Concordant Proficiency in Measurement of T-Cell Immunity in Human Immunodeficiency Virus Vaccine Clinical Trials by Peripheral Blood Mononuclear Cell and Enzyme-Linked Immunosot Assays in Laboratories from Three Continents

Mark J. Boaz,1†‡ Peter Hayes,2† Tony Tarragona,2 Laura Seamons,2 Andrew Cooper,2 Josephine Birungi,3 Paul Kitandwe,4 Aloysius Segamanda,5 Pontiano Kaleebu,3 Gwynneth Stevens,1 Omu Anzala,4 Bashir Farah,4 Simon Ogola,4 Jackton Indangasi,4 Patrick Mhlanga,5 Melanie Van Eeden,5 Madhuri Thakar,6 Ashwini Pujari,6 Shadri Mishra,6 Nilu Goonéilleke,7 Stephen Moore,7 Abdul Mahmoud,7 Pattabiraman Sathyamoorthy,8 Jayashri Mahalingam,8 Paranj R. Narayanan,8 Vadakkuppatu D. Ramanathan,8 Josephine H. Cox,1* Len Dally,9 Dilbinder K. Gill,2 and Jill Gilmour2

International AIDS Vaccine Initiative, New York, New York1; International AIDS Vaccine Initiative Core Laboratory, Imperial College, London, United Kingdom2; Uganda Virus Research Institute, Entebbe, Uganda3; Kenya AIDS Vaccine Initiative, Nairobi, Kenya4; Contract Laboratory Services, Johannesburg, South Africa5; National AIDS Research Institute, Pune, India6; Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford, Oxford, United Kingdom7; Tuberculosis Research Centre, Chennai, India8; and EMMES Corporation, Rockville, Maryland9

Received 8 September 2008/Returned for modification 9 October 2008/Accepted 9 December 2008

The gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay is used routinely to evaluate the potency of human immunodeficiency virus (HIV) vaccine candidates and other vaccine candidates. In order to compare candidates and pool data from multiple trial laboratories, validated standardized methods must be applied across laboratories. Proficiency panels are a key part of a comprehensive quality assurance program to monitor inter- and intralaboratory performance, as well as assay performance, over time. Seven International AIDS Vaccine Initiative-sponsored trial sites participated in the proficiency panels described in this study. At each laboratory, two operators independently processed identical sample sets consisting of frozen peripheral blood mononuclear cell (PBMC) samples from different donors by using four blind stimuli. PBMC recovery and viability after overnight resting and the IFN-γ ELISPOT assay performance were assessed. All sites demonstrated good performance in PBMC thawing and resting, with a median recovery of 78% and median viability of 95%. The laboratories were able to detect similar antigen-specific T-cell responses, ranging from 50 to >3,000 spot-forming cells per million PBMC. An approximate range of a half log in results from operators within or across sites was seen in comparisons of antigen-specific responses. Consistently low background responses were seen in all laboratories. The results of these proficiency panels demonstrate the ability of seven laboratories, located across three continents, to process PBMC samples and to rank volunteers with differential magnitudes of IFN-γ ELISPOT responses. These findings also illustrate the ability to standardize the IFN-γ ELISPOT assay across multiple laboratories when common training methods, reagents such as fetal calf serum, and standard operating procedures are adopted. These results are encouraging for laboratories that are using cell-based immunology assays to test HIV vaccines and other vaccines.

Most human immunodeficiency virus (HIV) vaccines currently in development aim to induce cellular immune responses, since these have been shown previously to temporally correlate with the containment of virus in infected individuals and, more significantly, to be crucial in the suppression of virus during reactivation and, more significantly, to be crucial in the suppression of virus in infected individuals. The ability to measure and quantitate cellular immune responses has been facilitated through the development of enzyme-linked immunospot (ELISPOT) and flow cytometry assays which determine the number of antigen-specific cells through surrogate markers of effector function, such as cytokine production or the degranulation of lytic granules (1, 8, 23, 29), and are more quantitative and less labor-intensive than traditional assays that detect T-cell responses, such as 51Cr release and lymphoproliferation assays (19). The gamma interferon (IFN-γ) ELISPOT assay is a primary assay employed to measure vaccine immunogenicity in HIV vaccine clinical trials, in addition to trials in the cancer, malaria, and tuberculosis vaccine fields (23, 30, 31). Although data on the performance of the IFN-γ ELISPOT assay across multiple laboratories both within and across continents are critical to the generation of standardized data on vaccine immunopotency (14), little published data exist. The IFN-γ ELISPOT assay results can demonstrate whether a vaccine is able to induce a range of immune responses in a particular population,
therefore justifying further development. The value of standardized methods for determining vaccine immunopotency should not be diminished in spite of recent disappointing data from an HIV vaccine trial in which advancement to a phase IIb trial was based partly on IFN-γ ELISPOT data from phase I and II clinical trials (7, 26). Future modifications to the IFN-γ ELISPOT assay may increase its relevance to efficacy testing or allow it to correlate better with elaborate assays that yield critical effector functions such as the inhibition of viral replication (9, 24). The International AIDS Vaccine Initiative (IAVI), in collaboration with local partners, has developed good clinical laboratory practice (GCLP)-compliant clinical trial laboratories at trial units across Europe, Africa, and India. These GCLP guideline-compliant laboratories can be used for the comparative assessment of HIV vaccine candidates developed by IAVI and other organizations and partners, for example, the Division of AIDS (NIH, Bethesda, MD) and biotechnology firms, to facilitate the development of an HIV vaccine (10, 22). As part of the ongoing assessment of laboratory performance and assay result comparability, IFN-γ ELISPOT proficiency panels are conducted regularly at the IAVI-sponsored laboratories. Such proficiency panels have also been conducted among laboratories from different organizations within the HIV vaccine field and have recently been implemented at laboratories working within the Cancer Vaccine Consortium (3, 4, 11). In contrast to published data, the findings of the present study demonstrate that when standardized training and validated assay methods are followed, the results of the IFN-γ ELISPOT assay and the associated handling of test material are notably and highly concordant among laboratories. These data hold promise for the HIV vaccine field as a whole and also for cancer, malaria, and tuberculosis cell-based vaccines. It is possible that comparable data can be obtained across multicenter trials and continents, facilitating concordant and, if warranted, accelerated vaccine development efforts.

MATERIALS AND METHODS

Participating laboratories. The following laboratories are currently participating or have previously participated in IAVI-sponsored HIV vaccine trials: (i) the IAVI Core Laboratory London (hereinafter referred to as the IAVI Core Lab), Imperial College, London, United Kingdom, (ii) the Centre for Clinical Vacci-

**TABLE 1. Design of proficiency panels 1 to 3**

| Panel | Participating laboratories | No. of PBMC samples | Plate type(s) | Counting instruments employed (no. of labs) | Stimuli |
|-------|---------------------------|---------------------|--------------|------------------------------------------|---------|
| 1     | Core, Oxford, CLS, KAVI, UVRI | 6                   | 1 Self-coated | Z1 Coulter Counter (4), hemocytometer (1) | Mock; HIV, CEF, and CMV peptides; PHA |
| 2     | Core, Oxford, CLS, KAVI, UVRI, NARI | 3                   | 1 Precoated, 1 self-coated | Z1 Coulter Counter (1), hemocytometer (1), Vi-CELL XR counter (3), Guava counter (1) | Mock, CEF and CMV peptides, PHA |
| 3     | Core, Oxford, CLS, KAVI, UVRI, NARI, TRC | 8                   | 1 Precoated, 1 self-coated | Z1 Coulter Counter (1), hemocytometer (1), Vi-CELL XR counter (4), Guava counter (1) | Mock, CEF and CMV peptides, PHA |

* To obtain viable cell counts with the Z1 Coulter Counter, a hemocytometer and trypan blue staining were also used.
Spearman's correlation coefficient.
groups (e.g., samples within each panel). Measures of correlation are based on
tween operators), and the Kruskal-Wallis test was used for comparing multiple
The signed-rank test was used for paired observations (e.g., comparisons be-
CV) of results were performed by the EMMES Corporation (Rockville, MD).
PBM, the numbers of SFC per million PBMC, and the coefficients of variation
AID ELISPOT reader (AutoImmun Diagnostika, Germany). The ELISPOT
biotin complex (Vector Laboratories, Burlingame, CA) for 1 h, and development
body (Mabtech, Sweden) for 2 to 4 h, the addition of ABC peroxidase-avidin-
previously (22). In brief, 96-well Multiscreen HTS IP plates (MSIP4510; Milli-
stimulus (RPMI medium–10% FCS with dimethyl sulfoxide [DMSO] to give a
final concentration per well of 0.45% DMSO) to control for DMSO included in
 according to the ELISPOT templates provided. Blind stimuli included a mock
37°C and 5% CO₂, the production of IFN-γ was assessed by the addition of 100
μl of 1-μg/ml filtered biotinylated clone 7-B6-1 mouse anti-human IFN-γ anti-
body (Mabtech, Sweden) for 2 to 4 h, the addition of ABC peroxidase-avidin-
biotin complex (Vector Laboratories, Burlingame, CA) for 1 h, and development
with filtered AEC (3-amino-9-ethylcarbazole) substrate solution (Vector Lab-
atories, Burlingame, CA) for 4 min. Plate results were read using an automated
AID ELISPOT reader (AutoImmun Diagnostika, Germany). The ELISPOT
data are expressed as the numbers of spot-forming cells (SFC) per million
PBMC.

CX500 (Jencons, United Kingdom). Following receipt, the PBMC continued to
be stored in vapor-phase liquid nitrogen until use. Prior to use, the PBMC were
thawed by being warmed in a water bath at 37°C until one small ice crystal
remained and then washed in RPMI medium–20% fetal calf serum (FCS) and
allowed to rest overnight in RPMI medium–20% FCS at 1.5 to 2 million
PBMC/ml in an atmosphere of 5% CO₂ at 37°C. The following morning, viable
cells were counted and placed onto the ELISPOT assay plates. All PBMC counts
and recovery and viability results were recorded on batch records.

ELISPOT assay. The IFN-γ ELISPOT assay was performed as described
previously (23). In brief, 96-well Multiscreen HTS IP plates (MSIP4510; Milli-
apore, United Kingdom) were incubated overnight with 10 μg/ml of clone 1-D1K
mouse anti-human IFN-γ monoclonal antibody (Mabtech, Sweden). The next
day, after being washed and blocked with RPMI medium–10% FCS, the PBMC
were plated at 2 × 10⁷ viable PBMC per well and stimulated in quadruplicate
according to the ELISPOT templates provided. Blind stimuli included a mock
stimulus (RPMI medium–10% FCS with dimethyl sulfoxide [DMSO] to give a
final concentration per well of 0.45% DMSO) to control for DMSO included in
the peptide stimuli, CEF and CMV peptides at 1.5 μg/ml, and PHA (Sigma,
Poole, Dorset, United Kingdom) at 10 μg/ml. Following overnight incubation at
37°C and 5% CO₂, the production of IFN-γ was assessed by the addition of 100
μl of 1-μg/ml filtered biotinylated clone 7-B6-1 mouse anti-human IFN-γ anti-
body (Mabtech, Sweden) for 2 to 4 h, the addition of ABC peroxidase-avidin-
biotin complex (Vector Laboratories, Burlingame, CA) for 1 h, and development
with filtered AEC (3-amino-9-ethylcarbazole) substrate solution (Vector Lab-
atories, Burlingame, CA) for 4 min. Plate results were read using an automated
AID ELISPOT reader (AutoImmun Diagnostika, Germany). The ELISPOT
data are expressed as the numbers of spot-forming cells (SFC) per million
PBMC.

Statistical analysis. Analyses of the recovery and viability results for thawed
PBMC, the numbers of SFC per million PBMC, and the coefficients of variation
(CV) of results were performed by the EMMES Corporation (Rockville, MD).
The signed-rank test was used for paired observations (e.g., comparisons be-
between operators), and the Kruskal-Wallis test was used for comparing multiple
groups (e.g., samples within each panel). Measures of correlation are based on
Spearman’s correlation coefficient.

RESULTS

Recovery and viability of PBMC. All PBMC were received at
the participating laboratories at a temperature below −170°C. Two operators in each laboratory independently recorded the
total number of viable cells following thawing and overnight
resting, in addition to the percent viability and the calculated
recovery percentage (Fig. 1). All recordings were received ex-
cept those from one laboratory which did not provide viability
data for panels 1 and 2. The median values (and ranges) for
recovery were 81.2% (46 to 163%) in the first panel, 96.3% (60
to 155%) in the second panel, and 69.8% (35 to 170%) in the
third panel.

Comparisons between donors. The Kruskal-Wallis test re-
sults showed variability in recovery rates between PBMC sam-
ple from different donors and were significant in panels 1 (P =
0.0005) and 3 (P = 0.0013) and borderline in panel 2 (P =
0.0657). This outcome may relate to natural variation in the
propensity of cells for freezing and thawing or to the large
volumes of blood handled (approximately 200 to 500 ml) in the
processing blood bank samples, leading to inaccurate counts
upon freezing. Data from the IAVI partner laboratory network
revealed a median recovery of 70% (median viability of 92%)
from 992 clinical trial samples frozen during 2006, of which the
common blood draw volume was between 40 and 80 ml of
blood (some of these data are shown in Table 2; also see
below). No difference was seen between donors in the viability
percentages (P, >0.23 for each of the three panels), which
ranged from 80 to 100%, with medians of 95, 96, and 95% for
panels 1, 2, and 3, respectively.

Comparisons between sites. Significant differences between
sites in the total recovery of viable cells (P values of 0.0005,
0.0079, and <0.0001 in panels 1, 2, and 3, respectively) and also
the viability of PBMC (P values of 0.0050, 0.0003, and <0.0001,
respectively), were noted (Fig. 1). This finding may relate to
the difference in the counting methods employed at the sites
(Table 1), since it has been reported previously that the levels
of viability determined by automated counters are lower than
that determined by manual counting (12).
Comparisons between operators. To compare the observations of operators at each site, the paired differences in recovery and viability for each donor were tested using Wilcoxon’s signed-rank test. Recovery data differed between operators at one lab in panel 1 (P = 0.031) and at three labs in panel 3 (P = 0.0156, 0.0078, and 0.0078). The viability percentages differed between operators at two labs in panel 1 (P = 0.031 for both) and one lab in panel 3 (P = 0.0156). With samples from only three volunteers, panel 2 had very low statistical power, and no one lab in panel 1, 2, and 3. No other correlations were observed.

Correlation with ELISPOT assay responses. Overall, there was a statistically significant though not very strong correlation (20.5%; P = 0.0067) between the percentages of viability and the magnitudes of PHA responses in the ELISPOT assay. Among panels 1, 2, and 3, the correlations were inconsistent in magnitude and direction, being −19% (P = 0.201), 49% (P = 0.006), and 29% (P = 0.005), respectively. Similarly, there was a weak though statistically significant negative correlation (−24.4%; P = 0.0007) overall between recovery rates and magnitudes of CMV responses in the ELISPOT assay. Again, the correlations were inconsistent, being 0% (P = 1.0), −44% (P = 0.008), and −26% (P = 0.012), respectively, for panels 1, 2, and 3. No other correlations were observed.

ELISPOT assay performance. Two operators per laboratory independently set up each ELISPOT assay by following the SOPs and template instructions for adding the blind stimuli. Responses for each donor sample in each laboratory to the different stimuli are expressed as the numbers of SFC per million PBMC and are shown in Fig. 2B. Mock (i.e., background or medium) responses are the well counts, whereas CMV, CEF, and PHA responses are the well counts after the subtraction of mock response values. Only 3 (1%) of the 323 responses to the mock stimulus were above 55 SFC/10⁶ cells, indicating an excessive background count that would result in assay failure and subsequent retesting of the sample in present IAVI clinical trials. For the IAVI proficiency panels in the present study, the mean background level at the seven sites ranged from 2.3 to 13.6 SFC/10⁶ PBMC and was 7.7 ± 15.2 (standard deviation [SD]) overall. If the three specimens with >55 SFC/10⁶ PBMC are excluded, then the mean background level ± SD was 6.6 ± 8.0 SFC/10⁶ PBMC.

The CEF and CMV stimuli allow the assessment of concordance in the magnitude of antigen-specific responses from donor PBMC across laboratories and, furthermore, for a given definition of a response classification (e.g., nonresponder or responder), the issue of whether responses from different laboratories would be classified equally.

The variation across laboratories in the responses of each donor sample to CEF peptides is shown in Fig. 2B. In general, the responses from the different labs are similar, with a range of about half a log for each sample. However, the figure also shows that any response classification (a horizontal line drawn from any point on the y axis) would result in at least one sample falling into more than one category. In IAVI clinical trials, the definition of CEF positivity is a response of >38 SFC/10⁶ cells from multiple samples evaluated over time. By this definition, the data across laboratories show 2 samples (no. 4 and 13) with only negative responses, 2 samples (no. 11 and 14) with mostly negative responses, one sample (no. 9) with mostly positive responses, and the remaining 12 samples with all positive responses. In panel 1, five of the six lowest CEF responses were analyzed by the same operator. Further investigation revealed that the operator had previously used only fresh PBMC for ELISPOT assays and had little experience with thawing PBMC. Revised instructions and training on the

| Specimen type and site | No. of samples | % Recovery Mean | SD | Median percentile | 5th Percentile | 95th Percentile | % Viability Mean | SD | Median percentile | 5th Percentile | 95th Percentile |
|------------------------|---------------|----------------|----|------------------|---------------|----------------|-----------------|----|------------------|---------------|----------------|
| **Proficiency panel specimens** | | | | | | | | | | | |
| CLS                    | 34            | 77             | 23 | 75               | 50            | 120            | 91              | 3.9 | 91               | 82            | 95            |
| TRC                    | 16            | 65             | 25 | 64               | 35            | 121            | 95              | 2.1 | 96               | 90            | 98            |
| Core lab               | 34            | 91             | 25 | 83               | 60            | 142            | 95              | 2.7 | 96               | 89            | 98            |
| KAVI                   | 34            | 81             | 28 | 73               | 50            | 136            | 91              | 4.4 | 92               | 81            | 98            |
| NARI                   | 22            | 91             | 21 | 89               | 58            | 127            | 98              | 1.6 | 98               | 96            | 100           |
| Oxford                 | 33            | 88             | 27 | 78               | 56            | 130            | 94              | 3.4 | 96               | 86            | 98            |
| UVRI                   | 34            | 76             | 26 | 69               | 46            | 123            | 96              | 1.5 | 96               | 93            | 99            |
| **Total**              | 207           | 82             | 26 | 78               | 48            | 130            | 94              | 3.9 | 95               | 86            | 99            |
| **Clinical trial specimens** | | | | | | | | | | | |
| CLS                    | 512           | 62             | 33 | 60               | 20            | 120            | 90              | 7.5 | 91               | 75            | 98            |
| TRC                    | 431           | 61             | 18 | 60               | 33            | 93             | 93              | 4.4 | 93               | 86            | 98            |
| KAVI                   | 426           | 74             | 21 | 70               | 40            | 110            | 92              | 4.4 | 93               | 85            | 97            |
| NARI                   | 233           | 77             | 30 | 70               | 40            | 130            | 91              | 6.1 | 93               | 77            | 97            |
| Oxford                 | 121           | 58             | 21 | 60               | 20            | 90             | 93              | 7.3 | 95               | 82            | 98            |
| UVRI                   | 309           | 65             | 17 | 60               | 40            | 90             | 92              | 6.0 | 93               | 82            | 97            |
| **Total**              | 2,092         | 66             | 25 | 65               | 30            | 110            | 91              | 6.0 | 93               | 81            | 97            |
The use of cryopreserved PBMC were provided for the subsequent panels.

The variation across laboratories in the responses of each donor sample to CMV pp65 is shown in Fig. 2C. Again, the range of responses is about half a log per sample (except for sample 4 from panel 1), showing the consistency in results across labs. If CMV-positive responses are arbitrarily defined as those with >50 SFC/10⁶ cells, then the CMV results would be categorized as six negative samples, two borderline responders (samples 4 and 9), and nine positive samples.

PHA responses are shown in Fig. 2D. The majority are >1,000 SFC/10⁶ cells, as expected, except for some panel 3 responses of ≥450 SFC/10⁶ cells and four panel 1 responses of <108 SFC/10⁶ cells. Three of the low panel 1 responses were measured by the same operator who obtained five of the six lowest CEF responses.

**ELISPOT assay variation in each panel.** In IAVI trials, a typical analysis of ELISPOT data uses the mean count from replicate wells for each peptide on a plate. Since the number of replicates is generally small (usually three or four), the mean can easily be influenced by extreme values. Thus, as one of the criteria for defining positive responses, IAVI requires that the variation among the replicates be small relative to the mean. That is, the CV, defined as the SD divided by the mean, must not be greater than 70%. Since the three panels were conducted in sequence, with an interval of 6 to 9 months between each one, we wanted to investigate whether there was any change in the CV across quadruplicate wells. Typical examples are shown in Fig. 3. Clearly, as the mean spot counts increased, the CV decreased; it remained below 70% (in general ≤30%) for counts greater than 50 SFC per 10⁶ cells, and there was little variation among the three panels.

**Concordance between HIV type 1 vaccine trial responses from two laboratories.** The conducting of ELISPOT assay proficiency panels is a critical tool for ensuring comparable laboratory performances both within and across networks and also for identifying and troubleshooting reasons for differences, if they exist. When dealing with actual volunteer samples during a clinical trial, laboratory personnel may be under increased pressure from the prioritization of work, the time at which samples are drawn, or late changes to scheduled visits. Table 2 shows the viability and recovery of PBMC thawed at the proficiency panel laboratories for panel participation (also see Fig. 1) and at the IAVI Core for the assessment of immunological responses from clinical trial specimens. Excellent recovery and viability of the PBMC shipped to the sites and of the PBMC cryopreserved on site and then shipped to the IAVI Core were seen. One vaccine trial in which the same PBMC samples were tested both fresh following blood drawing on site at KAVI in Nairobi and frozen after shipment to the IAVI Core in London...
provided ideal data with which to compare and assess performances in real time under real conditions (J. Bwayo et al., unpublished results). Trial donor PBMC samples assessed in the two laboratories exhibited concordant responses (Spearman’s correlation coefficients ranged from 50 to 81% for six HIV peptide pools \([P < 0.05 \text{ for all but one}])

Despite the use of either freshly isolated or thawed cryopreserved PBMC, providing further assurance that proficiency panel data are useful to indicate actual trial performance. These two laboratories also had concordant results in the proficiency panels (Fig. 2 and 3). Figure 4 shows typical examples of IFN-γ ELISPOT assay responses to CEF and HIV peptide pools used in the trial. There is a slight trend toward fresh samples scoring higher than frozen samples for vaccine-induced responses (those to HIV peptide pools), in contrast to CEF responses, which likely represent memory T-cell responses to previous CMV and EBV exposure. In addition, the CEF response should be entirely CD8 restricted (8- to 10-mer peptides) whereas the responses to Env, Pol, and Nef are mediated by both CD4 and CD8 T cells (15-mer peptides).

**DISCUSSION**

The IAVI Core and partner laboratories regularly participate in ELISPOT assay internal proficiency panels and external quality assurance (EQA) panels, with the aim of comparing their abilities to process PBMC, to evaluate the CMV and CEF ELISPOT assay responses of donor samples, and to identify and rectify any technical issues. Using standardized equipment and instructions and SOPs, with the only difference being the method of cell counting, the laboratory teams conducting the three panels analyzed to date have yielded remarkably concordant ELISPOT assay results. We have shown that IAVI partner laboratories are able, in the majority of cases, to successfully categorize samples across a range of low, medium, and high spot counts, to achieve low background values, and to correctly identify nonresponders. Cell viability and recovery results were much tighter and the minimum recoveries were much higher than those reported previously. These laboratories, with one exception, had never done ELISPOT testing prior to its implementation to support IAVI-funded clinical trials.

These types of results have not been achieved in previous proficiency panels among laboratories across organizations, either in the HIV vaccine field or in other fields such as cancer research (4, 11). The ability to determine whether a response is either positive or negative is critical for assessing vaccine immunopotency, i.e., the ability to induce an immune response (7, 8). When multiple laboratories are able to categorize samples in a consistent manner, comparative assessment and decision making for multiple vaccine candidates become easier both across and within networks. The use of multiple laboratories will accelerate the testing of vaccines and, hence, vaccine development and will furthermore provide robust ELISPOT data capable of distinguishing different response rates and magnitudes. The salient details that enabled concordant performances across seven laboratories based on three continents, an objective not achieved previously, were the standardized methods employed and the operators’ familiarity with these methods (11). These included not only the methods and reagents used in the assay but also the ELISPOT assay reader model and settings, which are critical for counting spots with the same morphology (12). Methods of shipping, storage, thawing, and overnight resting of PBMC have been shown previously to affect measures of antigen sensitivity and assay performance (4, 6, 8, 17, 18, 27). Indeed, other panels have shown an improvement in sensitivity and general performance when some of these factors are standardized in successive panels (11).

In addition to the standardized methods used, another significant difference affecting performance in these panels versus other panels was the quality systems of participating laboratories. All IAVI partner laboratory personnel involved in the testing of IAVI- or other network-sponsored HIV vaccines undergo carefully integrated training, operate in a GCLP environment, and follow detailed SOPs that necessitate active
These aspects result in a highly controlled environment that may not be achieved in all laboratories. In support of this prospect, it is prudent to highlight that operator variation, a well-known factor in ELISPOT assay variability, was not of note in these panels (12), although consistent differences in ELISPOT assay counts from the two operators at one laboratory in panel 1 and another in panel 3 were obtained (data not shown). The background values observed in the panels described in this study were very low, with an overall mean of 6.6 SFC/10^6 cells (determined by excluding 3 samples, of the total of 323, which had >55 SFC/10^6 cells), whereas in other proficiency panels, numerous laboratories produced high background values that clearly affected the determination of positive responses (11). A possible explanation for high background levels may be the serum source. At IAVI, a standardized FCS is purchased in a large volume after prescreening to ensure that both low background and antigen-specific responses are supported.

Differences across laboratories with respect to viable cell counts were noted, even though the counts were obtained from the same donor PBMC isolated from the same blood draw. These differences were due most likely to the use of different automated counting equipment, some of which performed integrated viable cell counts and some of which did not. The number of laboratories per panel using each particular counter does not permit the statistical evaluation of this variable, though we note that the different viable cell counts did not in general correlate with the SFC values, indicating that perhaps differences in recovered cells were related to disparity in the numbers of cryopreserved PBMC per vial. In particular, cell counting should be standardized across laboratories and the use of automated counters should be encouraged. The cell-counting procedures for these automated counters can be validated and carried out under GCLP guidelines. We looked at the CV among replicate wells as a measure of the performances of those laboratories that participated in all three consecutive panels. No marked decrease or improvement in the CV was noted, probably due to the optimized methods in use since the first panel was conducted. This conclusion is supported by the values observed in the first panel, in which 36 (95%) of the 38 CEF and CMV ELISPOT assay counts in the range of 50 to 250 SFC/10^6 cells had a CV below 50%, which is low for a biological assay of low magnitude (16, 21).

Upon the review of the panel 1 data, it was revealed that one operator was inexperienced at thawing frozen PBMC and en-
countered difficulties. As a result, improved instructions relating to these aspects, often considered routine in most laboratories, were provided. In the subsequent panels, no difficulties with thawing were observed.\(^{13}\)

Regular independent quality assurance testing is a key component of the quality systems required for any test being conducted by IAVI-sponsored GCLP guideline-compliant laboratories. Given that there is no independent EQA program such as the United Kingdom National External Quality Assessment Service CD4 program for ELISPOT assays at present, the proficiency panel provides a step toward such assurances within the IAVI program. In addition, frozen samples from all clinical trial sites are routinely shipped to the IAVI Core in London for independent testing. There remains a need across multiple programs for EQA panels.

The disappointing lack of efficacy of the Merck adenovirus-based HIV vaccine candidate led previously to a discussion concerning the utility of the IFN-γ ELISPOT assay \((26)\). It is worth noting that the performance and robustness of this assay continue to make it a valid assay of T-cell vaccine immunopotency in early clinical development \((7)\). This paper provides encouraging evidence that when applied using standardized methods, the ELISPOT assay is sensitive and discriminatory and that highly concordant results can be obtained across laboratories located on three different continents. This finding is encouraging for multicenter vaccine trials across disciplines and also for the possibility of obtaining comparable results in the detection and discernment of cellular immune responses of differential magnitudes.

**ACKNOWLEDGMENTS**

This work was made possible with funding from IAVI, including funding through USAID cooperative agreement number GPO-A-00-06-00005-00. The contents of this paper are the responsibility of IAVI and do not necessarily reflect the views of USAID or the U.S. government.

We acknowledge N. Baskaran and T. Sekar from the TRC site.

**REFERENCES**

1. Betts, M., J. Casazza, and R. Koup. 2001. Monitoring HIV-specific CD8+ T cell responses by intracellular cytokine production. Immunol. Lett. 79:117–125.

2. Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. 3:205–211.

3. Britten, C. M., S. Janetzki, S. H. van der Burg, C. Gouttefangeas, and A. Hoos. 2008. Toward the harmonization of immune monitoring in clinical trials: quo vadis? Cancer Immunol. Immunother. 57:285–288.

4. Cox, J. H., G. Ferrari, S. Kalams, W. Lapczynski, N. Oden, and M. P. D’Ouza. 2005. Results of an ELISPOT proficiency panel conducted in 11 laboratories participating in international human immunodeficiency virus type 1 vaccine trials. AIDS Res. Hum. Retrov. 21:68–83.

5. Currier, J., E. Kuta, E. Turk, L. Earhart, L. Loomis-Price, S. Janetzki, G. Ferrari, D. Birx, and J. Cox. 2002. A panel of MHC class I restricted viral peptides for use as a quality control for vaccine trial ELISPOT assays. J. Immunol. Methods 268:157–172.

6. Disis, M. L., C. dela Rosa, V. Goodell, L. Y. Kuan, J. C. Chang, K. Kuus-Reichel, T. M. Clay, H. Kim Lyerly, S. Bhatia, S. A. Ghanekar, V. C. Maino, C. Delarosa, and M. L. Disis. 2003. Moving to HIV-1 vaccine efficacy trials: defining T cell phenotype assays and QAs. Cancer Immunol. Immunother. 52:2748–2878.

7. D’Souza, M. P., and M. Alfeld. 2008. Measuring HIV specific T cell immunity: how valid are current assays? J. Infect. Dis. 197:337–339.

8. Dubey, S., J. Clair, T. M. Fu, L. Guan, R. Long, R. Mogg, K. Anderson, K. B. Collins, C. Gaunt, V. R. Fernandez, L. Zhu, L. Kierstead, S. Thaler, S. G. Gupta, W. Straus, D. McHortia, T. W. Tobery, D. R. Casimiro, and J. W. Shiver. 2007. Detection of HIV vaccine-induced cell-mediated immunity in HIV-tergencrative clinical trial participants using an opti-
26. Sekaly, R. P. 2008. The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? J. Exp. Med. 205:7–12.
27. Smith, J. G., H. R. Joseph, T. Green, J. A. Field, M. Wooters, R. M. Kaufhold, J. Antonello, and M. J. Caulfield. 2007. Establishing acceptance criteria for cell-mediated-immunity assays using frozen peripheral blood mononuclear cells stored under optimal and suboptimal conditions. Clin. Vaccine Immunol. 14:527–537.
28. Stiles, T., V. Grant, and T. Mawbey. 2003. Good clinical laboratory practice (GCLP). A quality system for laboratories that undertake the analyses of samples from clinical trials, p.1–18. British Association of Research Quality Assurance, Ipswich, United Kingdom.
29. Tobery, T. W., S. A. Dubey, K. Anderson, D. C. Freed, K. S. Cox, J. Lin, M. T. Prokop, K. J. Sykes, R. Mogg, D. V. Mehrtra, T. M. Fu, D. R. Castimiro, and J. W. Shiver. 2006. A comparison of standard immunogenicity assays for monitoring HIV type 1 gag-specific T cell responses in Ad5 HIV type 1 gag vaccinated human subjects. AIDS Res. Hum. Retrovir. 22:1081–1090.
30. Vuola, J. M., S. Keating, D. P. Webster, T. Berthoud, S. Dunachie, S. C. Gilbert, and A. V. S. Hill. 2005. Differential immunogenicity of various heterologous prime-boost vaccine regimens using DNA and viral vectors in healthy volunteers. J. Immunol. 174:449–455.
31. Whiteside, T. L., Y. Zhao, T. Tsukishiro, E. M. Elder, W. Gooding, and J. Baar. 2003. Enzyme-linked immunospot, cytokine flow cytometry, and tetramers in the detection of T-cell responses to a dendritic cell-based multipeptide vaccine in patients with melanoma. Clin. Cancer Res. 9:641–649.