Standardization of Sensitive Human Immunodeficiency Virus Coculture Procedures and Establishment of a Multicenter Quality Assurance Program for the AIDS Clinical Trials Group

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An independent quality assurance program has been established by the Division of AIDS, National Institute of Allergy and Infectious Diseases, for monitoring virologic assays performed by nearly 40 laboratories participating in multicenter clinical trials in the United States. Since virologic endpoints are important in evaluating the timing and efficacy of therapeutic interventions, it is imperative that virologic measurements be accurate and uniform. When the quality assurance program was initially created, fewer than 40% of the laboratories could consistently recover human immunodeficiency virus (HIV) from peripheral blood mononuclear cells (PBMCs) of HIV-infected patients. By comparing coculture procedures in the more competent laboratories with those in laboratories who were struggling to isolate virus, optimal conditions were established and nonessential reagents and practices were eliminated. Changes were rapidly introduced into a laboratory when experience dictated that such modifications would result in a favorable outcome. Isolation of HIV was enhanced by optimizing the numbers and ratios of patient and donor cells used in cultures, by standardizing PBMC separation procedures, by using fresh rather than frozen donor PBMCs, by processing whole blood within 24 h, and by using natural delectinated interleukin 2 instead of recombinant interleukin 2 products in existence at that time. Delays of more than 8 h in the addition of phytohemagglutinin-stimulated donor cells to freshly separated patient PBMCs reduced recovery. Phytohemagglutinin in cocultures and the addition of Polybrene and anti-human alpha interferon to media were not important in HIV isolation. The introduction of a consensus protocol based on this information brought most laboratories quickly into compliance. In addition, monthly monitoring has successfully maintained proficiency among the laboratories, a process that is critical for the scientific integrity of collaborative multicenter trials. Problems which might not be appreciated for months are now being resolved early, before data can be compromised unknowingly. This consensus protocol is recommended for any laboratory attempting to isolate HIV for the purpose of standardizing recovery and for accessing virologic endpoints in clinical trials.

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Contemporary virology laboratories perform many functions, including virus isolation, molecular virology, and immunodiagnosis. As part of a clinical service, a laboratory must maintain competence in each of these areas if accurate clinical information is to be provided to clinicians. It is assumed that standardized tests performed strictly according to protocol will produce the same results in all laboratories. However, for many assays, there is little information on interlaboratory variation. The consequences of such interlaboratory variation in clinical practice are unknown, but the variation could result in different management strategies for similar patients in different hospitals. The problem is magnified when products or procedures have not been licensed or are in the evolutionary (research) stage of development by a commercial company.

For laboratories involved in multicenter clinical trials, validation of assay results across institutions is essential if comparability and uniformity of data are to be achieved. This can be accomplished through a quality assurance (QA) program that assesses all aspects of quality control, including specimen collection and storage, instrumentation, the quality of assays and standards, the appropriateness of negative and positive controls, technical proficiency, and the mechanisms used by the laboratory to interpret and report results for data analysis. Both internal quality control and external multicenter proficiency-testing programs for monitoring interlaboratory variability are important.

In 1988, the Division of AIDS of the National Institute of Allergy and Infectious Diseases (NIAID) established an independent Virology Reference Laboratory (VRL) at Baylor College of Medicine in Houston, Tex., for the purpose of developing and implementing a system-wide quality control program for virologic measurements related to the human immunodeficiency virus (HIV). This program was to complement the multicenter AIDS Clinical Trials Group (ACTG) involved in national drug treatment trials. From its inception, the VRL recognized that the AIDS diagnostic field was in a state of flux. New assays were being developed at a rapid pace at the same time that existing methods were being fine-tuned. While clinical investigators focused on which dosage schedule, combination of drugs, or new therapeutic modalities represented the best means for controlling HIV infection, virologists were exploring those approaches that might provide appropriate, objective models for quantifying the antiviral response. Decisions concerning which virologic endpoints to use and how to control interlaboratory variations needed to be addressed.

Isolation of HIV from clinical specimens was the first QA program to be established by the ACTG, since the assessment of virologic endpoints following therapy is a desirable goal of clinicians. This report describes the development by the ACTG of the NIAID of a consensus protocol for optimizing the isolation of HIV from clinical specimens and the establishment of an ongoing quality control program.

MATERIALS AND METHODS

Prestandardization assessment. Whole-blood specimens (15 ml) obtained from HIV-infected individuals were mailed every month for 6 months from the virology laboratory at Memorial Sloan-Kettering Cancer Center to each of the 19 original ACTG virology laboratories for processing and coculturing. Data were collected on reporting forms and were analyzed for positive and negative agreement as well as for the time needed to obtain a positive result.

Development of HIV coculture questionnaire. To assist

those laboratories that were unable to consistently isolate HIV from peripheral blood mononuclear cells (PBMCs), a questionnaire prepared by the VRL was distributed to all ACTG virology laboratories (Fig. 1). Data from this questionnaire dealt with four major areas of concern: (i) processing of patient cells, (ii) processing and stimulation of donor PBMCs, (iii) the coculture procedure, and (iv) preparation of culture supernatants for HIV (p24) antigen enzyme immunoassay (EIA) testing. Data from 11 laboratories, referred to as the reference laboratories, that consistently were able to isolate virus in a pilot QA program were compared with information provided by 8 laboratories less successful in their attempts to isolate virus. Eleven other laboratories whose abilities to isolate virus were unknown were also invited to submit answers to the questionnaire.

Development of a multicenter QA program. During the evolution of a consensus protocol for the isolation of HIV from PBMCs, a QA program was initiated by the VRL for the ACTG. On the first Monday of each month, 420 to 450 ml of blood was collected in citrate-phosphate-dextrose (CPD) (anticoagulant) from each of three or four volunteers, aliquoted into 10-ml vacuum tubes, and shipped at ambient temperature by an overnight carrier to each ACTG laboratory. As a control, samples also were shipped to the VRL under identical conditions, and a comparison was made with cultures performed with fresh material. Volunteers were recruited from persons who were EIA anti-HIV and Western blot (immunoblot) positive, EIA anti-HIV positive but Western blot negative or indeterminate, or anti-HIV and Western blot negative. Hematologic parameters were assessed before the patients were enlisted for this service. To preserve the serologic identities of QA samples, randomized negative samples were sometimes spiked with HIV p24 antibody or HIV viral lysate, and some seropositive samples were intentionally inactivated. Each QA sample was processed individually, and the recovered PBMCs were used in their entirety for HIV isolation.

Shipping specifications. Blood samples were individually wrapped in absorbent material sufficient to retain all fluid should the tube break. The individually wrapped samples were placed in a Dual-Trans double mailing container (Baxter Healthcare Corp., McGaw Park, Ill.) that conforms with the Interstate Quarantine Regulation for Etiologic Agents (42 CFR, Part 72.25). This container is a combination of a seamless aluminum screw-cap inner container and a fiberboard screw-cap mailing case. The cap of the inner container is lined to prevent leakage. The outer cap is sealed with vinyl tape, and then the double mailing container is transferred to a heavy-duty, 4.5-mil, Kapak heat-sealable polyester-polyolefin transparent pouch that is sealed with an electrical sealer prior to being wrapped in bubble wrap and placed inside a sturdy corrugated fiberboard double-wall mailing package (Lawrence Packaging Supply Corp., Newark, N.J.). This packaging exceeds the new requirements by Federal Express, D.O.T. 173.387, and UN 6.13 (a and b).

Data collection and analysis. All virology laboratories participating in the ACTG are equipped with a data management-transmission program called the Retrovirus Laboratory Management Program developed by Dataworks Development, Inc. (Seattle, Wash.), specifically for the ACTG virology laboratories. Besides maintaining patient information, the program is capable of collecting the data directly from an EIA plate reader, analyzing it, and placing it into the appropriate patient files. Through this program, the QA data files are exported to a data collection site, Frontier Science

Processing of Patient PBMCs**Preparation of PBMCs:**

- Volume of blood processed and anticoagulant used?
- Is processing done the same day or next day?
- Is plasma removed and/or blood diluted before processing?
- Diluent used and dilution prepared?

Centrifugation specifications:

- Size of centrifuge tube and volume of diluted blood added?
- Brand and volume of lymphocyte separation solution used?
- Centrifugation temperature, time and g-force?
- Number of washes and wash reagent?

Processing and Stimulation of Donor PBMCs**Donor PBMC specifications:**

- Are normal donor PBMCs added to culture?
- Source of PBMCs, e.g. random, fresh or frozen and anticoagulant used?
- Are PBMCs from different donors pooled?
- Interval between collection of blood and processing?
- Is donor anti-HIV status known?
- Are donor cells cultured independently for HIV?
- Interval between stimulation of donor PBMCs and cocultivation?

Medium used for stimulation of donor PBMCs:

- Fetal bovine serum concentration?
- PHA-P and IL-2; brand and concentration?
- Polybrene concentration (if used)?
- Antibacterial and antifungal agent(s); brand and concentration (if used)?

Tissue culture features:

- Size of tissue culture flask and volume of media added?
- Concentration of donor PBMCs?

HIV Cocultivation Procedure**Cocultivation medium:**

- (See questions for donor medium)
- Anti-human alpha-interferon concentration (if used)?

Tissue culture features:

- Size of tissue culture flask and volume of media added?
- Concentration of donor and patient PBMCs?
- Donor-to-Patient PBMC ratio?

Incubation details:

- Are excess patient PBMCs cryopreserved?
- Are stimulated donor PBMCs added to flask within 8 hours?
- Incubation temperature and CO₂ concentration?
- Are fresh or frozen donor PBMCs added later to coculture?
- Are PBMCs from same donor used?
- Interval for adding supplemental donor PBMCs?
- Are coculture PBMCs cryopreserved? How often?

Preparation of Supernatant for HIV (p24) Antigen Assay**Sample processing:**

- Is sample clarified by centrifugation?
- Is supernatant frozen before being assayed?

Centrifugation specifications:

- Volume clarified; centrifugation temperature, time and g-force?

FIG. 1. Questionnaire for laboratories isolating HIV from whole blood.

(Buffalo, N.Y.), and then retrieved by Research Triangle Institute (Research Triangle Park, N.C.) for analysis.

HIV cocultures were initially assessed for positive-negative agreement (see below) on the basis of an 80% agreement among the reference laboratories. In addition to providing the correct answer, a laboratory's coculture results also were evaluated for sensitivity. The criteria for a positive coculture and determination of the days to first positive were based on HIV p24 antigen results obtained from biweekly culture samples according to the following schema: (i) two consecutive HIV p24 antigen values of ≥ 30 pg/ml, of which the second value is at least four times greater than the first value or is out of range (see below); (ii) two consecutive HIV p24 antigen values that are out of range; or (iii) three consecutive increasing HIV p24 antigen values of ≥ 30 pg/ml, where neither consecutive value is four times or more that of the previous sample but the third value is at least four times greater than the first.

Out of range means that the HIV p24 antigen EIA result is greater than the uppermost point on the assay's standard curve. In case i or case ii (above), the first day that the HIV p24 antigen value was ≥ 30 pg/ml was considered the day to first positive. For case iii (above), the days-to-first-positive value was that associated with the intermediate positive sample.

HIV antigen kits utilized. Because of the large quantities of HIV p24 antigen synthesized during replication, precision is less important in defining a positive culture than it is for determining changes in serum levels following treatment. An HIV p24 antigen QA program established by the VRL uses common reagents for a standard calibrator curve and quality control reagents at two concentrations to monitor inter- and intralaboratory variations. Both licensed and unlicensed (research) antigen capture kits from Abbott (Abbott Laboratories, Abbott Park, Ill.); E. I. du Pont de Nemours & Co., Inc. (Boston, Mass.); Cellular Products, Inc. (Buffalo, N.Y.); and Coulter Immunology (Hialeah, Pa.) were purchased by the laboratories.

RESULTS AND DISCUSSION

Analysis of survey questionnaire for isolation of HIV. (i) **Processing of patient cells.** The volume of blood from an HIV-infected patient that is processed is relevant, since the number of infected cells existing in a coculture is directly proportional to the volume of blood used. Most adult ACTG virology units queried were initially processing 30 ml of whole blood (range, 15 to 60 ml). At this volume, the potential suppressive effect of CD8 cells in the lymphocyte population (7, 12, 19, 20) appears to be of limited consequence (16, 18), since isolation of HIV from all confirmed seropositive patients is being accomplished regardless of the patient's clinical status. Subsequent studies by the VRL have shown that PBMCs obtained from 7.5 ml of blood also are sufficient to ensure isolation of HIV from virtually all HIV-infected patients.

The type of anticoagulant used to collect blood for the isolation of HIV from PBMCs is probably not important. Nevertheless, all but 2 of the 30 centers used heparin as the anticoagulant for their patients' blood samples. Among the 11 reference centers, 9 used sodium heparin, and 2 used lithium heparin at concentrations ranging from 14 to 19 U/ml. For donor cells, eight of the reference laboratories used blood collected in citrate-phosphate-dextrose or acid-citrate-dextrose solution, and three used heparinized blood.

In contrast to the PBMC findings, heparinized blood for

the direct isolation of HIV from whole blood has been found to result in poor recovery of virus (2) and thus should not be employed. Similarly, heparin should be avoided when the polymerase chain reaction procedure is being contemplated for the detection of HIV RNA in plasma, since heparin can inhibit cell-free protein translation *in vitro* (6). For each of these situations, it is recommended that samples be collected in EDTA, citrate-phosphate-dextrose, or acid-citrate-glucose. Conversely, attempts to isolate HIV from plasma containing anticoagulants that inhibit divalent cations often result in the development of fibrin clots when the sample is added to medium containing calcium. Thus, decisions concerning the appropriate use of an anticoagulant depend on the ultimate purpose for which the sample is being collected.

All but 1 of the 11 reference laboratories processed patient or donor cells on the day of collection. However, when time constraints intervened, some of these laboratories processed their samples the next day. To ascertain whether delays might affect coculture results, the VRL examined 69 samples from HIV-infected persons immediately after collection and after 24 h of storage at ambient temperatures. The mean number of days required to isolate HIV was 6.97 ± 2.28 for the fresh cocultures and 7.04 ± 2.30 for cocultures that were not started until 24 h after collection. When individual pairs were compared, virus was detected earlier in 13 of the fresh cultures and later in 15, while in 41 of the paired cultures, virus was isolated the same day. Thus, delays up to 24 h at ambient temperature did not appreciably affect recovery.

Removal of plasma from centrifuged whole blood ($200 \times g$ for 10 min at 20 to 24°C) prior to processing PBMCs also does not appear to affect recovery. About half of the 11 reference laboratories elected to do this because it provided a plasma sample for other studies. All but 6 of the 24 centers diluted the remaining whole blood from 1:2 to 1:3 prior to processing, with the vast majority using a 1:2 dilution. When this option was selected, plasma removal proceeded prior to dilution of the residual blood sample. The diluent employed did not appear to be important unless citrate-phosphate-dextrose or acid-citrate-dextrose was used, in which case a calcium-free diluent was essential to avoid clot formation. Most of the centers used phosphate-buffered saline (PBS) or a balanced salt solution (without calcium). Two of the reference laboratories used saline. The brand of lymphocyte separation solution (LSS) used also did not appear to be important. Among the reference laboratories, four used LSS from Organon, five used LSS from Pharmacia, and two used LSS from Sigma.

Recovery of PBMCs from the LSS gradient must be optimized to ensure that an adequate number of cells are obtained for coculture. Thus, conditions of time, temperature, *g* force, and volume of LSS and blood used must be standardized. As much as a 10-fold difference in cell recovery was observed among the various laboratories when comparable samples of blood were processed. Superior recovery was observed when a ratio of 4 parts blood was layered over 3 parts LSS, e.g., 16 ml of undiluted or diluted blood to 12 ml of LSS. However, up to three times more blood than LSS is acceptable. Most laboratories used a 50-ml conical centrifuge tube for sedimenting their cells. Six reference laboratories centrifuged the blood at 24 to 26°C, three used 20 to 21°C, and one used 37°C. The *g* force and time selected for centrifugation also were quite variable. To achieve standardization, 30 min at $400 \times g$ and 20 to 24°C was selected. Following removal of the PBMCs at the gradient-plasma (or diluent) interface, the cells were washed. The number of washes did not appear to be critical.

Seven of the reference laboratories used two washes, and four used three washes.

(ii) **Processing of donor cells.** Donor PBMCs should be obtained from the buffy coat of random, seronegative donors and processed within 12 h of collection. A delay of 24 h is inadvisable, because recovery is then suboptimal. Some laboratories pooled buffy coats from more than one individual, but most did not. The use of frozen donor PBMCs (1, 4), either to initiate a coculture or to add later, markedly reduced recovery of virus. Therefore, such cells should not be used. All but four of the centers were aware of the anti-HIV status of their donors prior to culturing the cells. Although it is recommended that donor PBMCs be cultured separately to confirm seronegativity, no laboratory has reported such an occurrence.

All laboratories but one used RPMI 1640 medium containing glutamine and 10 to 20% heat-inactivated fetal bovine serum. In addition, all laboratories stimulated their donor cells with phytohemagglutinin (PHA-P), usually purchased from Difco or Sigma. This purified plant lectin provides the stimulus for initiating a proliferative T-cell response (10) through the generation of interleukin 2 (IL-2) receptors and the synthesis of IL-2 (3, 9, 14). The median and mode PHA-P concentration used by the reference laboratories was 5 $\mu\text{g/ml}$ (range, 0.2 to 50 $\mu\text{g/ml}$). High concentrations of PHA-P were clearly detrimental. Nine of the 11 reference laboratories used natural, delectinated IL-2 obtained from Cellular Products; Electro-Nucleonics, Inc. (Silver Springs, Md.); or Boehringer Mannheim Corp. (Indianapolis, Ind.). Concentrations ranged from 2 to 10%, with a median of 5%. It is noteworthy that two of the reference laboratories did not use IL-2 in their donor stimulation medium if the cells were to be used within 24 h of culturing. After 24 h, these laboratories washed the donor PBMCs in Hanks balanced salt solution and replaced the PHA-P stimulation medium with growth medium containing IL-2 but no PHA-P. This additional step seems unwarranted, since recovery was not facilitated. In retrospect, the addition of IL-2 to the donor stimulation medium was not judged to be necessary if the cells are used within 48 h of collection. The use of recombinant IL-2 consistently resulted in suboptimal rates of HIV recovery and therefore is not recommended (see below).

The addition of Polybrene to the donor stimulation medium did not appear to affect recovery of HIV from the patient PBMCs. Only 3 of the 11 reference laboratories used Polybrene. Similarly, none of the laboratories used anti-human alpha interferon in the donor stimulation medium. Thus, both reagents can be excluded from this medium without affecting recovery during the cocultivation phase. Most laboratories used a combination of penicillin and streptomycin or gentamicin alone as the antibiotic of choice. No preference for one or the other could be established. Eight of the 30 laboratories (but only 1 of the reference centers) added an antifungal agent (amphotericin B) to their donor cultures at concentrations ranging from 0.25 to 2.5 $\mu\text{g/ml}$ (median, 1.25 $\mu\text{g/ml}$). While no specific detrimental effect on virus recovery was observed at these facilities, the use of an antifungal agent is not recommended, since HIV expression may be inhibited, resulting in a false-negative response if the virus burden is low (8, 11, 15).

The concentration of donor PBMCs per milliliter of medium for the reference laboratories ranged from 0.6×10^6 to 3×10^6 , with a median of 2×10^6 PBMCs per ml. Eight of the reference laboratories routinely used PHA-stimulated donor cells within 2 days of initiating culture. The remaining centers used them on the third day. Recovery of HIV from

patient PBMCs was diminished in those laboratories that cultivated donor cells for more than 3 days before adding them to freshly processed patient PBMCs.

(iii) **Coculture procedures.** The medium used by the reference laboratories for the cocultivation procedure was RPMI 1640 containing glutamine and 10 to 20% heat-inactivated fetal bovine serum. Only two of the reference laboratories used PHA-P in the cocultivation medium, implying that PHA-P is not essential for the eventual recovery of HIV. This is presumably because a significant proportion of T cells from infected patients are already activated and contain sufficient IL-2 receptors (5) or because a mixed lymphocyte reaction induced by the donor cells provides sufficient stimulation for these cultures. However, in assessing this issue, it also should be noted that some of the laboratories simply removed supernatant from the stimulated donor PBMCs prior to adding them to the patient cells. Thus, residual lectin present in the medium covering the cells may have been transferred to the coculture, where it exerted a stimulatory effect, leading to an increase in virus production (17, 21).

In contrast to the superfluous role of PHA-P in the coculture medium, IL-2 is an essential ingredient. As previously stated, the use of natural, delectinated IL-2 at a 5% concentration appeared to enhance HIV recovery, whereas isolation rates were substantially reduced when recombinant IL-2 was used. Only 5 of 11 reference laboratories used Polybrene (1 to 10 $\mu\text{g/ml}$, with a mode of 2 $\mu\text{g/ml}$), and only 2 of 11 used anti-human alpha interferon (0.4 to 200 U/ml) in the cocultivation medium. Thus, as noted above, these two products did not appear to be necessary for HIV recovery. Either penicillin (100 U/ml)-streptomycin (100 $\mu\text{g/ml}$) or gentamicin (50 $\mu\text{g/ml}$) was added to the medium at the concentrations described above. Only one reference laboratory added amphotericin B (1.25 $\mu\text{g/ml}$) to the PBMC coculture medium.

The total number of patient PBMCs in each coculture ranged from 6×10^6 to 120×10^6 cells, with a median of 10×10^6 PBMCs. The ratio of donor PBMCs to patient PBMCs ranged from 2:1 to 1:6, with a median ratio of 1 part donor cells to 2 parts patient PBMCs (1:2). As previously indicated, some laboratories elected to wash their donor PBMCs in PBS or Hanks balanced salt solution prior to use, while other centers did not. This step does not appear to be necessary. The initial concentration of PBMCs (donor plus patient) ranged from 0.3×10^6 to 5.6×10^6 cells per ml, with a median of 1.5×10^6 PBMCs per ml for the reference laboratories, e.g., 5×10^6 donor cells plus 10×10^6 patient cells in 10 ml of medium. However, the use of equivalent concentrations of donor and patient PBMCs appeared to enhance isolation. A delay of more than 8 h before the addition of PHA-stimulated donor cells to the patient PBMCs appears to significantly reduce recovery of HIV, especially if the interval exceeds 2 days.

All but two laboratories subsequently added fresh PHA-stimulated donor cells to the cocultures every week. At all but one center, PBMCs from different donors were employed. Those centers that used frozen PBMCs and/or added donor cells less frequently than every week were less successful in isolating HIV. Recovery was improved if approximately half of the coculture medium was removed every 3 to 4 days and replaced with an equal volume of fresh coculture medium. The supernatant collected from the culture can be assayed for HIV (p24) antigen either in the fresh state or after being frozen. Clarification of the supernatant

- A. **Processing of Donor and Patient PBMCs**
 1. Centrifuge anticoagulated blood at 200 x g for 10 minutes at 20 to 24°C and remove most of the plasma which should be aliquoted and frozen.
 2. To one part centrifuged blood, from which most of the plasma has been removed, add one part diluent (saline or PBS) e.g., 8 mL centrifuged blood to 8 mL diluent.
 3. Layer 4 parts of diluted blood over 3 parts lymphocyte separation solution (LSS: Organon or Pharmacia) e.g., 16 mL diluted blood over 12 mL LSS.
 4. Centrifuge at 400 x g for 30 minutes at 20 to 24°C. Remove PBMCs and wash twice in two volumes of PBS or Hanks BSS.
 5. Enumerate cells and adjust sample with culture medium to achieve a concentration of 2 million cells per mL.

- B. **Stimulation of Donor PBMCs**
 1. Stimulation Medium for donor PBMCs:
 - a. RPMI 1640 with glutamine; 20% fetal bovine serum (heat-inactivated); PHA-P (5 µg/mL, Difco or Sigma); 3% IL-2 (Cellular Products, Electro-Nucleonics, or Boehringer Mannheim); penicillin (100 units/mL)/streptomycin (100 µg/mL) or gentamicin (50 µg/mL).
 2. IL-2 should be a purified, delectinated human preparation, not a recombinant product. Amphotericin B should be avoided as it may inhibit HIV replication.
 3. Do not use frozen donor cells or pooled donor PBMCs.
 4. Process random donor buffy coats within 12 hours of collection.
 5. Maximum PBMC concentration in stimulation culture: 2 million PBMCs/mL.
 6. Use only anti-HIV negative donors; culture donor PBMCs separately to verify absence of HIV infectivity.

- C. **HIV Coculture Procedure**
 1. Coculture Medium for HIV isolation:
 - a. RPMI 1640 with glutamine; 20% fetal bovine serum (heat-inactivated); 5% natural, delectinated IL-2; penicillin/streptomycin or gentamicin (concentrations cited above).
 2. Coculture patient PBMCs within 8 hours of processing.
 3. Sediment 1 to 3 day old stimulated donor PBMCs at 200 x g for 10 minutes at 20 to 24°C, remove and discard supernatant, then resuspend cells in coculture medium and enumerate cells. Adjust sample with coculture medium to a concentration of 2 million PBMCs/mL.
 4. Maintain equivalent concentrations of donor PBMCs to patient PBMCs e.g., 10 million donor cells to 10 million patient cells in 10 mL medium (final PBMC concentration: 2 million PBMCs/mL).
 5. Remove one-half volume of coculture medium every 3 to 4 days and replace with an equal volume of fresh medium. The medium is saved at -30°C to -80°C for HIV p24 antigen determination.
 6. Once a week add 10 million, freshly pelleted and enumerated, 1 to 3 day old PHA-stimulated donor cells (2 million PBMCs/mL), resuspended in coculture medium.

- D. **Assaying Medium for HIV (p24) Antigen to Determine HIV Replication**
 1. Fresh or frozen medium may be tested and clarification is optional.

FIG. 2. Consensus culture protocol (developed by the VRL for the Division of AIDS of the NIAID from protocols used by the ACTG laboratories). BSS, balanced salt solution.

by centrifugation does not appear to affect the overall assay results.

Consensus protocol. As a direct result of the questionnaire, which was supplemented by an analysis of culture data from all laboratories, certain features were found to clearly enhance the isolation of HIV from PBMCs and improve proficiency among the laboratories. For example, a finite number of donor and patient cells were needed for optimal

recovery of virus in cocultures. Centrifugation parameters and volume of lymphocyte separation medium employed in processing whole blood were standardized. Delays of up to 24 h prior to processing cells, volume of blood processed, anticoagulant used, or brand of separation medium selected were found not to influence recovery. Data indicated that donor cells from single, random, seronegative donors should be processed within 12 h of collection and stimulated with

TABLE 1. Effect of shipping conditions on time of recovery of HIV from whole blood

Specimen	Time (days) to recovery of HIV following shipment at (°C) ^a :		Difference (days) observed (24°C vs 6°C)
	24 ± 4	6 ± 4	
MD1	21	14	+7
MD2	24	21	+3
MD3	21	18	+3
MD4	36	26	+10
MD5	Neg	36	+≥3
MD6	Neg	Neg	Unknown
MD7	22	15	+7
MD8	8	8	0
MD9	Neg	Neg	Unknown

^a Days in culture to initial positive result by the reverse transcriptase enzyme assay (13). Neg, negative at day 36 (culture terminated).

PHA-P and IL-2. Correspondingly, fresh PHA-stimulated donor cells should be added weekly to the cocultures. We also found that frozen cells and recombinant IL-2 products commercially available at the time of this study should not be used in cocultures, that Polybrene and anti-human alpha interferon were not essential, and that the use of antifungal agents in the medium should be discouraged. Equivalent concentrations of donor and patient PBMCs were recommended for the coculture procedure, and medium requirements were similar to those needed to maintain donor cells except that PHA-P was not essential. Data showed that recovery of virus was substantially reduced if the addition of PHA-stimulated donor cells to freshly processed patient cells was delayed for more than 8 h.

On the basis of this information, a consensus protocol for HIV isolation by coculture was formulated (Fig. 2). The protocol was validated at the VRL, which found no significant difference in the days-to-first-positive results obtained for HIV-infected patient samples tested using both its existing HIV coculture system and the consensus protocol (data not shown). The method was then distributed to all the participating ACTG virology laboratories. This consensus protocol is recommended for any laboratory attempting to

TABLE 2. Scoring system for evaluating QA HIV cultures

Numerical score	Description
0	Shipping problem
1	2-5 days to first positive
2	6-8 days to first positive
3	9-12 days to first positive
4	13-15 days to first positive
5	16-19 days to first positive
6	20-22 days to first positive
7	23-26 days to first positive
8	27-29 days to first positive
9	Indeterminate result when sample is consensus positive
10	False negative, contamination, or technical error when sample is consensus positive
15	Indeterminate result, contamination, or technical error when sample is consensus negative
20	False positive when sample is consensus negative or no data submitted

TABLE 3. HIV coculture performance of ACTG laboratories over time

Evaluation interval (mo/yr)	Avg no. of laboratories	% of laboratories with certification status of:		
		Certified	Provisional	Probation
4/87-7/88	25	54	24	22
8/88-2/89	34	61	19	20
3/89-9/89 ^a	36	75	10	15
10/89-4/90	37	84	9	7
5/90-12/90	38	88	5	6

^a Consensus protocol was introduced during this evaluation interval.

isolate HIV for the purpose of standardizing recovery and for assessing virologic endpoints in clinical trials.

Quality control program. (i) Shipping and virus stability. Optimal conditions for the interstate shipment of whole blood by overnight mail were investigated. Two sets of samples from nine anti-HIV EIA- and Western blot-positive subjects were collected in heparin by the Maryland Medical Laboratory, Inc. (courtesy of William Meyer III). These samples were sent overnight by Federal Express to the VRL in Houston. One set was mailed under ambient conditions (24 ± 4°C). The other set was packaged with frozen Koolit refrigerant in a styrofoam container in such a way that the temperature was maintained between 2 and 10°C during shipment. Upon arrival at the VRL in Houston, PBMCs were separated from each sample and placed in culture. Results of this study are shown in Table 1. Samples which had been shipped under refrigerated conditions became positive for reverse transcriptase (13) from 0 to 10 days earlier than those shipped under ambient conditions (median of 3 days, or 1 sampling period). This difference was not believed to be great enough to warrant adoption by the ACTG QA program, considering the increased cost that would be incurred by refrigerating the shipments.

(ii) Certification of laboratories. Until September 1990, all ACTG laboratories were evaluated monthly. A certification round consisted of QA coculture results from a 3- or 4-month evaluation period. The correct positive or negative result for each HIV QA coculture was based on an 80% consensus agreement among the reference laboratories (laboratories that had been certified for the three most recent consecutive certification periods). The positive or negative result, as well as the days to first positive, was identified for each culture by using the HIV p24 antigen criteria outlined above. Subsequently, a numeric score, based on a point system listed in Table 2, was assigned for each culture. An "ln mean score" for each laboratory was derived by transforming the numeric score for each culture to natural logarithms (ln), following which the mean of those ln was computed. A mean score (±1 standard deviation [SD]) was obtained for the reference laboratories (the triply certified laboratories excluding the top and bottom one or two laboratories, depending on the degree of variance).

The certification status of each laboratory is derived by determining how much their ln mean score deviates from the ln mean score of the reference laboratories. General guidelines used for evaluating culture data from each laboratory over a 3- or 4-month evaluation period or round were as follows: certified, <2.5 SD; provisional, 2.5 to 3.99 SD; probation, ≥4.0 SD. Thus, laboratories that deviated ≥4 SD from the ln mean score of the reference laboratories received probationary status for that round of cocultures.

(iii) **Performance of ACTG laboratories over time.** Virology laboratory proficiency for recovery of HIV from cocultures has increased significantly since the QA program was initiated. In 1987, only 38% of 21 ACTG virology laboratories successfully isolated HIV from each of six infected blood samples! Five other laboratories had an acceptable rate of recovery (>80%), but the interval to first positive response often was inordinately delayed, and three of these laboratories failed to isolate virus from one of the samples. The performance of eight other laboratories was less than satisfactory. Four were unable to detect virus in two of the six samples, and two laboratories recovered virus in only two samples. This initial evaluation emphasized the desperate need for a QA program designed to achieve consistent and optimal levels of isolation so that a more quantitative approach could eventually be developed. Table 3 compares the certification status for the various ACTG laboratories over five evaluation intervals from April 1987 through December 1990. The consensus protocol was introduced in the latter half of 1989. The percentage of laboratories fully certified to perform HIV cocultures increased from 54 to 88%, while the percentage of laboratories on probation steadily declined from 22 to 6%, a remarkable turnaround in proficiency. From this experience with HIV culture standardization and proficiency testing, we conclude that a similar approach is essential for monitoring newly introduced quantitative plasma and micrococulture assays, polymerase chain reaction assays, and HIV p24 antibody procedures for virologic endpoints in any clinical trial.

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