimens to central laboratories. In this study, we examine the effect of stimulant, anticoagulant, cell separation, storage, and transportation on LPA results. LPA responses of whole blood and separated peripheral blood mononuclear cells (PBMC) to different stimulants (cytomegalovirus, varicella-zoster virus, candida and tetanus toxoid antigens, and phytohemagglutinin) were measured using fresh specimens shipped overnight and frozen specimens collected in heparin, acid citrate dextrose (ACD), and citrate cell preparation tubes (CPT) from 12 HIV-infected patients and uninfected controls. Odds ratios for positive LPA responses were significantly higher in separated PBMC than in whole blood from ACD- and heparin-anticoagulated samples obtained from HIV-infected patients and from ACD-anticoagulated samples from uninfected controls. On separated PBMC, positive responses were significantly more frequent in fresh samples compared with overnight transportation for all antigens and compared with cryopreservation for the candida and tetanus antigens. In addition, viral antigen LPA responses were better preserved in frozen PBMC compared with specimens shipped overnight. CPT tubes yielded significantly more positive LPA results for all antigens, irrespective of the HIV patient status compared with ACD, but only for the candida and tetanus antigens and only in HIV-negative controls compared with heparin. Although HIV-infected patients had a significantly lower number of positive antigen-driven LPA responses compared with uninfected controls, most of the specimen processing variables had similar effects on HIV-positive and -negative samples. We conclude that LPA should be performed on site, whenever feasible, by using separated PBMC from fresh blood samples collected in either heparin or ACD. However, if on-site testing is not available, optimal transportation conditions should be established for specific antigens.

Human immunodeficiency virus (HIV) infection is associated with progressive loss of cell-mediated immune (CMI) responses that renders the host susceptible to opportunistic infections. The standard of care of patients with advanced AIDS has included prophylactic therapy for the most common opportunistic infections. The recent introduction of highly active antiretroviral therapy (HAART) has dramatically decreased the incidence of opportunistic infections in HIV-infected patients. The use of HAART has also resulted in predictable, potent, and durable suppression of HIV replication, elevation of the CD4 cell count, and increased survival of HIV-infected patients, including those with advanced disease (5). The extent of the immune reconstitution in these patients has been the object of several studies (1, 7). The findings showed a staggered improvement of immune function, including an increase in memory and naive CD4 cells, an increase in naive CD8 cells, a decrease in activated CD8 cells, and improvement of CD4 T-cell reactivity to recall antigens. In contrast, the Vβ repertoire remained abnormal (3), suggesting that in severely immunodepleted patients the immune restoration may not be complete. Furthermore, a minority of patients continue to experience opportunistic infections despite the increased number of CD4 cells (6). Functional CMI assays can provide prognostic information for HIV-infected patients (2). For patients on HAART, in addition to estimating the extent of immune reconstitution, pathogen-specific CMI assays might identify the individuals who will continue to require prophylactic therapy against specific agents.

Functional measurements of CMI responses commonly rely on antigen-specific lymphocyte proliferation assays (LPA). There is very little information on the effects of different methods of specimen collection, shipment, and processing on specific LPA results (4). However, this information is critically needed for the multicenter study designs currently used to acquire data on a large number of patients in a relatively short time. In these trials, specimens are frequently shipped fresh or after cryopreservation to central laboratories for special immunology studies such as LPA.

The objective of this study was to examine the effects of several variables—stimulant, anticoagulant, specimen storage and transportation, and cell separation—on LPA results for HIV-infected individuals.

MATERIALS AND METHODS

Study design. Informed consent was obtained from all study participants after the nature and possible consequences of this study had been fully explained to them. Five 8-ml tubes of blood were collected from HIV-infected patients and uninfected controls: two heparin tubes, two acid citrate dextrose (ACD) tubes, and one citrate cell preparation tube (CPT; Becton-Dickinson). One heparin tube and one ACD tube were processed on the day of collection. The remaining tubes were shipped overnight (Federal Express) and processed when received (after 24 h). Because CPTs are expensive, we did not use them for fresh cell assays, in which we did not expect a significant difference from heparin or ACD

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TABLE 1. LPA responses[a] by stimulant, HIV status, and cell separation for all anticoagulants

Cell type(s)	Response	SI of HIV-negative (HIV-positive) sample in response to:				
		CMV	Tetanus	VZV	Candida	PHA
Separated PBMC	Negative	2 (22)	12 (44)	2 (36)	2 (24)	0 (0)
	Positive	21 (33)	8 (7)	18 (18)	17 (30)	22 (55)
Whole blood	Negative	7 (26)	9 (32)	7 (32)	4 (29)	0 (5)
	Positive	5 (6)	3 (0)	5 (0)	8 (3)	12 (27)
Both	Negative	9 (48)	21 (76)	9 (68)	6 (53)	0 (5)
	Positive	26 (39)	11 (7)	23 (18)	25 (33)	34 (82)

[a] Positive, SI of ≥3; negative, SI of <3.

tubes. However, since CPTs are used to separate immune cells from erythrocytes shortly after collection, we considered that they might offer a significant advantage for overnight shipment, which would justify the additional expense. The CPTs were centrifuged at 1,500 × *g* for 20 min and also shipped overnight.

A fraction of the blood collected in heparin and ACD, both from fresh samples and from overnight samples, was used for whole-blood LPA. PBMC separated from all tubes (including CPTs) were used in the LPA. A matched aliquot of these cells was cryopreserved and assayed by LPA at a later time. Each specimen was assayed by using the following antigens and mitogens: cytomegalovirus (CMV) and varicella-zoster virus (VZV) antigens prepared in our laboratory as previously described (11), candida antigen (Greer Laboratory), tetanus toxoid (Connaught Laboratory), and phytohemagglutinin (PHA; Sigma). Unstimulated control wells were included. If there were insufficient cells for all 60 experimental conditions, then assays on cryopreserved cells were deleted in the following order: candida antigen, tetanus toxoid, VZV antigen, and CMV antigen.

LPA on separated PBMC. PBMC separated on Ficoll-Hypaque gradients (Sigma) or collected from CPTs were washed three times in phosphate-buffered saline, counted, and resuspended in RPMI 1640 medium (Gibco) with glutamine containing 10% human AB serum (Nabi) and 1% antibiotics (Gibco). PBMC, at 10^5/well, were added to triplicate wells containing the antigens (CMV and VZV at 1:50 and 1:200 dilutions, respectively; candida antigen at 20 μg/ml; and tetanus toxoid at 5 μg/ml), mitogen (PHA at 10 μg/ml), or no antigen (control). After 6 days of incubation at 37°C, wells were pulsed with 50 μCi of [^{3}H]thymidine per well (1 mCi/ml; Amersham) for 6 h, harvested, and counted in a Beckman scintillation counter using Biosafe II scintillation liquid (Research Product International Corp.). The stimulation index (SI) was calculated by dividing the average counts per minute in stimulated wells by the average counts per minute in unstimulated wells. The antigen concentrations used in this study were standardized to yield an SI of ≥3 for immune individuals and an SI of <3 for naive individuals. By using PBMC obtained from five normal donors and serial dilutions of the stimulants, we chose the highest antigen concentration that did not have an inhibitory effect on the LPA of seropositive volunteers and did not stimulate the proliferation of PBMC from seronegative donors.

Whole-blood LPA. Blood was diluted 1:5 in RPMI medium with 2% antibiotics and glutamine, and 0.1 ml of the suspension was added to triplicate wells containing the same panel of antigens, mitogen, or control as that described above. Cells were pulsed and harvested after 6 days of incubation, and the SI was calculated as described above.

LPA on cryopreserved cells. Separated PBMC were frozen by a step-down procedure in fetal calf serum containing 10% dimethyl sulfoxide. Cells were stored in liquid nitrogen until used for LPAs identical to those described for separated PBMC.

Statistical analysis. The analysis outcome was the SI. Because of the large variability in this assay and because neither the SI nor the log SI appeared to be sufficiently normal to justify the use of an analysis of variance model, the SI was treated as a qualitative measurement and dichotomized into positive and negative responses. An SI greater than or equal to 3 was used to define a positive response because antigen concentrations were standardized to this threshold. We also examined the results under an alternative threshold of 5.

Conditional logistic regression models (10) with the dichotomized SI as the outcome and the other experimental conditions entered as nominal variables (i.e., every condition was entered into the model as an indicator variable) were fitted. In addition, indicator variables identifying each individual were put into the model to adjust for individual differences. They were then conditioned out, and thus their coefficients were not estimated.

Model selection techniques were used to identify the important two-way interactions among the conditions of specimen manipulation, anticoagulant, cell separation, and HIV status. Odds ratios of interest were estimated based on this interaction model. Because the conditional logistic model cannot estimate the main effects of cluster level covariates, such as HIV status, a population-averaged model (GEE) was fitted to estimate this effect (9). Confidence intervals and *P* values reported from this model were adjusted for the clustering of the data. Wald tests and *P* values are reported for odds ratios. Unless stated otherwise, the significance level was taken to be 0.05.

RESULTS

Demographics. There were nine HIV-infected patients and three uninfected controls. The HIV-infected patients were males between 28 and 48 years of age (median, 41); seven were Caucasians, one was an African American, and one was Hispanic. Their CD4 counts varied from 164 to 403, with a mean and median of 238 and 235, respectively. Seven patients were on protease inhibitor-containing therapeutic regimens, one was on nucleoside analogue monotherapy, and one was on no therapy. Eight HIV-infected patients were seropositive for CMV, and one had unknown CMV status. All HIV-infected patients had prior chickenpox. Among the three uninfected controls, there were one male and two females, 40, 41, and 58 years of age. All were Caucasians and seropositive for both CMV and VZV. All study participants had been vaccinated against tetanus.

Effect of stimulant. The distribution of LPA-positive results (SI, ≥3) for each antigen and mitogen for HIV-infected patients and uninfected controls including all assay conditions is summarized in Table 1. Adjusting for all experimental factors, the odds ratio for a positive SI response to any antigen for HIV-positive versus HIV-negative individuals was 0.09, with a 95% confidence interval of 0.04 to 0.20. For the HIV-negative individuals, 20 to 30% of the assays had negative CMV, VZV, and candida results, and 66% of the tetanus-specific LPA had negative results. No negative assay results were observed for PHA. For the HIV-positive patients, 50 to 92% of the assays had negative results for CMV, VZV, candida, and tetanus. In contrast, only 6% of the assays had negative results for PHA. This shows that the proliferative response to PHA significantly differs from the antigen-induced proliferation. For this reason, for the analysis of the remaining variables (cell separation, transportation, and cryopreservation), we excluded the results of PHA-induced stimulation and used only the microbial antigen SI. The proportions of negative assays are slightly lower when restricting to separated peripheral blood mononuclear cell (PBMC) assays (Table 1). After adjusting for all specimen manipulations and anticoagulants, the odds ratios of a positive SI response for HIV-positive patients versus HIV-negative individuals were not significantly different among microbial antigens ($P = 0.566$).

Effect of cell separation. Whole-blood assays had a very small number of positive results after antigen stimulation: 30 (17%) of 176. In contrast, there were 152 positive results among 296 separated PBMC assays (51%) (Table 1). The odds of obtaining a positive response from separated PBMC were

TABLE 2. Odds ratios for a positive LPA response[a] in separated-PBMC versus whole-blood assays

Anticoagulant	Storage	HIV positive		HIV negative	
		OR	*P*	OR	*P*
Heparin	Fresh	19.61	<0.001	3.89	0.071
Heparin	Overnight	6.99	0.002	1.39	0.650
ACD	Fresh	153.62	<0.001	30.49	<0.001
ACD	Overnight	54.74	<0.001	10.86	<0.001

[a] SI, ≥3.

significantly higher than the odds of obtaining a positive response from whole-blood assays for HIV-infected patients for all microbial antigens and regardless of the anticoagulant or transportation (Table 2). This finding was also true for uninfected patient samples collected in ACD, regardless of transportation. The differences observed between separated PBMC and whole-blood samples collected in heparin from HIV-negative patients were marginally significant for fresh assays ($P = 0.071$) and not significant after overnight shipment ($P = 0.650$).

Effect of storage and transportation on separated-PBMC assays. The odds of obtaining a positive response from a fresh sample were significantly higher than the odds of obtaining a positive response from an overnight sample for all patients (Table 3). The model selection techniques did not identify any significant interactions between HIV status and storage or transportation, indicating that HIV-infected patients and uninfected controls behaved similarly with respect to these comparisons. The odds of obtaining a positive response from a fresh sample were significantly higher than those of obtaining one from a frozen sample for candida and tetanus but not for CMV and VZV in all patients. The odds of obtaining a positive response from frozen samples were significantly higher than those of obtaining one from shipped samples for CMV and VZV but not for tetanus and candida for all patients.

Effect of anticoagulant on separated PBMC assays. The odds of obtaining a positive response from a heparin-treated sample were significantly higher than those of obtaining one from an ACD-treated sample only for CMV in HIV-infected patients (Table 3). The odds of obtaining a positive response from a CPT tube were significantly higher than those of obtaining one from an ACD-treated blood sample for all antigens for both HIV-infected patients and uninfected controls. The odds of obtaining a positive response were significantly higher for CPT than heparin in uninfected controls for VZV, tetanus, and candida but not for CMV. In HIV-infected patients, the odds of obtaining a positive SI were similar for heparin and CPT. Confirming our original hypothesis, in fresh separated PBMC assays, there was no significant difference between heparin and ACD (odds ratio, 1.74; $P = 0.37$).

The analysis of the effects of specimen storage and transportation, anticoagulant choice, and cell separation resulted in the same conclusions as those described above when a positive result was defined as an SI of ≥5.

DISCUSSION

In this study, we showed that LPA results were greatly influenced by stimulant, cell transportation, storage, and cell separation. Separated PBMC yielded a significantly higher number of positive results than whole-blood assays, independently of anticoagulant and for all microbial antigens in HIV-infected patients. There was a similar effect in non-HIV-infected controls, but the differences were statistically significant only for ACD-anticoagulated samples. These results indicate that removal of the anticoagulant, erythrocytes, granulocytes, and/or patient plasma from the PBMC culture favors in vitro proliferation. Autologous serum has been used in LPA in previous studies (8) testing samples from healthy individuals, and it did not seem to inhibit proliferative responses. However, plasma from HIV-infected patients may contain cytokines or viral products that impair lymphocyte function. There was no difference between fresh and shipped samples in whole-blood assays (data not shown), but this was probably due to the low number of positive responses observed in whole-blood LPA. Although whole-blood assays are simpler and less time-consuming and therefore seem advantageous for the immunology laboratory work load, this method cannot be relied upon for CMI measurement in HIV-infected patients.

Fresh samples of separated PBMC yielded higher odds of obtaining positive LPA results for tetanus and candida compared with frozen PBMC and for all antigens compared with samples shipped overnight. This result was not surprising, and it probably reflects better preservation of cell viability and/or function in samples that were manipulated less. Frozen PBMC had a significantly higher number of positive LPA results than overnight specimens for the viral antigens and PHA (data not shown) but not for candida or tetanus.

There was no significant difference among anticoagulants in assays of fresh separated PBMC. Across all cell manipulations, heparin preserved only CMV responses significantly better than ACD. In contrast, CPT specimens had significantly higher odds ratios than ACD specimens in all patients and for all antigens. The difference between CPT and heparin was significant only in HIV-uninfected patients and only for tetanus,

TABLE 3. Odds ratios for a positive LPA response in separated-PBMC assays

Comparison	OR (*P* value) with stimulation by:			
	CMV	Tetanus	VZV	Candida
Fresh vs ON[a]	3.72 (<0.001)	3.72 (<0.001)	3.72 (<0.001)	3.72 (<0.001)
Fresh vs. Cryo[b]	0.55 (0.466)	3.93 (0.020)	0.83 (0.796)	3.93 (0.020)
ON vs Cryo	0.15 (0.016)	1.06 (0.918)	0.22 (0.030)	1.06 (0.918)
Heparin vs ACD (HIV^+)	5.17 (0.008)	2.05 (0.098)	2.05 (0.098)	2.05 (0.098)
Heparin vs ACD (HIV^-)	1.61 (0.565)	0.64 (0.475)	0.64 (0.475)	0.64 (0.475)
CPT vs heparin (HIV^+)	0.61 (0.455)	1.55 (0.365)	1.55 (0.365)	1.55 (0.365)
CPT vs heparin (HIV^-)	1.97 (0.443)	4.96 (0.020)	4.96 (0.020)	4.96 (0.020)
CPT vs ACD	3.17 (0.008)	3.17 (0.008)	3.17 (0.008)	3.17 (0.008)

[a] ON, blood shipped overnight.
[b] Cryo, cryopreserved cells.

VZV, and candida antigens. Superior preservation of LPA responses in CPT tubes may be ascribed to separation of PBMC from erythrocytes and granulocytes during transport. Note that cell number or viability tested by trypan blue exclusion (data not shown) did not significantly vary with the type of anticoagulant or separation method.

The impact of different conditions on LPA results significantly differed among antigens and between mitogen and antigens. Mitogen responses were also better preserved than the antigen ones in HIV-infected patients. This finding is not surprising because mitogens activate a broad population of mononuclear cells and, unlike antigens, do not require processing, presentation, or functional memory T cells. Our results indicate that mitogen-stimulated LPA is not representative of the host immune status with respect to antimicrobial responses, nor can it substitute for antigen-specific proliferation when optimizing LPA conditions.

Overall, specimen handling affected LPA responses from HIV-infected patients and uninfected controls similarly. This observation suggests that the effect of specific factors on LPA responses in healthy individuals can be used to predict the effect of similar factors on specimens from HIV-infected patients.

In conclusion, when designing specimen processing for LPA as part of a multicenter study, preference should be given to on-site testing of fresh samples. If on-site testing is not a viable alternative, cryopreserved and overnight PBMC assays (particularly using CPT tubes) should be compared for the relevant antigens.

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