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Epstein-Barr Virus Infection of Cryopreserved Umbilical Cord Blood Lymphocytes (40047)

THOMAS C. SHOPE, MARTHA K. SELL, STEPHEN T. SMITH, WARD D. PETERSON, JR. AND CYRIL S. STULBERG

Department of Pediatrics, Wayne State University School of Medicine and Child Research Center, Children's Hospital of Michigan, 3901 Beaubien Blvd., Detroit, Michigan 48201

Infectious Epstein–Barr virus (EBV) will infect and transform lymphocytes from several primate species (1–4). The most sensitive primary culture system for the detection of infectious EBV is human neonatal lymphocytes, usually obtained from umbilical cord blood specimens (5, 6). Each milliliter of umbilical cord blood will yield up to 3×10^6 lymphocytes; from a single cord blood specimen, 10^8 lymphocytes or more can be obtained. Cells to be infected may be kept at 37° in appropriate medium for several days before being used, but eventually, through attrition, become unsuitable for use. Generally, an investigator obtains a fresh supply of cells each time EB virus infection studies with primary lymphocytes are undertaken. The availability of a cell storage method which preserves cells, in a state comparable to fresh cultures, would remove one of the major limitations concerning studies of the biology of primary lymphocytes infected with EBV.

Cryopreservation of tissue culture cells has been described and practiced for years with monolayer cell lines (7). Freezing of primary leukocytes has likewise been described (8–10) but we are unaware of studies which compare EBV transformation sensitivities of cryopreserved lymphocytes with fresh lymphocytes. We here describe preservation of lymphocytes in the vapor phase of liquid nitrogen and present data indicating that, compared to autologous fresh cells, cryopreserved cells remain sensitive to infection and transformation with EB virus.

Materials and methods. Cell collection and preparation. Neonatal lymphocytes were obtained by venipuncture of recently delivered placentas (up to 30 min post delivery). Whole blood was mixed with preservative-free sodium heparin (10 units/ml blood) to prevent coagulation. Lymphocytes were separated in a Ficoll-hypaque gradient (11), washed free of plasma in three 1:5 volumes of Puck's saline A and counted in 1% acetic acid. Cells for fresh culture were adjusted to 5×10^5 cells/ml in maintenance medium; cells for cryopreservation were adjusted to 1×10^7 cells/ml in freeze medium.

Maintanence medium consisted of RPMI 1640 supplemented with 20% heat inactivated (56°, 30 min) fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Freeze medium consisted of RPMI 1640, 20% heat inactivated fetal bovine serum and 10% analytical reagent grade dimethyl sulfoxide (DMSO) (Mallinckrodt Chemical Works).

Feeder layers used in the transformation assay consisted of cell strains of human placental fibroblasts, established as described by Miller (12). Assays were carried out in maintenance medium.

EBV preparation. Virus stock was prepared from supernatant fluid of an EBV-transformed marmoset cell line (B95-8) (2). Fluid was centrifuged at 400*g* to sediment cells, frozen and thawed three times and passed through a 0.45 nm filter (Millipore Corp.). The virus stock was determined to be free of bacteria and mycoplasma by culture, and was stored at −70°.

Cryopreservation. Lymphocytes suspended in 1 ml of freeze medium and transferred to plastic vials (Cooke Pro-vial) were placed in a polystyrene plug (Linde biological freezer, type BF-5, Union Carbide Corp.) fitted to the neck tube of a liquid nitrogen refrigerator (type LNR25B, Union Carbide Corp.) (13). By placing the "O" ring on the plug at level "F", the temperature decrease was constant at 1.6° per min until the sample changed from a liquid to a

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solid (18 min) after which the temperature decrease followed a sigmoid curve reaching −50° by 60 min. The vials were then stored at −170° in the vapor of a liquid nitrogen storage tank (type LR-36, Union Carbide Corp.).

In experiments depicted in Tables I and II, lymphocytes were stored frozen overnight. Cell stocks for other experiments (Table III) were stored frozen up to 12 months.

Virus inoculation and transformation assay. Frozen lymphocytes were rapidly thawed (37° water bath) and resuspended in medium (5×10^5 cells/0.9 ml). Fresh lymphocytes, stored in medium overnight at 37° in 5% CO_2, were resuspended at a concentration of 5×10^5 cells/0.9 ml. Both culture sets were exposed to serial tenfold dilutions of EBV (0.1 ml), or to culture medium (0.1 ml), four replicates per set, and cultivated in combination with human placental fibroblast feeder layers in the bottom of plastic capped, 13 × 100 mm glass test tubes. Medium was changed (1/2 vol) twice weekly and cultures were examined with the aid of an inverted microscope for signs of cell transformation. Transformation was recognized by the appearance of clusters of rapidly growing, enlarged lympho-

TABLE III. Repeated Titrations of a Single Stock Preparation of EBV[a] on Cryopreserved Human Umbilical Cord Blood Lymphocytes.

Cord blood preparation	Feeder layers[b]	Duration of cryopreservation (days)	Infectivity titer[c]
1	Yes	23	4.0
2	Yes	360	3.5
3	Yes	1	3.0
4	Yes	1	3.0
5	Yes	1	3.5
5	No	1	2.8
6	No	17	1.8
7	No	1	2.6

[a] Stock prepared from supernatant fluid of EBV infected cotton-top marmoset lymphocytes (B95-8), stored at −70° until used.
[b] Human placental fibroblasts (see Ref. 12).
[c] Expressed as 50% transforming doses ($\log_{10}TD_{50}$) per 0.1 ml, calculated by Reed-Muench formula.

TABLE I. Comparison of EB Virus Transforming Titers Detected by Fresh or Autologous Cryopreserved Lymphocytes.

	Sample A[b]		Sample B		Sample C		Cumulative	
Virus dilutions[a]	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
10^{-2}	3/4[c]	4/4	4/4	4/4	4/4	4/4	11/12	12/12
10^{-3}	1/4	3/4	1/3	2/4	3/4	1/4	5/11	6/12
10^{-4}	0/4	0/4	1/4	0/4	1/4	2/4	2/12	2/12
10^{-5}	0/4	0/4	0/4	0/4	0/4	0/4	0/12	0/12
Infectivity titer[d]	2.5	3.3	3.0	3.0	3.5	3.0	3.0	3.2
No virus	0/4	0/4	0/4	0/4	0/4	0/4	0/12	0/12

[a] Aliquots from a single virus stock (B95-8, lot #5) used throughout these experiments.
[b] Human cord blood lymphocytes from three separate placentas designated A, B, and C. In each sample cells were either frozen overnight (frozen) or incubated overnight (fresh) before infection with EBV.
[c] Number of cultures showing transformation/number of cultures in test.
[d] Expressed as 50% transforming doses ($\log_{10}TD_{50}$) per 0.1 ml, calculated by Reed-Muench formula.

TABLE II. Comparison of EB Virus Transforming Titers Detected by Fresh or Dimethylsulfoxide (DMSO) Treated Fresh or Cryopreserved Lymphocytes.

		Virus Dilution					
Condition of cells[a]	Percent DMSO	No Virus	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Infectivity titer[c]
Fresh	none	0/4[b]	4/4	3/4	1/4	0/4	3.5
Fresh	1	0/4	4/4	4/4	1/4	0/4	3.7
Fresh	10	0/4	4/4	4/4	0/4	0/4	3.5
Frozen	10	0/4	4/4	1/4	2/4	0/4	3.0

[a] Human cord blood lymphocytes from sample C, Table I. Cells were either cryopreserved overnight (frozen) or incubated overnight (fresh) before infection with EBV.
[b] Number of cultures showing transformation/number of cultures in test.
[c] Expressed as 50% transforming doses ($\log_{10}TD_{50}$) per 0.1 ml, calculated by Reed-Muench formula.

cytes. Endpoint for transformation was determined 60 days after cultures were initiated. Infectivity titers were calculated by the Reed-Muench formula (14) as a function of the number of culture tubes showing transformation compared to the number inoculated with virus.

Results. Transformation of lymphocytes before and after cryopreservation. In two experiments, using cord blood lymphocytes obtained from three separate placentas, comparisons were made between autologous lymphocyte sets which had been either cryopreserved overnight or were incubated fresh overnight (Table I). Serial dilutions of virus from a single virus stock yielded transformation endpoints ranging between $10^{2.5}$ TD_{50}/0.1 ml (TD = transforming dose) and $10^{3.5}$ TD_{50}/0.1 ml in the three fresh lymphocyte sets and between $10^{3.0}$ TD_{50}/0.1 ml and $10^{3.3}$ TD_{50}/0.1 ml in the three cryopreserved lymphocyte sets. Cryopreserved lymphocytes are as sensitive to transformation by EBV as unfrozen, fresh cultured lymphocytes. Neither fresh nor cryopreserved lymphocytes transformed unless EBV was added. Cell viability of recovered cryopreserved lymphocytes ranged between 85–88% by trypan blue dye exclusion.

Transformation of fresh lymphocytes exposed to DMSO before infection with EB virus. To establish whether preincubation of lymphocytes with DMSO could alter the sensitivity of cell cultures to transformation with EB virus, fresh cells from cord blood sample "C" were incubated with 1% or 10% DMSO for one hour prior to exposure to EB virus. Table II shows that preexposure of lymphocytes to two concentrations of DMSO neither enhanced nor suppressed the sensitivity of lymphocytes to EBV transformation.

Reproducibility of the transformation assay using cryopreserved lymphocytes. Over a three year period, we have stored, by cryopreservation, thirty samples of umbilical cord blood lymphocytes prepared as described. Titrations of a single lot of EBV have been performed on seven different cryopreserved umbilical cord blood lymphocyte samples. Table III shows that the infectivity titer on cryopreserved cells varies according to the presence of feeder layers; cultures with feeder layers detected tenfold more virus than cultures without. However, infectivity titers remain relatively reproducible from sample to sample of cord blood lymphocytes. The longest cryopreservation period for a specific cord blood preparation was 12 months; when thawed that sample's virus sensitivity detected an infectivity titer of $10^{3.5}TD_{50}$/0.1 ml (preparation 2, Table III).

Transformation of adult lymphocytes. The cryopreservation technique has been applied to lymphocytes from sources other than cord blood. Although, we have not compared infectability of cryopreserved cells with fresh cells, cryopreserved lymphocytes from adult humans and cotton-top marmosets can be readily transformed and will support EB viral replication. Cryopreserved lymphocytes from EBV-antibody negative adult sources, cultured without addition of EB virus, have not transformed.

Discussion. Considerable experimental evidence indicates that the efficiency of recovery of cells after cryopreservation depends not only on the rate of temperature change and the cryoprotective additives used, but also on the type of cell being frozen (for reviews see 15, 16). Cells recovered by Ficoll-hypaque gradient separation of heparinized peripheral blood are a heterogenous collection of mononuclear cells containing not only morphologically different cells (lymphocytes and monocytes) but also cells which, although morphologically quite similar, carry a wide variety of membrane components as well as different biologic functions. EB virus infects lymphocytes with B-cell surface characteristics (17). While EB virus receptors have been demonstrated to be common to all B-lymphocytes (18), studies of the efficiency of transformation in B-lymphocyte enriched cultures, indicate that only 10% of the virus exposed cells establish permanent lines (6). Thus, there appears to be a very small subpopulation of cells in each sample of peripheral blood lymphocytes destined for transformation induced by EB virus.

The presently reported studies were undertaken to establish that even though peripheral blood lymphocytes can be preserved through freezing with relatively high

efficiency, the small, and as yet unidentified, population of cells which are the target of EBV infection and transformation, can also be adequately preserved by this freezing procedure. Our studies indicate that, compared to autologous fresh cells, cryopreserved lymphocytes remain sensitive to infection and transformation by EB virus.

Dimethyl sulfoxide, used here as a cryoprotective additive, is also known to enhance the transport of infectious nucleic acid across cell membrane (19). Since we saw no enhancement of virus induced transformation when fresh lymphocytes were pretreated with DMSO, we conclude that the similarity of infectivity titers seen in cryopreserved cells and fresh cells is due to the cryoprotective effect of the chemical, not to enhanced transport of viral information across the target cell membrane.

Miller and Lipman (20) showed good reproducability of the transformation assay of infectious EBV using fresh primary cord blood lymphocytes. We here present comparable data on cord blood lymphocytes after cryopreservation. Recently, Henderson *et al.* (6) have shown that EB virus titration, using fresh lymphocytes cocultivated with feeder layer tissues, leads to enhanced sensitivity of the transformation assay. Our data using cryopreserved lymphocytes (Table III) supports that conclusion: lymphocytes cocultivated with feeder layers resulted in approximately tenfold more virus transformation activity than lymphocytes cultured without feeder layers.

The technology required to freeze and store primary umbilical cord blood lymphocytes is simple to apply, has been extended to include peripheral blood lymphocytes of adult human and nonhuman primates and has led to major reduction in time spent in delivery rooms waiting for umbilical cord blood specimens. In addition, large samples of lymphocytes from the same infant can be obtained, preserved and referred to as often as needed, making possible repeated investigations on the same sample of lymphocytes over a long series of experiments. Finally, having a cell system sensitive to EBV infection preserved for use whenever needed has increased the flexibility of the clinical diagnostic virology laboratory which is now able to process specimens for isolation of EB virus as they sporadically arrive.

Summary. We have described the application of cryopreservation technology to freeze storage of human primary umbilical cord blood lymphocytes and have shown that the cryopreserved cells remain as sensitive to EB virus infection and transformation as the fresh autologous lymphocytes. The methodology has been satisfactorily applied to primary peripheral blood lymphocytes from adult human sources, and has been a convenient time and resource saving advance.

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