Effective Use of Frozen Donor Peripheral Blood Mononuclear Cells for Human Immunodeficiency Virus Type 1 Isolation from Vertically Infected Pediatric Patients

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In this study, we examined variables related to human immunodeficiency virus (HIV) isolation utilizing samples from 51 HIV-infected (153 plasma and 122 peripheral blood mononuclear cell [PBMC] samples) and 57 uninfected (182 plasma and 163 PBMC samples) infants. Our chief observation was that cryopreservation of donor PBMCs does not significantly alter their sensitivity or specificity for isolation of HIV from patient PBMCs or plasma.

Despite the emergence of PCR as one of the most sensitive tests for the detection of human immunodeficiency virus (HIV) in clinical specimens (7, 8, 11, 14, 24, 27), isolation of the virus by coculture with donor peripheral blood mononuclear cells (PBMC) has been the “gold standard” for evidence of such infection in infants born to HIV-seropositive mothers (15, 20, 23, 24). However, methodologies for virus isolation vary with respect to the constituents and volume of the maintenance medium and the number (and density) of donor cells, as well as the number of patient cells used as the inoculum and their ratio to donor cells (18, 26). A consistent recommendation for performing virus isolation has been the use of fresh donor PBMC for coculture with patients’ cells (3, 16, 18). Because of this requirement, patient samples often need to be frozen until freshly prepared donor cells become available for coculture. In the present study, we have examined the issues, and here we present a comprehensive account of our strategies for successful isolation of HIV from infants, with particular reference to the use of unfrozen and frozen donor PBMC as targets for such isolation.

Peripheral blood was drawn from infants in three institutions, North Shore University Hospital, Harlem Hospital, and SUNY Downstate Hospital, between August 1990 and July 1992. Blood samples were collected within 3 months of birth, and then every 2 to 3 months thereafter, until a definitive clinical diagnosis of “infected” or “not infected” was made according to Centers for Disease Control and Prevention guidelines. Clinically infected children were designated P-2 (symptomatic) or P-1 (asymptomatic with HIV antibody persisting beyond 15 months). Those children who seroreverted were considered uninfected and were arbitrarily designated P-3 for this study. Heparin was initially used as the anticoagulant. This was replaced from March 1992 with acid citrate glucose. Plasma samples were separated after centrifugation of heparinized or citrated blood, and PBMC were obtained by standard Ficoll-Hypaque density gradient techniques. On the basis of the samples examined, specimens were divided into two groups, A and B. Group A consisted of sequential samples

from 108 children ranging in age from 0.1 to 213 months, whose plasma and PBMC samples were investigated without prior freezing of either specimen. Test samples were processed and inoculated into donor cells within 24 h after collection for the majority of specimens (80%) and after a holding period at 4°C for 1 to 6 days for the remainder. Group B consisted of plasma samples, frozen at -80°C , from 37 infants and children whose PBMC obtained at the same time as the plasma yielded positive virus cultures.

Donor PBMC from HIV-seronegative and hepatitis B surface antigen-negative donors were prepared. The techniques used were similar to those described by Castro et al. (6) and Jackson et al. (21, 22). The donor PBMC were used either without freezing or after cryopreservation. Donor PBMC that were not frozen were maintained at 37°C , at a concentration of 1.5×10^6 to 2.0×10^6 cells per ml, in IL-2 medium (RPMI with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] and 20% fetal calf serum, 5% IL-2 [Advanced Biotechnologies, Inc.], 2 μg of Polybrene [Sigma] per ml, penicillin, and streptomycin). For cryopreservation, PBMC were prepared by techniques described previously (1) and stored at -80°C in 10% dimethyl sulfoxide in IL-2 medium at a concentration of 1.0×10^7 cells per ml in round-bottomed screw-cap vials (Nunc). For long-term storage, vials were transferred to liquid nitrogen within a week after storage at -80°C . When needed, the content of each vial was rapidly thawed in a water bath set at 40°C , added to 5 ml of IL-2 medium, sedimented by centrifugation, and resuspended in 5 ml of fresh IL-2 medium. The viability of thawed cells was between 90 and 100% as judged by the trypan blue exclusion method. The count of these revived cells was adjusted before use to 2×10^6 cells per ml.

For HIV isolation, PBMC (1×10^6 to $10 \times 10^6/\text{ml}$) and plasma (0.1 to 0.5 ml) were inoculated into 1.0×10^7 donor PBMC, unfrozen or after cryopreservation, in 5 ml of IL-2 medium in T-25 flasks or 1 ml in 24-well microtiter plates. With group B samples, the plasma specimens were inoculated simultaneously into phytohemagglutinin (PHA)-stimulated donor PBMC that had been maintained for 3, 7, and 12 days either in culture, at 37°C , or by cryopreservation at -80°C . Culture supernatants (3 ml per flask or 1 ml per well) were withdrawn and tested for HIV p24 antigen (HIV p24 antigen assay kits; Coulter, Hialeah, Fla.) for 1 to 3 days and subsequently once weekly after inoculation. At such times, the

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TABLE 1. Comparison of unfrozen and frozen donor PBMC specimens of group A P-2 (HIV-infected) patients for HIV-1 isolation

Age (mo) of patient	No. (%) of plasma samples positive/no. tested with:		<i>P</i>	No. (%) of PBMC samples positive/no. tested with:		<i>P</i>
	Unfrozen PBMC	Frozen PBMC		Unfrozen PBMC	Frozen PBMC	
0.8–12	14/16 (88)	13/19 (68)	0.24	13/14 (93)	9/12 ^a (75)	0.31
13–24	1/6 (17)	5/13 (38)	0.60	7/7 (100)	11/12 (92)	1.00
25–48	11/17 (65)	9/21 (43)	0.31	13/14 (93)	11/11 (100)	1.00
49–72	7/14 (50)	1/8 (13)	0.17	14/15 (93)	5/6 (83)	0.50
73–213	15/23 (63)	9/16 (56)	0.82	18/19 (95)	12/12 (100)	1.00
Total	48/76 (64)	37/77 (48)	0.09	65/69 (94)	48/53 (91)	0.50

^a The three negative samples were from three patients, two of whom tested positive when frozen donor PBMC samples taken at earlier time points were used. The third specimen was obtained from a 0.8-month-old infant. Subsequent samples from this patient, taken at ages 12.5 and 18.3 months, were positive when fresh and frozen donor PBMC, respectively, were used.

medium was replaced with fresh IL-2 medium. In addition, fresh IL-2 medium was added to each flask (3 ml) or microplate well (1 ml) once weekly. A culture was regarded as positive if the supernatant was positive on two consecutive samplings, with a concomitant increase in optical density value. Once positive, culture supernatants were harvested and the cultures were terminated. The time to positivity for such cultures was the number of days from culture initiation to the first positive result. Specimens with supernatants determined to be positive only once were regarded as equivocal. Cultures consistently negative for HIV were terminated after 4 weeks of incubation.

A total of 620 specimens from 108 patients were examined. One hundred fifty-three plasma and 122 PBMC samples from 51 patients subsequently classified as P-2 were examined, and 182 plasma and 163 PBMC samples were from 57 patients designated P-3. The isolation rates for plasma and PBMC specimens obtained from P-2 patients were 56 and 93%, respectively. All 345 plasma and PBMC samples from P-3 patients were negative for HIV type 1 (HIV-1).

We first examined the effect of cryopreservation of donor cells on rates of virus isolation and time to positive cultures. Between 70 and 95% of patient PBMC and plasma samples were inoculated within a day after collection and processing. Isolation rates for this group were almost identical to those for fresh and frozen donor PBMC. As shown in Table 1, isolation rates for plasma were usually lower for the different age groups with cryopreserved donor PBMC compared with those for groups with PBMC that were not frozen. Overall, this difference (48 versus 63%) was not statistically significant ($P = 0.09$). Virus isolation rates for patient PBMC were comparable, regardless of whether PBMC were frozen or not. With both unfrozen and cryopreserved donor cells, virus cultures of PBMC specimens from 75% of the infected infants became positive within the first week, with only 3% or less becoming positive after periods of greater than 2 weeks of incubation (Table 2). With plasma samples as well, the unfrozen and cryopreserved donor PBMC were equivalent in sensitivity; however, in comparison with that for PBMC specimens, the time to positive culture for the plasma samples was occasionally longer, and virus recovery occurred after 2 weeks in 9 to 12% of infected specimens.

The duration for which donor PBMC could be effectively stored at -80°C without a loss of sensitivity was examined for periods of 4 days up to 50 days, and results were equivalent at

TABLE 2. Time to positivity of HIV-1 cultures of sequential plasma and PBMC samples of P-2 patients inoculated into unfrozen and frozen donor PBMC

Time (days) to positivity	No. (%) of total of plasma samples tested with:		No. (%) of total of PBMC samples tested with:	
	Unfrozen PBMC ^a	Frozen PBMC ^b	Unfrozen PBMC	Frozen PBMC
1–7	24 (55)	21 (66)	32 (70)	30 (79)
8–14	14 (32)	8 (25)	13 (28)	7 (18)
≥15	5 (12)	3 (9)	1 (2)	1 (3)
Total	43	32	46	38

^a Information on five isolates missing.

^b Information on four isolates missing.

different time points for both patient plasma and PBMC samples (Fig. 1). Additionally, different batches of donor PBMC were stored in liquid nitrogen for 6 and/or 18 months. These cells were found to be equivalent in sensitivity and specificity for isolation of virus from frozen plasma samples inoculated into the same batch of fresh donor PBMC used for initial testing of plasma samples.

Next, we examined the effect of holding donor PBMC at 37°C after PHA stimulation. Donor PBMC were inoculated with patient plasma or PBMC at various times starting at day 0, up to 14 days after PHA stimulation; the sensitivities of these cells for recovery of virus from plasma were 56% at day 0, 47% at 1 to 2 days, 81% at 3 to 7 days, and 55% at 8 to 14 days poststimulation. Although data are limited, the observed higher sensitivity of donor PBMC at 3 to 7 days after PHA stimulation for isolation of virus from plasma is noteworthy. For patient PBMC, no difference in isolation rate was observed after inoculation into donor cells kept at 37°C for various periods up to 14 days after PHA stimulation.

We also examined the effect of holding patient samples at 4°C for various periods (Table 3). For a holding period at 4°C , for up to 6 days, there was no consistent difference in virus isolation rates for patient PBMC or plasma. The one exception was a drop in sensitivity of frozen donor cells for recovery of virus from plasma samples which had been held at 4°C for 1 day. The significance of this observation is difficult to ascertain because the trend did not continue when plasma samples were held for longer periods.

For group B specimens, which consisted of frozen plasma samples from known infected children with positive PBMC virus cultures, isolation rates were not significantly different for PHA-stimulated donor PBMC of the same batch after storage (-80°C) or after maintenance at 37°C for 3, 7, or 12 days. Optical density ratios for specimens, when the specimens were first determined to be positive, correlated very well for fresh and donor frozen PBMC ($r = 0.98$), with median values of 2.25 and 2.15, respectively.

A majority (82%) of patient PBMC samples used for coculture with donor cells contained either 1×10^6 or 2×10^6 cells. The number of patient PBMC, between 1 million and 20 million cells, had no significant effect on the isolation rate when a constant number (10×10^6) of either unfrozen or cryopreserved donor lymphocytes was used. Also, neither heparin nor acid citrate glucose significantly affected the isolation rate for patient plasma or PBMC in fresh or cryopreserved donor PBMC ($P > 0.05$) (data not shown).

In this report, we demonstrate that frozen donor PBMC are comparable to unfrozen donor PBMC in the ability to support HIV replication. The sensitivities were almost the same for

FIG. 1. Effect of storage period of donor PBMC, frozen at -80°C , on the rate of isolation of HIV-1 from fresh plasma (■) and PBMC (▨) of clinically infected (P-2) children.

PBMC samples of P2 patients in the two systems, regardless of the parameters tested, i.e., age of patient, time to positivity of isolates, and holding period of specimens (patient PBMC or plasma) before inoculation. Donor PBMC, frozen at -80°C for almost 2 months and frozen in liquid nitrogen (-183°C) for 18 months, still supported HIV growth without any apparent loss of sensitivity.

Rates of isolation of HIV from plasma of infants have been reported infrequently, presumably because of the limitations in blood volume obtained by venipuncture. However, the mean sensitivities, 64 and 48%, of plasma inoculated into fresh and frozen donor PBMC, respectively, compare favorably with that of 60% reported by Alimenti et al. (2) for pediatric patients and with the range published for adults, 49 to 100% (9, 13, 17). Although the overall sensitivity of frozen donor PBMC was slightly less than that of unfrozen cells for isolation of HIV from fresh plasma, our study shows that for infants of up to 12

months of age, isolation rates for plasma were higher than those for patients of other ages whether unfrozen or frozen donor PBMC were utilized (Table 1). The rate of isolation from plasma then declined, especially in infants between 13 and 24 months of age.

Of crucial importance to this project has been the early determination of HIV infection in infants by the gold standard of virus culture. Viral culture of PBMC and plasma by our techniques identified all of nine infected infants before they reached the age of 6 months when we utilized either fresh or frozen donor PBMC (data not shown). These findings are in agreement with other published reports (4, 5, 7, 14, 19, 23). As observed in other studies (4, 7, 8, 12), results of virus culture with patient PBMC were 95% concordant with those of DNA PCR for a subset of 200 specimens examined concurrently by both methods (kappa [measure of agreement between the two assays] = 0.88).

It appears, therefore, that frozen donor PBMC, which retain T- and B-cell characteristics (10, 25), can be a useful, economical and convenient adjunct or alternative for HIV isolation and characterization in circumstances in which the supply of fresh cells is suboptimal.

Our results are similar to those obtained with other more cumbersome and elaborate protocols (5, 13). The success of our simple yet effective procedures was probably a reflection of the relatively high donor cell density (2×10^6 cells per ml) in a small (5-ml) volume (6). This highly concentrated donor cell suspension conceivably presented a high density of permissive cells. The additional step of changing the medium within 1 to 3 days after inoculation, which perhaps eliminated substantial amounts of soluble antibody and nonspecific factors inhibitory to virus adsorption and replication, also facilitated virus isolation. The techniques allowed for efficient HIV detection with

TABLE 3. Influence of holding period at 4°C on isolation of HIV-1 from sequential specimens of P-2 patients inoculated into unfrozen and frozen donor PBMC

Time (days) to inoculation	No. (%) of plasma samples positive/ no. tested with:		<i>p</i>	No. (%) of PBMC samples positive/ no. tested with:		<i>p</i>
	Unfrozen PBMC	Frozen PBMC		Unfrozen PBMC	Frozen PBMC	
0	22/35 (63)	26/43 (60)	0.99	26/28 (93)	20/22 (91)	1.00
1	12/18 (67)	8/30 (27)	0.02	20/21 (95)	21/24 (88)	0.61
2-3	11/17 (65)	2/3 (67)	1.00	10/10 (100)	6/6 (100)	
4-6	3/6 (50)	1/1 (100)	1.00	9/10 (90)	1/1 (100)	1.00
Total (0-20)	48/76 (64)	37/77 (48)	0.09	65/69 (94)	48/53 (91)	0.50

minimal numbers of patient cells and relatively small quantities of plasma, which are critical factors in the acquisition of specimens from infants and young children.

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