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Use of Cryopreserved Normal Peripheral Blood Lymphocytes for Isolation of Human Immunodeficiency Virus from Seropositive Men

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The possibility was investigated of using frozen stocks of phytohemagglutinin (PHA)-stimulated normal human peripheral blood lymphocytes (PBL) in cocultivation with human immunodeficiency virus (HIV)-infected lymphocytes for the isolation of HIV. Fresh and cryopreserved PBL from eight healthy volunteers were compared for their susceptibility to HIV infection in vitro. Fresh lymphocytes, as well as lymphocytes that were stimulated with PHA before or after cryopreservation, displayed comparable susceptibilities to HIV infection in vitro. In addition, HIV was recovered in all cases when lymphocytes stimulated with PHA before or after cryopreservation were cocultured in parallel with PBL from 15 patients with acquired immune deficiency syndrome. However, the cryopreserved PBL were less efficient in isolating HIV from asymptomatic men.

Human immunodeficiency virus (HIV) is the causative agent of the acquired immune deficiency syndrome (AIDS) and AIDS-related complex (1, 15, 19). HIV has been isolated from lymphocytes, macrophages, lymph nodes, and various body fluids (6–8, 10). This virus is generally isolated by cocultivation of peripheral blood lymphocytes (PBL) from seropositive individuals with freshly isolated seronegative PBL that have been stimulated with phytohemagglutinin (PHA) for 72 h (4, 12, 16). After initiation of the culture, PHA-stimulated normal PBL are added once or twice weekly for a period of 4 weeks.

Routine processing of normal donor lymphocytes 72 h before cocultivation is time-consuming and costly and may result in variable culture conditions. Qualitative differences in different batches of PHA-stimulated cells may also eventually lead to inconsistent isolation and detection of the virus. Moreover, the availability of large amounts of fresh PBL on demand might pose a logistical problem for many laboratories. Hence, to overcome the difficulties associated with routine processing of the PHA-stimulated fresh PBL, we compared the efficiency of virus production obtained by cocultivation of lymphocytes from HIV-seropositive patients with PHA-stimulated freshly obtained PBL, cryopreserved PHA-stimulated PBL, and cryopreserved PBL that were thawed and subsequently stimulated with PHA for 72 h. The results reported here indicate that cryopreserved PHA-stimulated cells can be used to isolate HIV from AIDS patients. However, these cells are less efficient in isolating virus from asymptomatic HIV-seropositive men.

Since the efficiency of virus production in cocultivation depends upon the relative susceptibility of the donor cells to HIV, we have compared the susceptibility to in vitro HIV infection of PHA-stimulated fresh cells, cryopreserved PHA stimulated cells, and cryopreserved cells that were PHA stimulated after thawing. For this purpose, peripheral blood was obtained from eight healthy HIV-seronegative volunteers, and the lymphocytes were separated by Ficoll-Hypaque density-gradient centrifugation. One-third of the cells was immediately cryopreserved, and the remainder were cultured in the presence of PHA (PHA-P, 5 μg/ml; Burroughs Wellcome Co., Research Triangle Park, N.C.) for 72 h. One-half of the PHA-stimulated cells was directly used for in vitro infection with HIV, and the other half was cryopreserved. The cryopreserved, unstimulated cells were thawed 1 month later, PHA stimulated for 72 h, and then infected in vitro with HIV. Concordantly, the PHA-stimulated cells that were cryopreserved for the same length of time were thawed and then infected in vitro with HIV. The release of HIV into the culture medium was measured by the reverse transcriptase (RT) assay or the antigen capture assay. These assays are equally sensitive in detecting HIV in culture supernatants (9).

Representative results with PBL from one such volunteer are shown in Fig. 1. There was no significant difference either in the reaction kinetics or in the amount of virus produced with fresh or cryopreserved lymphocytes, regardless of PHA stimulation. In most cases, the RT activity reached a peak 8 to 10 days postinfection. The development of cytopathic effect was observed within 4 to 6 days postinfection. The kinetics and the amount of virus produced by PBL (fresh or frozen) were found to vary between donors. However, with the same virus inoculum and with the same donor PBL (fresh or frozen), similar amounts of virus were obtained at multiple occasions.

We next determined whether the variation in sensitivity to HIV infection was due to differences in the level of various T-cell subpopulations. Fresh cells from the above eight volunteers were examined by flow cytometric analysis for the levels of the CD4 (Leu-3), transferrin receptor, and interleukin-2 (IL-2) receptor (Becton Dickinson, Mountain View, Calif.) before infection. The CD4 and the IL-2 receptors are the cell surface molecules essential for HIV infection and multiplication (3, 14). Transferrin receptor was used as an indicator of generalized physiology of cells (13, 17). There was no correlation between the amount of HIV production and the various cell surface markers tested (Table 1).

We then tested directly the feasibility of using cryopreserved donor lymphocytes in cocultivation with patients' lymphocytes for the isolation of HIV. For this purpose, a large quantity of HIV-seronegative lymphocytes was obtained from a local blood bank-derived leukapheresis pack. The cells were divided into two parts; one-half was cryopreserved immediately and subsequently thawed and stimulated

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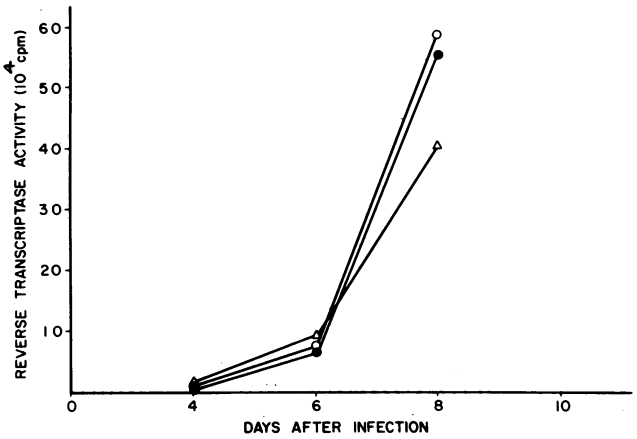

FIG. 1. Kinetics of appearance of HIV in culture fluids of PHA-stimulated fresh and cryopreserved PBL that have been infected with HIV in vitro. The cryopreservation of cells was done by a standard procedure used in our laboratory. Briefly, cells were suspended in ice-cold RPMI 1640 growth medium containing 20% fetal bovine serum and 10% dimethyl sulfoxide and were put inside freezing vials. The vials were then placed in a styrofoam box and transferred to a −70°C freezer. After overnight incubation, vials were stored in liquid nitrogen until ready to use. Infection of PHA-stimulated PBL (fresh or cryopreserved) was carried out as described by Gupta et al. (9). Briefly, 6×10^6 PHA-stimulated freshly obtained or cryopreserved lymphocytes were washed with RPMI 1640 without serum and treated with 5 μg of Polybrene per ml for 1 h. Cells were washed and suspended in 1 ml (2.5×10^5 RT units) of cell-free HIV obtained from the culture medium of an infected H9 cell line and incubated for 1 h at 37°C. After incubation, the cells were washed, suspended in 6 ml of RPMI 1640 medium containing 20% fetal bovine serum, 1 μg of Polybrene per ml, and 10% IL-2 (Cellular Products, Buffalo, N.Y.), and incubated at 37°C. Culture fluids were harvested every 2 days and tested for the presence of HIV by the RT assay (2). Symbols: △, PHA-stimulated fresh cells; ○, frozen PHA-stimulated cells; ●, frozen cells stimulated with PHA after thawing.

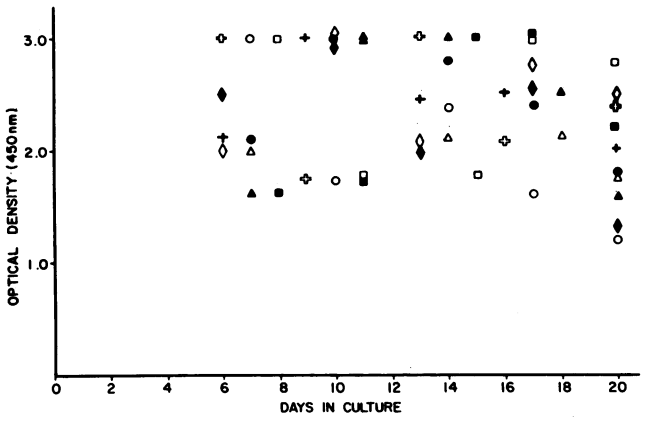

FIG. 2. Kinetics of HIV induction in culture fluid of lymphocytes from AIDS patients cocultivated with PHA-stimulated frozen lymphocytes. The cocultivation was performed as described by Levy and Shimabukuro (16) with slight modification. Briefly, 8×10^6 PBL from an AIDS patient were cocultivated in 10 ml of RPMI 1640 medium containing 20% fetal bovine serum and 10% IL-2 with 3×10^6 of either cryopreserved mitogen-stimulated normal cells or cryopreserved cells that had been stimulated with PHA after thawing. Thereafter, 3×10^6 normal cells of the same kind were added to the culture twice a week for a period of 4 weeks. Culture fluids were harvested every 4 days and tested for the presence of HIV by the antigen capture assay (Cellular Products, Buffalo, N.Y.). Parallel cultures were run for five representative samples (●, ○; ■, □; ▲, △; ◆, ◇; ✚, ✜); in each set, the closed symbol represents PBL cells cocultured with cryopreserved mitogen-stimulated cells and the corresponding open symbol represents the parallel culture with cryopreserved donor cells that had been stimulated with PHA after thawing.

with PHA, whereas the remaining half was stimulated with PHA for 72 h and then cryopreserved. Freshly donated PBL from 15 AIDS patients were cocultivated in parallel cultures with cryopreserved donor lymphocytes that had been stimulated by either procedure. Both cell types were cryopreserved for the same length of time. Kinetics of appearance of HIV from five representative parallel cultures are shown in Fig. 2. Results indicate that there was no significant difference in the kinetics of appearance of the virus in the culture medium or in the quantitative levels of the virus among the parallel cultures. Virus was isolated from all 15 AIDS patient samples cocultured with cryopreserved lymphocytes that had been stimulated before or after cryopreservation.

TABLE 1. Cell surface markers and viral expression in fresh lymphocytes infected with HIV

Normal lymphocyte donor	IL-2 receptor (%)	Leu-3 (%)	Transferrin receptor (%)	RT activity[a] (10^5 cpm/ml)
1	51	49	41	3.8
2	78	62	72	1.5
3	—[b]	—	—	4.2
4	78	51	67	2.6
5	66	32	60	4.0
6	79	78	78	2.8
7	50	18	58	1.5
8	68	34	77	6.0

[a] Maximum RT activity detected 8 days after infection. Extensive cell death occurs 8 to 10 days after infection.
[b] —, Not done.

We have also compared fresh and cryopreserved (without mitogen stimulation) normal lymphocytes in isolating HIV from lymphocytes of 13 asymptomatic HIV-seropositive men. Both types of lymphocytes were mitogen stimulated for 3 days and then used directly for cocultivation in parallel culture. Of the 13 cultures in which fresh normal donor lymphocytes were used, 11 were positive for HIV. In contrast, in cultures with frozen donor lymphocytes, only 3 of 13 were found to be positive for HIV.

Data presented here indicate that cryopreserved normal lymphocytes are equally susceptible to in vitro HIV infection when compared with fresh lymphocytes. Moreover, PHA-stimulated normal cells can be cryopreserved without a loss in susceptibility to HIV infection. The variation in the susceptibility to HIV infection of lymphocytes from different subjects is in accord with the results of Folks et al. (5). Such variation, however, does not appear to be related to the presence or absence of $CD4^+$ cells, the major target for HIV (3).

Although frozen normal PBL were found to be quite effective in the isolation of HIV from AIDS patients, they seem to be less efficient than freshly obtained PBL in the isolation of HIV from asymptomatic men ($P < 0.05$). Currently, using frozen donor lymphocytes, virus is isolated routinely from 80 to 90% of AIDS patients enrolled in our AIDS Treatment Evaluation Unit. However, using the freeze-thawed donor cells, we observed only approximately

a 20 to 30% isolation rate with samples from asymptomatic HIV-seropositive men enrolled in the Multicenter AIDS Cohort Study in Pittsburgh. Isolation rates from asymptomatic men were increased to 70% when their lymphocytes were cocultured with fresh PBL. Of course, these fresh donor cells yielded a 90% isolation rate with samples from AIDS patients (P. Gupta, unpublished results). These data are in accordance with our more comprehensive multicenter study (Farzadegan et al., submitted for publication).

The relatively lower HIV isolation rate from PBL of asymptomatic men cocultured with frozen donor cells may reflect a lower percentage of infected cells in asymptomatic men than in symptomatic seropositive men. Alternatively, it has been suggested that monocytes present in donor PBL may act as efficient targets for replication of HIV (7, 11, 18). Although monocytes survive freeze-thawing (20), such cells may be less efficient in amplifying virus from PBL of asymptomatic men, who may have fewer infected lymphocytes and monocytes than do AIDS patients.

The data presented here indicate that frozen donor PBL can be used effectively in isolating HIV from AIDS patients. This could be important in cases where routine supplies of fresh PBL are limited. Moreover, use of a stock of frozen normal cells from a single donor that has been previously tested to be highly susceptible to infection may result in less variation in isolation of HIV.

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