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Shipment Impairs Lymphocyte Proliferative Responses to Microbial Antigens

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Lymphocyte proliferation assays (LPAs) are widely used to assess T-lymphocyte function of patients with human immunodeficiency virus infection and other primary and secondary immunodeficiency disorders. Since these assays require expertise not readily available at all clinical sites, specimens may be shipped to central labs for testing. We conducted a large multicenter study to evaluate the effects of shipping on assay performance and found significant loss of LPA activity. This may lead to erroneous results for individual subjects and introduce bias into multicenter trials.

Assays of lymphocyte proliferation (LPAs) in response to mitogens, microbial antigens, and allogeneic cells are performed to evaluate cellular function in patients with primary immunodeficiency (e.g., severe combined immunodeficiency or DiGeorge syndrome) and secondary immunodeficiency (e.g., human immunodeficiency virus [HIV] infection or malnutrition) (2, 4–6)). These assays are useful for monitoring immune reconstitution of severe combined immunodeficiency after bone marrow transplantation (1) and immune reconstitution in HIV-infected patients after initiation of highly active antiretroviral therapy (8). Finally, LPAs may provide information about pathogen-specific T-lymphocyte responses to supplement information obtained from delayed-type hypersensitivity skin tests (7, 11).

LPAs are time-consuming and labor-intensive and are not widely available except at major medical centers and referral laboratories. The needs of physicians at outlying locations who participate in multicenter clinical trials therefore require shipping of blood specimens to central laboratories. The AIDS Clinical Trials Group (ACTG) has developed a standardized protocol for the performance of LPAs within a network of specialized immunology laboratories. This study was designed to assess the effects of shipping and handling on lymphocyte proliferative responses to mitogens and recall antigens.

MATERIALS AND METHODS

Sample collection. Ten ACTG immunology laboratories participated in this study to examine the effects of storage and shipment, anticoagulant, and HIV status on LPA response. Each lab tested blood from up to eight HIV-infected patients with CD4 counts of between 200 and 400/mm^3 and from between two and four normal controls. Blood was collected into three 7-ml Vacutainer tubes containing heparin, three Vacutainer tubes containing acid citrate dextrose (ACD), and three ACD cell preparation tubes (CPT tubes) (Becton-Dickinson). One set of each tube type was processed immediately (fresh), one set was held overnight in the laboratory at room temperature (bench), and one set was shipped to and from the same lab via overnight courier, being certain that the tubes were flown through the courier's hub (shipped).

LPA. The assay was performed by the ACTG consensus methodology (http://actg.s-3.com/labs.htm). Briefly, peripheral blood mononuclear cells (PBMC) were purified from heparin and ACD tubes by centrifugation over Ficoll-Hypaque; CPT tubes were centrifuged as per the manufacturer's instructions. Cells were resuspended to a final concentration of 5×10^5 cells/ml in RPMI 1640 containing 10% AB$^+$ human serum. Two hundred microliters (10^5 cells) was cultured in quadruplicate wells of round-bottom 96-well culture plates containing pokeweed mitogen (5 μg/ml), tetanus toxoid (1 μg/ml), *Candida albicans* antigen (10 μg/ml), streptokinase (10 μg/ml), or no antigen (control). On day 6 of culture, cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 6 h and harvested onto glass fiber filters. The [^{3}H]thymidine incorporated into DNA was counted and expressed as median counts per minute for each stimulation condition. A stimulation index (SI) was calculated as median counts per minute for stimulated wells/median counts per minute for control wells.

Statistical Analysis. Because of the large variability associated with SI and because neither SI nor log SI appeared to be sufficiently normal to justify analysis in an analysis-of-variance model, SI was treated as a qualitative outcome and dichotomized into positive and negative responses. A positive response was defined to be an SI of >5 for antigens and of >10 for mitogens. Several of the analyses were repeated for alternative cutoffs of 3 and 10 for antigens.

Conditional logistic regression models (10), with the dichotomized SI as the

TABLE 1. Distribution of positive results by HIV status

Stimulant	No. positive/total (% positive)[a]		
	HIV-negative subjects	HIV-positive subjects	All subjects
Pokeweed mitogen	163/172 (94.8)	94/101 (93.1)	257/273 (94.1)
Candida	167/188 (88.8)	102/145 (70.3)	269/333 (80.8)
Tetanus toxoid	132/188 (70.2)	36/144 (25.0)	168/332 (50.6)
Streptokinase	92/162 (56.8)	24/101 (23.8)	116/263 (44.1)
All antigens	391/538 (72.7)	162/390 (41.5)	553/928 (59.6)
Total	554/710 (78.0)	256/491 (52.1)	810/1,201 (67.4)

[a] Positive is defined as an SI of >10 for pokeweed mitogen and an SI of >5 for antigens.

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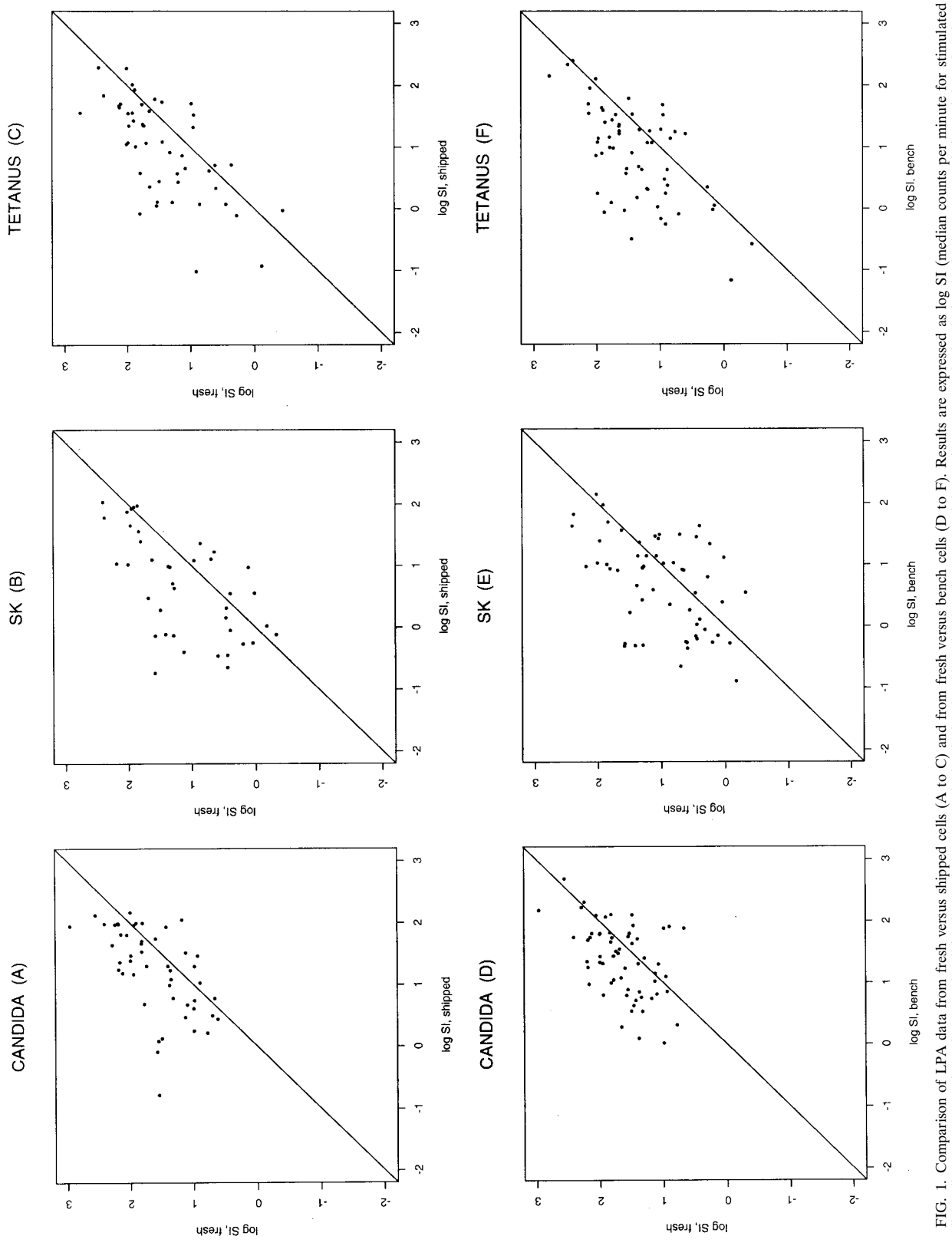

FIG. 1. Comparison of LPA data from fresh versus shipped cells (A to C) and from fresh versus bench cells (D to F). Results are expressed as log SI (median counts per minute for stimulated wells/median counts per minute for control wells). The *x* axis represents the log SI for the shipped or bench assays, and the *y* axis represents the log SI for the fresh assays. Deviations of the points from the 45° line depicted in each panel indicate a lack of agreement between the log SI from fresh assays and the log SI from shipped or bench assays. SK, streptokinase.

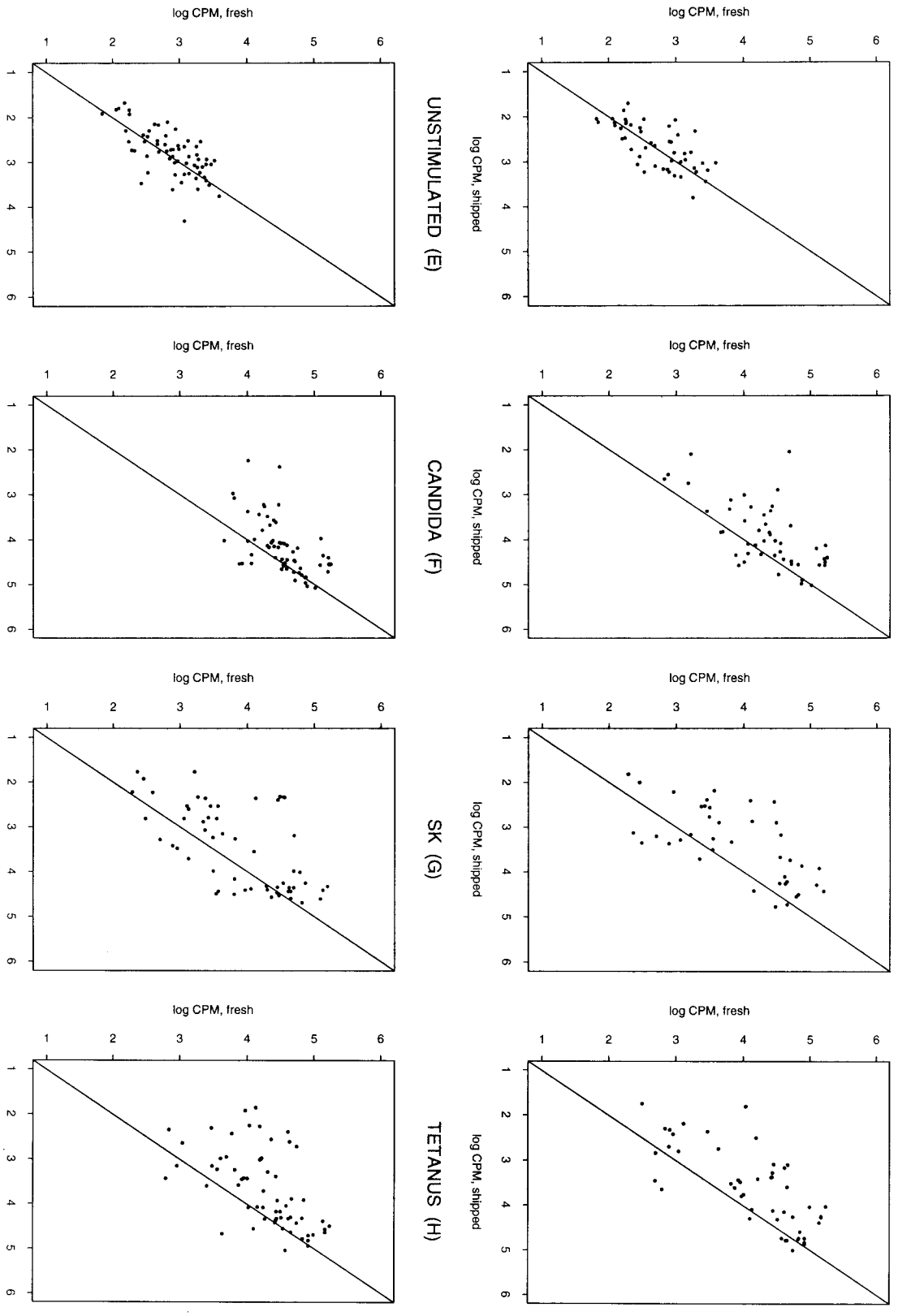

FIG. 2. Comparison of LPA data from fresh versus shipped cells (A to D) and from fresh versus bench cells (E to H). Results are expressed as log counts per minute. The *x* axis represents the log counts per minute for the shipped or bench assays, and the *y* axis represents the log counts per minute for the fresh assays. Deviations of the points from the 45° line depicted in each panel indicate a lack of agreement between the log counts per minute from fresh assays and the log counts per minute from shipped or bench assays. SK, streptokinase.

TABLE 2. Odds ratios for a positive SI response[a]

Comparison	Results with the following stimulant:							
	Candida		Tetanus toxoid		Streptokinase		Pokeweed mitogen	
	OR	*P*	OR	*P*	OR	*P*	OR	*P*
Fresh vs shipped (HIV−)	11.92	—[b]	3.62	—	3.62	—	0.52	0.429
Fresh vs shipped (HIV+)	4.32	0.003	1.31	0.491	1.31	0.491	0.29	0.141
Fresh vs bench (heparin, HIV−)	7.16	0.003	4.66	0.002	1.78	0.196	2.72	0.017
Fresh vs bench (heparin, HIV+)	1.18	0.786	0.77	0.606	0.29	0.013	0.59	0.272
Fresh vs bench (ACD, HIV−)	6.04	0.006	3.93	0.003	1.50	0.339	1.48	0.327
Fresh vs bench (ACD, HIV+)	1.00	0.994	0.65	0.394	0.25	0.005	0.32	0.020
Fresh vs bench (ACD-CPT, HIV−)	17.01	—	11.08	—	4.23	0.001	2.70	0.018
Fresh vs bench (ACD-CPT, HIV+)	2.80	0.100	1.82	0.250	0.70	0.465	0.58	0.274
Shipped vs bench (heparin, HIV−)	0.60	0.380	1.29	0.610	0.49	0.149	5.25	0.055
Shipped vs bench (heparin, HIV+)	0.27	0.026	0.59	0.312	0.22	0.005	2.02	0.427
Shipped vs bench (ACD, HIV−)	0.51	0.227	1.09	0.861	0.42	0.061	2.86	0.220
Shipped vs bench (ACD, HIV+)	0.23	0.012	0.49	0.182	0.20	0.002	1.10	0.914
Shipped vs bench (ACD-CPT, HIV−)	1.43	0.525	3.06	0.026	1.17	0.747	5.21	0.056
Shipped vs bench (ACD-CPT, HIV+)	0.65	0.445	1.39	0.539	0.53	0.235	2.00	0.434
Heparin vs ACD (fresh, shipped)	1.40	0.208	1.40	0.208	1.40	0.208	1.32	0.277
Heparin vs ACD (bench)	1.18	0.656	1.18	0.656	1.18	0.656	0.72	0.328
Heparin vs ACD-CPT (fresh, shipped)	1.04	0.890	1.04	0.890	1.04	0.890	1.20	0.493
Heparin vs ACD-CPT (bench)	0.88	0.775	0.88	0.775	0.88	0.775	0.66	0.320
ACD-CPT vs ACD (fresh, shipped)	1.34	0.294	1.34	0.294	1.34	0.294	1.10	0.732
ACD-CPT vs ACD (bench)	0.48	0.040	0.48	0.040	0.48	0.040	0.60	0.138

[a] A positive response is an SI of >5 for antigens and an SI of >10 for pokeweed mitogen. Abbreviations: OR, odds ratio; HIV−, HIV negative; HIV+, HIV positive.
[b] —, $P < 0.001$.

dependent variable and the other experimental conditions as predictors, were fit. In addition, separate intercepts for each individual were put into the model to adjust for individual differences. Since each individual defined a matched set, or cluster, these intercepts dropped out of the likelihood for the conditional logistic regression and were not estimated. Model selection techniques were used to identify the important two-way interactions among the conditions of specimen manipulation, stimulant, anticoagulant, and HIV status. These were included in a model with the main effect terms without any attempt to reduce the model further. Odds ratios of interest were estimated based on the interaction model.

Because the conditional logistic regression model cannot estimate the main effects of individual-specific features such as HIV status, a population-averaged model (generalized estimating equations) was fit to estimate this effect (9). Inferences based on this model are adjusted for the clustering of the data due to the multiple assays from each individual.

Wald tests and *P* values are reported for odds ratios. The significance level was taken to be 0.05. No adjustment was made for multiple testing.

RESULTS

The 10 participating laboratories performed 1,317 LPAs on 53 subjects, 23 of whom (43%) were HIV seropositive. Of these, 116 assays were eliminated from analysis because they did not conform to the protocol (due to problems with blood collection, shipping, or assay conditions), leaving a total of 1,201 assays for analysis.

Overall, 810 (67.4%) of 1,201 LPA results were positive (i.e., SI of >5 for tetanus toxoid, candida, and streptokinase antigens and SI of >10 for pokeweed mitogen). Several of the analyses were repeated for alternative SI cutoffs of >3 or >10 for the antigens; the results were comparable to those based on the cutoff of >5, and only the latter results are discussed in detail. Positive LPA results were obtained for pokeweed mitogen in 257 of 273 tests (94.1%), for candida in 269 of 333 tests (80.8%), for tetanus toxoid in 168 of 332 tests (50.6%), and for streptokinase in 116 of 263 tests (44.1%) (Table 1). After adjustment for specimen handling, stimulant, and anticoagulant, as well as the multiple assays from each individual, HIV-positive individuals were found to have significantly lower odds of a positive assay response than HIV-negative individuals (odds ratio = 0.23; $P < 0.0001$).

LPA results for fresh versus shipped and fresh versus bench specimens were compared in terms of SI. Responses were higher in fresh specimens than in shipped specimens (Fig. 1A to C) and higher in fresh specimens than bench specimens (Fig. 1D to F). This effect was due to decreased counts per minute in antigen-stimulated wells and not to increased counts per minute in unstimulated wells (Fig. 2).

The odds of a positive response were significantly higher for fresh specimens than for shipped specimens for candida, tetanus toxoid, and streptokinase in HIV-negative individuals (Table 2). The odds ratios for all stimulants except candida were not significant for HIV-positive individuals, most likely because there were fewer positive SI responses and thus the power for comparison was reduced. Similarly, the odds of a positive response were significantly higher for fresh specimens than for bench samples for candida, tetanus toxoid, and pokeweed mitogen in HIV-negative individuals. All but one of these odd ratios were not significant for HIV-positive individuals, most likely because there were fewer positive SI responses.

For all cutoffs, no differences in the odds of positive SI responses were observed between heparin and ACD and between heparin and CPT tubes for any of the stimulants, for any of the methods of specimen handling, or for HIV status. For specimens held on the bench, the odds of a positive SI response were significantly lower in CPT tubes than in ACD.

DISCUSSION

The LPA is a standard method for evaluating cell-mediated immune function. Because it is a labor-intensive test for which extensive training and careful quality control are required, LPAs are generally available only at large referral laboratories and major medical centers. This study documents some of the problems encountered when samples are shipped to such laboratories.

Regardless of anticoagulant or stimulant, there was a loss of LPA activity whenever the assay setup was delayed, either by shipment or by overnight storage of whole blood. We did not test whether the addition of medium before storage or shipment would improve lymphocyte function. This large, multicenter trial confirms and extends previous studies. Fletcher et al. (3) reported that 24 h of storage at room temperature had only marginal effects on mitogen-induced proliferation of PBMC from eight normal individuals, but they did not test responses to microbial antigens and did not test responses of immunodeficient subjects. Weinberg et al. (12) studied nine HIV-infected patients and three uninfected controls at a single center. They found that the odds of obtaining a positive response from a fresh blood sample were significantly higher than the odds of obtaining a positive response from a sample held at room temperature overnight or from a cryopreserved sample. The small number of uninfected controls made direct comparison with the study of Fletcher et al. (3) difficult.

To simulate the issues faced in multicenter collaborative trials, this study was performed at 10 laboratories following a consensus protocol. The data from this large study emphasize that loss of LPA activity occurs even when PBMC from normal subjects are tested. These differences are reflected in decreased net counts per minute (stimulated counts per minute − unstimulated counts per minute) and decreased percent positive responses as defined by SI. Strong responses (e.g., to mitogens) are generally better preserved than weak responses. Loss of activity in shipped specimens may lead to erroneous results for individual subjects (e.g., diagnosis of cell-mediated immune deficiency or lack of pathogen-specific immunity) and may introduce bias into multicenter studies in which some specimens are shipped and others are delivered to an on-site laboratory for immediate processing.

There is no obvious solution to the problem of shipping. Not all laboratories will be able to perform LPAs. Cryopreservation also leads to loss of functional activity (12). Neither we nor others have found that shipping conditions or the choice of anticoagulant can eliminate the fresh-shipped bias. Whenever possible, patients, not blood specimens, should be sent to the site of the laboratory when LPAs are required. When specimens are shipped, a specimen from a normal subject always should be included as a quality control. Finally, the systematic error introduced by shipping must be considered when planning large multicenter trials.

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