Sensitive Microculture Method for Isolation of Human Immunodeficiency Virus Type 1 from Blood Leukocytes

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A study was conducted to compare our standard culture with a new microculture procedure for isolation of human immunodeficiency virus type 1 (HIV-1) from blood leukocytes. A total of 137 blood specimens from 102 HIV-1 antibody-positive individuals (52 were asymptomatic, 31 were symptomatic, and 19 had AIDS) were cultured in a microculture system in which 10^6 of the patients' peripheral blood mononuclear cells (PBMC) were cocultured with 10^6 phytohemagglutinin (PHA)-stimulated PBMC from an HIV-1 antibody-negative blood donor in 1.2 ml of culture medium. Results were compared with those of a historical control group of 139 standard HIV-1 cultures from 108 HIV-1 antibody-positive subjects (58 were asymptomatic, 36 were symptomatic, and 14 had AIDS). For standard cultures, 10×10^6 of the patients' PBMC were cocultured with 5×10^6 PHA-stimulated PBMC from an HIV-1 antibody-negative blood donor in 15 ml of culture medium. HIV-1 was isolated in 128 (93%) microcultures and 133 (96%) standard cultures. Both methods identified more than 75% of the positive cultures within 7 days and 100% of the positive cultures within 14 days. The isolation rates for HIV-1 in microcultures compared with standard cultures were 91 versus 93% (specimens from asymptomatic individuals), 93 versus 96% (specimens from symptomatic individuals), and 97 versus 100% (specimens from patients with AIDS). The median time to positivity for both culture methods was 7 days, and this correlated significantly with symptoms and CD4⁺ cell counts. The microculture method is a sensitive and less expensive system for isolation of HIV-1 from PBMC of HIV-1 antibody-positive individuals, and we recommend it as the culture method of choice, especially for children and patients with AIDS and severe anemia or leukopenia whose blood volume is an important consideration.

Human immunodeficiency virus type 1 (HIV-1) has been identified as the causative agent of AIDS (1, 19, 20). The hallmark of AIDS is severe immunodeficiency resulting from quantitative depletion and functional abnormalities of the CD4⁺ T-cell subset (7, 9). In addition to CD4⁺ lymphocytes, HIV-1 also infects cells of the monocyte-macrophage lineage (12). Although early studies suggested that only a small proportion of the peripheral blood mononuclear cells (PBMC) of patients with AIDS were infected with the virus (10), more recent studies suggest that the proportion of infected cells is much higher, ranging from 1 cell in 50,000 PBMC in asymptomatic individuals to 1 cell in 400 PBMC in patients with AIDS (11).

HIV-1 has been isolated from a variety of tissues and body fluids (3, 6, 13, 14, 19, 22, 24). In our laboratory, we use a sensitive culture method (HIV-1 standard culture) for isolation of HIV-1 from PBMC of HIV-1 antibody-positive individuals. With this method, it is possible to isolate HIV-1 from 98% of HIV-1-seropositive asymptomatic individuals, 99% of patients with AIDS-related complex, and 100% of patients with AIDS (16, 17). One disadvantage of the standard culture method is that a relatively large amount of blood (30 ml) is required to obtain the 10×10^6 PBMC required for the culture.

On the basis of previous studies indicating that HIV-1 antibody-positive individuals harbor a high number of infected cells, we evaluated a microculture method for HIV-1 isolation that uses fewer PBMC from infected patients ($1 \times$

10^6 instead of 10×10^6 cells) and compared the results with those obtained by our standard culture technique for HIV-1.

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MATERIALS AND METHODS

Patients and specimens. Between February and July 1990, all blood specimens from study participants in National Institutes of Health-sponsored AIDS Clinical Trials Unit treatment protocols processed in the University of Minnesota HIV Laboratory were cultured for HIV-1 by using the microculture method described below. Results were compared with those of a similar number of standard HIV-1 cultures previously performed in our laboratory, which were used as historical controls. All study participants were HIV-1 seropositive and were classified by their clinical symptoms when blood specimens were drawn as asymptomatic, symptomatic (HIV-1-related symptoms in patients not fulfilling AIDS definition criteria), or having AIDS. Other data collected included absolute CD4⁺ cell count, CD4⁺/CD8⁺ ratio, anti-HIV-1 therapy, and number of weeks since enrollment in study protocols.

Standard HIV-1 cultures. PBMC were separated from 30 ml of heparinized blood on a Ficoll-Paque (Pharmacia, Inc., Piscataway, N.J.) gradient, and 10×10^6 cells were placed in 50-ml tissue culture flasks containing 15 ml of culture medium (RPMI 1640 medium, 20% fetal calf serum, 5% natural interleukin 2 [Cellular Products, Buffalo, N.Y.], penicillin [160 U/ml], streptomycin [160 µg/ml]), and cocultured with 5×10^6 phytohemagglutinin (PHA)-stimulated PBMC from an

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HIV-1 antibody-negative blood donor. Cocultures were incubated at 37°C in a 5% CO₂ atmosphere for a maximum of 42 days. Approximately 7 ml of supernatant above the settled PBMC was removed every 3 to 4 days for HIV-1 antigen detection and replaced with an equal volume of fresh culture medium. An additional 3×10^6 PHA-stimulated donor PBMC were added weekly. Culture supernatant fluids were tested for the presence of HIV-1 antigen with a commercially available enzyme immunoassay (Abbott Laboratories, North Chicago, Ill.) that detects primarily the core p24 antigen of HIV-1 (8). A culture was considered positive if two serial supernatant fluid samplings were above the cutoff value of the assay (mean optical density value of three negative controls plus 0.05), with the later sampling demonstrating at least a fourfold increase in reactivity.

Microculture method for HIV-1. PBMC were prepared from 30 ml of heparinized blood as indicated above. A cell suspension containing 10^6 PBMC from a patient and 10^6 PHA-stimulated PBMC from an HIV-1 antibody-negative blood donor (in 1.2 ml of culture medium) was placed in one well of four-well tissue culture plates (Nunc, Naperville, Ill.). One additional well in the plate was filled with sterile water to minimize evaporation. Cultures were incubated at 37°C in a 5% CO₂ atmosphere for a maximum of 42 days. Every 3 to 4 days, 0.5 ml of supernatant above the settled PBMC was removed for HIV-1 antigen testing and replaced with fresh culture medium. Once a week, an additional 3×10^5 PHA-stimulated donor PBMC were added to the cultures. Culture positivity was defined by the criteria used for standard HIV-1 cultures.

Standard HIV-1 cultures inoculated with less than 10×10^6 PBMC. Results of standard HIV-1 cultures inoculated with fewer than 10×10^6 PBMC from patients (because of a low yield of PBMC after gradient separation of the specimen) performed in our laboratory between January 1987 and July 1990 were also reviewed.

Parallel microcultures and standard HIV-1 cultures. To compare the two culture methods further, a group of blood specimens were inoculated in microcultures and standard cultures in parallel. Two types of specimens were included: (i) freshly obtained PBMC and (ii) thawed aliquots of PBMC that had been previously microculture negative when freshly cultured.

Statistical methods. Summaries of statistics presented are with respect to samples, not subjects, unless otherwise indicated. All of the analyses were weighted (1/number of observations per subject). The two culture methods were compared by two-sample *t* tests with respect to continuous variables (CD4⁺ counts per mm³, CD8⁺ counts per mm³, and CD4⁺/CD8⁺ ratio). The distribution of the number of days required for cultures to become positive by the two methods were compared by nonparametric tests (Kolmogorov-Smirnoff test). Chi-square tests were used to compare proportions, and McNemar's test was used to examine the symmetry of tables.

RESULTS

Between February and July 1990, a total of 137 blood specimens obtained from 102 individuals (52 were asymptomatic, 31 were symptomatic, and 19 had AIDS) were cultured by using the microculture method described in Materials and Methods. The historical control group included 139 standard HIV-1 cultures obtained between October 1989 and February 1990 from 108 subjects (58 were asymptomatic, 36 were symptomatic, and 14 had AIDS). Cumulative propor-

TABLE 1. Cumulative proportion of HIV-1-positive cultures by day of supernatant sampling after inoculation

Day(s) sampled after inoculation	Cumulative no. (%) of cultures positive by:	
	Microculture (n = 137)	Standard method (n = 139)
3	70 (51)	59 (42)
7	108 (79)	115 (83)
10	125 (91)	132 (95)
14	128 (93)	133 (96)
17,21,24,28,35,42	128 (93)	133 (96)
Negative cultures	9 (7)	6 (4)

tions of cultures positive by the two culture methods are shown in Table 1. The cumulative proportions of cultures positive by both methods, grouped by the symptoms present when blood specimens were drawn and mean CD4⁺ cell counts per mm³ are shown in Tables 2 and 3. Overall, HIV-1 was isolated in 128 (93%) microcultures and 133 (96%) standard cultures. The isolation rates for HIV-1 in microcultures compared with standard cultures were 91 versus 93% (cultures from asymptomatic individuals), 93 versus 96% (cultures from symptomatic individuals), and 97 versus 100% (cultures from patients with AIDS). The cumulative distribution functions of days to positivity were similar for the two culture methods (Kolmogorov-Smirnoff test, *P* > 0.2), with

TABLE 2. Cumulative proportions of HIV-1-positive microcultures by day in culture based on symptoms present when cultures were obtained^a

Patient group and no. of days in culture	Cumulative no. (%) of positive cultures	Mean no. of CD4 ⁺ cells/mm ³ (± SD)
Asymptomatic		
3	19 (33)	365 ± 131
7	39 (68)	508 ± 268
10	49 (86)	505 ± 235
14	52 (91)	557 ± 206
Negative	5 (9)	788 ± 293
Total	57	487 ± 245
Symptomatic		
3	23 (55)	244 ± 347
7	33 (78)	308 ± 160
10	39 (93)	542 ± 286
Negative	3 (7)	625 ± 144
Total	42	329 ± 314
AIDS		
3	28 (74)	86 ± 115
7	36 (95)	78 ± 131
10	37 (97)	15
Negative	1 (3)	10
Total	38	80 ± 115

^a Cultures were maintained for a maximum of 42 days, and supernatants were tested for the presence of HIV antigen every 3 or 4 days.

TABLE 3. Cumulative proportions of standard HIV-1-positive cultures by day in culture based on symptoms present when cultures were obtained^a

Patient group and no. of days in culture	Cumulative no. (%) of positive cultures	Mean no. of CD4 ⁺ cells/mm ³ (± SD)
Asymptomatic		
3	14 (24)	391 ± 148
7	42 (72)	499 ± 203
10	53 (91)	564 ± 276
14	54 (93)	794
Negative	4 (7)	728 ± 380
Total	58	506 ± 233
Symptomatic		
3	23 (49)	381 ± 437
7	40 (85)	352 ± 242
10	45 (96)	455 ± 230
Negative	2 (4)	556 ± 206
Total	47	386 ± 346
AIDS		
3	22 (65)	135 ± 160
7	33 (97)	176 ± 188
10	34 (100)	19
Negative	0	
Total	34	145 ± 167

^a Cultures were maintained for a maximum of 42 days, and supernatants were tested for the presence of HIV antigen every 3 or 4 days.

a maximum difference of 9% more cultures positive at day 3 in the microculture group. The median time to positivity by both culture methods was 7 days, with more than 75% of the positive cultures identified within 7 days and 100% of the positive cultures identified within 14 days. In both systems, virus was detected in positive cultures from asymptomatic individuals within 14 days and from symptomatic patients and those with AIDS within 10 days. There was a statistically significant association between days to positivity, CD4⁺ counts, and clinical status, with longer time to positivity for cultures obtained from patients with higher CD4⁺ counts ($P < 0.001$; analysis of variance test for linearity). Similarly, cultures from asymptomatic individuals took longer to become positive than did cultures from symptomatic patients, which in turn took longer to become positive than did those from patients with AIDS ($P < 0.001$).

Cultures from subjects in both groups were similar with respect to CD4⁺ counts, CD8⁺ counts, and CD4⁺/CD8⁺ ratios ($P > 0.05$). In the microculture group, the mean (± the standard deviation [SD]) CD4⁺ count was 393 ± 280, the mean CD8⁺ count was 984 ± 550, and the mean CD4⁺/CD8⁺ ratio was 0.38 ± 0.22. In the standard culture group, the mean (± SD) CD4⁺ count was 417 ± 287, the mean CD8⁺ count was 1,015 ± 511, and the mean CD4⁺/CD8⁺ ratio was 0.44 ± 0.28. The average numbers (± SD) of weeks that the patients had been enrolled in study protocols when blood specimens were collected were 86 ± 55 in the microculture group and 76 ± 41 in the standard culture group. Ninety-

TABLE 4. Microcultures and standard cultures grouped by anti-HIV-1 therapy when cultures were obtained

Treatment	Microcultures			Standard cultures		
	No. (%)	Median no. of days to positivity (range)	No. negative	No. (%)	Median no. of days to positivity (range)	No. negative
Zidovudine	66 (48)	7 (3–14)	3	74 (53)	7 (3–10)	3
Didanosine	27 (20)	3 (3–10)		12 (9)	3 (3–7)	
None	7 (5)	3 (3–7)	1	12 (9)	7 (3–10)	1
Unknown	37 (27)	7 (3–14)	5	41 (29)	7 (3–14)	2
Total	137		9	139		6

three (68%) microcultures and 86 (62%) standard cultures were collected from patients treated with zidovudine or didanosine. Seven (5%) microcultures and 12 (9%) standard cultures were from patients not receiving specific anti-HIV-1 therapy. Thirty-seven (27%) microcultures and 41 (29%) standard cultures were obtained from participants in trials that remained blinded. Both culture groups were similar with respect to days to positivity when antiviral therapy was considered (Table 4). There were no changes in the clinical status of subjects who had more than one specimen cultured. Fifty-nine subjects had blood specimens (collected at different dates) cultured by both methods; in 25 cases, days to culture positivity were the same. In 15 (25%) individuals, standard cultures took longer to become positive, whereas in 19 (32%) cases the microcultures were positive later. These data indicate that the two culture methods were similar ($P > 0.5$ in the McNemar test).

We identified 44 standard HIV-1 cultures that were inoculated with fewer than 10×10^6 PBMC from HIV-1 antibody-positive individuals. These included 16 cultures from asymptomatic individuals, 11 from symptomatic patients, and 17 from patients with AIDS. The average numbers (± SD) of PBMC inoculated were $6.2 \times 10^6 \pm 2.3 \times 10^6$ in the asymptomatic group, $6.3 \times 10^6 \pm 1.4 \times 10^6$ in the symptomatic group, and $6.5 \times 10^6 \pm 2.2 \times 10^6$ in the AIDS group. All cultures were positive within 14 days.

HIV-1 was isolated from the 10 blood specimens cultured in parallel in microcultures and standard cultures using freshly obtained PBMC. Nine specimens were positive by both culture methods. Of these, three specimens were simultaneously positive on day 3 by microculture and standard culture, two specimens were positive by microculture on day 3 and positive by standard culture on day 7, and four specimens were positive by the two culture methods on day 7. The only specimen that was microculture negative became positive by standard culture on day 35.

Of the 15 specimens cultured in parallel using aliquots of frozen PBMC (previously microculture negative when freshly cultured), 7 (47%) were positive, including 3 (20%) positive by the two culture methods and 4 (27%) positive by standard culture only. The remaining eight (53%) specimens were negative by both culture methods.

DISCUSSION

We have described a sensitive microculture method for isolation of HIV-1 from PBMC of HIV-1 antibody-positive individuals. By using this assay, HIV-1 was isolated from 93% (128 of 137) of the blood cultures tested, including 91% (52 of 57) of the cultures from asymptomatic individuals,

93% (39 of 42) of the cultures from symptomatic individuals, and 97% (37 of 38) of the cultures from patients with AIDS. These results were similar to those obtained with the historical control group of standard HIV-1 cultures, in which 96% (133 of 139) of the cultures were positive, including 93% (54 of 58) of the cultures from asymptomatic individuals, 96% (45 of 47) of the cultures from symptomatic patients, and 100% (34 of 34) of the cultures from patients with AIDS. The HIV-1 recovery rate of our microculture method was also similar to the recovery rate of the standard culture method in previous reports from this institution (16, 17). Furthermore, statistical analysis of our data indicated that both culture systems were essentially identical for HIV-1 isolation.

Overall, both culture systems identified more than 75% of the positive cultures within 7 days. In the two methods, 100% of the positive cultures became so within 14 days, with those from symptomatic patients and those with AIDS being positive within 10 days. A significant correlation was found between fewer days to culture positivity by both culture systems, presence of symptoms, and low CD4⁺ cell counts. This shorter time to positivity in cultures from symptomatic patients and those with AIDS suggests that they harbor a larger viral load or more virulent strains of HIV-1 with better *in vitro* replication (17).

In an earlier report from this institution, a small proportion of standard HIV-1 cultures from antibody-positive individuals took longer to become positive (up to 17, 35, and 38 days for cultures from persons with AIDS and symptomatic and asymptomatic individuals, respectively) (17). Since then, the only change in the methodology of standard cultures was elimination of hexadimethrine bromide (Polybrene; 5 µg/ml; Sigma) from the culture medium (this change was introduced a few weeks before collection of the first standard culture in the control group). Although we cannot be absolutely certain, this change may have contributed to the earlier isolation of HIV-1 observed in the present study. Also, earlier isolation of HIV-1 in the microculture group may have resulted from the combined effect of a higher cellular density ($2 \times 10^6/1.2$ ml instead of 1×10^6 /ml), a different culture surface (one well of a four-well tissue culture plate instead of the bottom area of a 25-cm² tissue culture flask), and a lower patient-donor PBMC ratio (1:1 instead of 2:1). As indicated by other investigators, these are all variables that could affect HIV-1 recovery in culture (2, 4, 5, 21).

HIV-1 was isolated from all 44 standard cultures that were inoculated with less than 10×10^6 PBMC from HIV-1 antibody-positive individuals. This further supports the utility of using fewer than 10×10^6 PBMC from patients for isolation of HIV-1 in blood cultures.

Results from the group of microcultures and standard cultures that were inoculated in parallel with freshly obtained PBMC from patients also confirmed that the two culture methods were similar. All of these cultures were positive by the standard method. Interestingly, the only microculture-negative specimen was from an asymptomatic individual with 874 CD4⁺ lymphocytes per mm³; the standard culture became positive unusually late (35 days), suggesting a low viral load in the specimen. When thawed aliquots of frozen PBMC from patients (previously microculture negative when freshly cultured) were inoculated in microcultures and standard cultures in parallel, HIV-1 was isolated in 20% (3 of 15) of the microcultures and 47% (7 of 15) of the standard cultures. This confirms the observation made by others that freezing-thawing PBMC from patients prior to culture might increase HIV-1 isolation rates (21). In view of these results, in our laboratory negative microcul-

tures are confirmed by standard cultures using thawed aliquots (10×10^6 cells) of PBMC from patients.

A variety of microculture assays for HIV-1 isolation have been evaluated in previous studies. Gupta et al. (9) used a semiquantitative assay in which sequential twofold dilutions of PBMC (10^5 to 10^2 cells) from patients were cocultured for 21 days with 10^6 PHA-stimulated donor PBMC. In their study, HIV-1 was isolated in 100% of 18 blood specimens from patients with AIDS and in 80% of 49 blood specimens from seropositive asymptomatic individuals. Dimitrov et al. (5) compared a microculture assay, in which decreasing ratios of PBMC (2.5×10^6 to 4×10^3 cells) from patients were cocultured with donor cells for 14 days, with a standard culture method for HIV-1. Eight specimens from symptomatic individuals were included in their study; HIV-1 was isolated in six microcultures containing the highest number of PBMC from patients (2.5×10^6). Similarly, Burke et al. (2) reported HIV-1 isolation in blood specimens from 75% of 65 asymptomatic and 90% of 41 symptomatic persons and 97% of 31 patients with AIDS when 3×10^5 or 3×10^6 from PBMC patients were cocultured with 2×10^6 PHA-stimulated donor PBMC. These data, together with our results, strongly support the use of a microculture assay for isolation of HIV-1 from the blood of infected individuals.

The major limitations of our study were that a group of standard HIV-1 cultures was used as a historical control and the microculture and the standard culture methods were compared in parallel in only a small fraction of samples. However, because blood specimens included in the microculture and standard culture groups were obtained from patients at similar stages of disease, immunologic status, and anti-HIV-1 therapy, we believe that our data provide an acceptable comparison of the two culture methods.

The microculture method offers two major advantages over the standard HIV-1 culture. (i) It requires substantially fewer PBMC, therefore decreasing the volume of blood needed for culture. This is particularly important for pediatric patients and other HIV-1-infected persons with leukopenia or anemia. (ii) The method is less expensive because smaller volumes of laboratory reagents, particularly culture medium, are used. The costs (reagents only) of a microculture versus a standard culture are \$53 versus \$66 for 14-day cultures and \$66 versus \$78 for 28-day cultures. Thus, microcultures are 16 to 17% less expensive than standard cultures. For a laboratory like ours, where an average of 10 HIV-1 cultures are performed per week, use of the microculture method would save between \$5,500 and \$6,300 per year.

On the basis of these results, we recommend use of the microculture method described here for isolation of HIV-1 from blood leukocytes of HIV-1 antibody-positive individuals. The yield of this method for isolation of HIV-1 from infected HIV-1 antibody-negative individuals merits further evaluation (15, 23).

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