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Use of Bone Marrow Fibroblasts to Prepare Targets for an HLA Restricted-Cytotoxicity Assay System

RALEIGH A. BOWDEN,†* LORIS McGAVREN, ANTHONY R. HAYWARD, AND MYRON J. LEVIN
University of Colorado School of Medicine and Children's Hospital of Denver, Denver, Colorado 80262

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Methods are described for obtaining and growing fibroblasts from bone marrow for use as virus-infected targets. Fibroblasts obtained at the time of routine marrow examination were maintained through 12 to 18 passages as confluent monolayers. Bone marrow fibroblasts could be infected with varicella-zoster virus, and these infected cells were suitable targets for a ^{51}Cr-release cytotoxicity assay. Since these virus-infected cells retain their HLA-A and -B antigens, they are readily available to study the immune cells which mediate virus-specific cytotoxicity.

Varicella-zoster virus (VZV) is an important pathogen for immunosuppressed patients. When it causes chicken pox in susceptible children, an appreciable number of them develop visceral involvement, and some may develop fatal disease (6). Herpes zoster virus can also cause severe morbidity and mortality in immunosuppressed patients of any age (5, 7, 23). A number of cell-mediated immune responses have been measured after VZV infection, although their relative roles in recovery are unknown (2, 3, 8, 9, 11, 14, 19, 26, 27). Lymphocyte-mediated cytotoxicity has been demonstrated against targets infected with each of the human herpesviruses (21, 25, 29, 30). Furthermore, the cytotoxic response against cytomegalovirus-infected target cells, among a variety of cellular immune responses measured in bone marrow (BM) transplant recipients infected with cytomegalovirus, best correlated with the clinical outcome (20).

Cytotoxic response to VZV infection has been investigated only once (29). In that report the small number of patients did not permit firm clinical correlations, and the potential importance for the assay system of the HLA type of the target cell was ignored. To define the role of specific cytotoxic T cell responses to VZV infection in leukemic children, we developed an assay utilizing fibroblasts from BM that had been obtained during diagnostic aspirations from these children. In this paper we detail the optimal growth conditions for BM fibroblasts, the kinetics of their growth, permissiveness for VZV infection, and their use in a ^{51}Cr-release cytotoxicity assay system.

MATERIALS AND METHODS

Subjects. Patients from our pediatric oncology clinic had BM aspirated for diagnosis or treatment monitoring. BM was also aspirated from three healthy adults. Informed consent was obtained from all subjects.

HLA typing. Subjects were HLA typed by using fresh peripheral blood mononuclear cells (MNC) and National Institutes of Health basic trays (1).

Assay for antibody of VZV. Serum was assayed for antibody to VZV membrane antigen by using a fluorescence technique (32).

VZV. Cell-free VZV (strain CP5,262 obtained from the Centers for Disease Control) was prepared in human embryonic lung fibroblasts which were obtained from J. Waner (University of Oklahoma) and used between passages 13 and 21. Infected cells were harvested at 75% cytopathic effect, sonicated (Braun Sonic model 1510; 100 W; 25% of maximum power) for 40 s at 4°C in 5 to 10 ml of medium, and clarified at 630 × *g* for 5 min at 4°C. The supernatant was brought to 7% sorbitol by adding 50% sorbitol, and the virus was stored at −70°C at a concentration of 5.2×10^5 PFU/ml.

Cell-associated virus was used to prepare target cells. This inoculum was obtained by infecting BM fibroblasts with cell-free VZV at a multiplicity of 0.1 PFU per cell. When 75% cytopathic effect was observed (3 to 5 days), these cells were added to fresh BM fibroblasts at a ratio of 2×10^5 infected cells per 10^6 uninfected cells. Target cells were harvested 48 h later by trypsinization. The infected cells in the inoculum were autologous to the cells used to prepare targets.

Preparation of BM fibroblasts. BM (1 to 2 ml) was aspirated into a syringe containing 300 U of preservative-free heparin in 0.1 ml and placed in 20 ml of Dulbecco minimal essential medium (D-MEM) with 15% fetal calf serum (FCS). This was divided into two 25-cm^2 flasks (no. 62-350-07; Flow Laboratories, Inc.) which were held at 37°C in a 5% CO_2 incubator for 5 to 7 days, washed twice with Hanks balanced salt solution (HBSS) to remove nonadherent cells, and reincubated with D-MEM plus 15% FCS until a confluent monolayer formed. These were split 1:2 by washing with 5 ml of Pucks Saline A and trypsinizing with 3 ml of 0.5% trypsin–EDTA. BM fibroblasts were maintained in D-MEM plus 10% FCS.

Freezing BM fibroblasts. Trypsinized cells were resuspended at a concentration of 10^6/ml in D-MEM containing 30% FCS and 8% dimethyl sulfoxide, and 1-ml aliquots in pro-vials (no. 235-1; Dynatech Laboratories, Inc.) were frozen at −70°C and transferred to a liquid nitrogen flask. Cells were recovered by rapid thawing at 37°C and adding them to 10 ml of D-MEM with 15% FCS in a 25-cm^2 tissue culture flask. The medium was changed after 24 h. Approximately 50 to 75% of the frozen cells were recovered, and a confluent monolayer was reached by 5 to 7 days.

Chromosomal analysis of BM fibroblasts. When dividing monolayers of the fibroblasts were two-thirds confluent, either colcemid (final concentration, 0.04 μg/ml) or nocodazole (final concentration, 0.04 μg/ml) was added to the flasks for 3 h. Actinomycin was added 1 h before harvest (final concentration, 2 μg/ml) to inhibit chromosome condensation (31). The cells were removed from the flask by

* Corresponding author.
† Present address: Fred Hutchinson Cancer Research Center, Seattle, WA 98104.

trypsinization and harvested by using standard cytogenetic techniques. The metaphase spreads were G-banded by a standard Giemsa-trypsin G-banding method (24). Twenty mitotic figures were analyzed per culture.

Fluorescent staining of BM fibroblasts. Fluorescent staining of VZV-infected fibroblasts was accomplished with varicella-zoster immune globulin (Massachusetts State Laboratory Institute) which had been rhodamine conjugated by standard methods (13). The conjugate (E_{OD} 515/280 of 1.2) was used at 0.05 mg/ml. Infected fibroblasts were cytocentrifuged, fixed in methanol, stained for 10 min, washed three times in phosphate-buffered saline, and viewed by incident UV light and phase-contrast microscopy.

Infectious center assay. Human embryonic lung fibroblast monolayers were prepared in six-well 35-mm plastic tissue culture plates (no. 3046; Falcon Plastics) by seeding each well with 3×10^5 cells in 3 ml of D-MEM with 10% FCS. Four days later 10-fold dilutions of infected BM fibroblasts were added in 0.1 ml of medium. After 2 h at 37°C the plates were fed with 3 ml of D-MEM plus 2% FCS. Five days later, the monolayers were fixed with a 3:1 mixture of 95% ethanol and glacial acetic acid and stained with 1% crystal violet. Typical VZV plaques were counted with an inverted microscope at a 50-fold magnification.

^{51}Cr labeling of targets. VZV-infected or uninfected BM fibroblasts were suspended at 10^6/0.2 ml of RPMI 1640 medium plus 10% FCS containing 100 μCi of ^{51}Cr (1 mCi/ml; New England Nuclear Corp.). After 40 min at 37°C, the cells were washed three times in RPMI 1640 medium plus 20% FCS. More than 90% of the labeled targets excluded eosin Y.

MNC. Effector cells for the cytotoxicity assay were MNC obtained from HLA-matched and unmatched donors. The blood was defibrinated and separated on a Ficoll-Hypaque gradient. The interface was harvested, washed twice with HBSS, and resuspended in RPMI 1640 medium with 10% FCS to give effector/target (E/T) ratios of 50:1, 20:1, 10:1, and 2:1.

T cell subset depletion by panning. A total of 10^7 MNC was incubated with 25 μl of monoclonal antibodies OKT4, OKT8, OKM1 (Ortho Pharmaceuticals), or HNK-1 (Becton, Dickinson & Co.) on ice for 30 min to selectively remove helper-inducer, cytotoxic-suppressor, natural killer-monocyte, or natural killer cells, respectively. The cells were washed twice with HBSS, resuspended in 3 ml of HBSS plus 5% FCS, and then added to petri dishes (Falcon 1002) precoated with 1.0 ml containing 10 μg of purified goat anti-mouse immunoglobulin G (Tago 4150) per ml in 0.1 M sodium bicarbonate for 24 h. MNC and plates were kept on ice together for 2 h, and nonadherent cells were recovered by gentle swirling and pipetting. Less than 5% of the cells adhered when control cells (cells not incubated with subset antibody) were exposed to the coated plates (16, 22).

^{51}Cr-release cytotoxicity assay. An 18-h ^{51}Cr-release cytotoxicity assay was performed by standard methods (28) in a round-bottom 96-well microtiter plate (Linbrow 76-013-05; Flow Laboratories). A total of 5×10^3 target cells in 0.1 ml were mixed with 0.1 ml of effectors to give the desired E/T ratios. Cultures were run in triplicate at 37°C. After 18 h the plates were centrifuged ($150 \times g$) at 4°C for 5 min, and 100 μl of supernatant was harvested and counted in a gamma counter.

The specific lysis was calculated from mean values according to the following formula: percent specific lysis = [(test counts per minute − control counts per minute)/(maximum counts per minute − control counts per minute)] × 100.

TABLE 1. Effect of harvest conditions on the preparation of BM fibroblast cultures

Expt[a]	Method of separation	Time of plating	Cell yield per ml of BM ($\times 10^6$)	Time until confluence of culture (days)
1	Ficoll-Hypaque	Immediate	0.26	37
2	Ficoll-Hypaque	Immediate	0.40	35
3	None	After refrigeration at 4°C for 12 h	3.3	35
4	None	Immediate	1.4	20
5	None	Immediate	4.3	17

[a] BM aspirate (1 to 5 ml) was obtained from patients of comparable age, cellularity, and interval from last chemotherapy.

Test counts per minute are ^{51}Cr counts released from targets in the presence of effector cells, control counts per minute are from targets in the absence of effector cells, and maximum counts per minute are from frozen-thawed target cells. Uninfected targets were run in parallel at identical E/T ratios.

RESULTS

Growth kinetics. In preliminary experiments the BM aspirate was centrifuged on a Ficoll-Hypaque gradient to deplete it of erythrocytes. However, this step reduced the number of nucleated cells recovered and increased the time elapsed before a confluent monolayer formed (Table 1). An aspirate that was refrigerated overnight also showed a prolongation of the time required for formation of a confluent monolayer. For subsequent studies we prepared cultures within 2 h of obtaining the sample.

BM spicules readily adhered to the culture flask and were not removed by washing with HBSS at 5 days. Serial observations revealed extensive fibroblast growth radiating from the spicules until these areas subsequently became confluent (Fig. 1A and B). However, even when few spicules were seen, a sufficient number of individual fibroblasts adhered to the plastic to produce a confluent culture, and the time required to achieve confluence was not related to the number of spicules present in the initial plating. All specimens yielded a complete monolayer.

Of the media and growth factors examined, D-MEM was superior to RPMI 1640 medium in supporting the growth of BM fibroblasts, and growth was better in cultures supplemented with 15% FCS than in those containing 10% FCS or 10% human serum. The supernatant from phytohemagglutinin-stimulated tonsillar lymphocytes did not enhance growth.

The age of the donor did not appear to be a factor in the time required for cultures to become confluent initially (Table 2). However, in the two adult donors there was a trend for the subsequent rate of growth to be slower. All lines which were adequately studied were viable for at least 12 passages.

Chromosomal analysis. Cytogenetic studies were performed from passage numbers 4, 9, and 13 from one BM fibroblast culture. The karyotype of each passage was 46, XX, a normal female. No abnormalities of chromosome structure were observed. The number of aneuploid cells observed in each passage and the number of chromosome breaks were within the background levels observed in this laboratory.

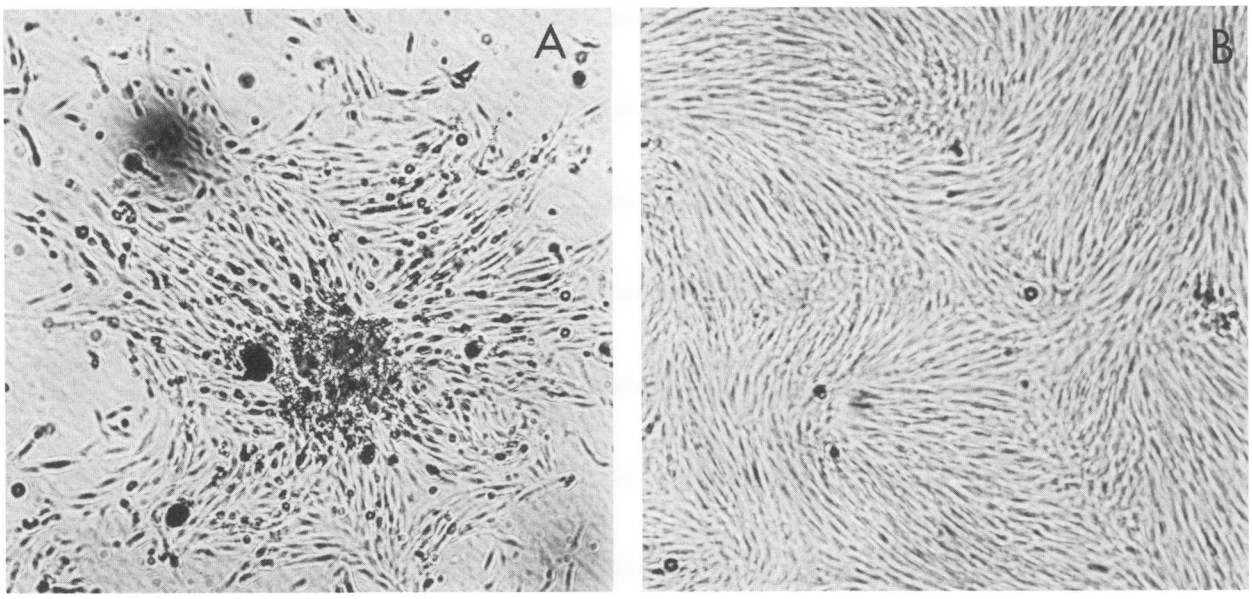

FIG. 1. Photograph of BM fibroblasts in culture. (A) Fibroblasts extending from bony spiculae. (B) Confluent fibroblast monolayer. Magnification, ×50.

VZV infection of BM fibroblasts. Optimal conditions for the preparation of VZV-infected BM fibroblasts were determined by adding a cell-associated VZV inoculum at a multiplicity of infection of 0.1 to 0.2. Cytopathic effect was apparent at 24 h, and the cells detached between 48 and 60 h. Infected fibroblasts in the monolayer were determined by an infectious center assay, and the rate of appearance of cell surface-associated VZV antigen was determined by immunofluorescence (Fig. 2A). More than 90% of cells were infectious and contained VZV-specific antigen at 48 h after infection.

Cytotoxicity assay with VZV-infected BM fibroblasts. VZV-infected BM fibroblasts prepared as described for Fig. 2A were detached and labeled with ^{51}Cr for use in a cytotoxicity test with autologous blood MNC as effectors (Fig. 2B). The percent specific lysis of the fibroblasts, which was a function of the duration of the infection, was maximal at 48 h after infection. Mean spontaneous lysis was 25% at 48 h and then greatly increased. Because of this, and because the monolayer was detaching at later times, further experiments with BM fibroblasts used as targets for cytotoxicity were ^{51}Cr labeled at 48 h after infection. At E/T ratios of 100:1 or greater a significant increase in specific lysis did not reliably occur. For this reason, and because of the limited number of effector cells, the experiments reported were performed with a maximum E/T ratio of 50:1.

TABLE 2. Growth of BM fibroblasts as a function of the age of the donor

Donor age (years)	Time to confluence[a] (days)		Highest passage number[b]
	Initial[c]	Subsequent[d]	
3	16	4	ND[e]
4	18	4	12
6	15	5	17
8	16	4	18
10	15	5	12
11	15	6	15
13	21	6	14
16	15	5	17
39	21	12	12
44	15	10	13

[a] Time required after plating 5×10^6 BM aspirate cells in a 25-cm^2 flask until a confluent monolayer was achieved.
[b] Attempted passage beyond the number shown did not result in sustained growth and formation of a confluent monolayer.
[c] Initial culture of the aspirate.
[d] Third to fifth passage level at a 1:2 split with 10^6 cells in a 25-cm^2 flask.
[e] ND, Not done.

Specificity of cytotoxicity assay. The VZV-infected targets were readily lysed only by MNC from an immune donor, and this lysis was a function of the E/T ratio (Table 3). The specificity of the assay was demonstrated by the failure of MNC from an immune donor to lyse uninfected BM fibroblasts and the failure of MNC from a nonimmune individual to lyse either VZV-infected or uninfected BM fibroblasts (Table 3). These results were obtained with autologous effector cells. Lysis of VZV-infected targets was less when the BM fibroblasts and the effector cells were from different individuals with no HLA-A or -B locus antigens in common (Table 3). Comparable results were obtained in three further cultures, suggesting that HLA-restricted antigen recognition contributed to lysis of the infected fibroblasts. The contributions of cytotoxic T lymphocytes and natural killer cells to the lysis of infected fibroblasts were further analyzed by selective removal of T cell subsets or NK cells by panning. The effect of these manipulations at the 20:1 E/T ratio is shown in Table 4. Proportional reductions in target cell lysis were also found at the 50:1 and 10:1 E/T ratios. A more detailed analysis of the cells responsible for VZV-infected fibroblast lysis, and their activation, forms the basis of another communication (R. A. Bowden, M. J. Levin, R. H. Giller, D. G. Tubergan, and A. R. Hayward, submitted for publication).

DISCUSSION

Fibroblasts which were suitable for use in preparing HLA-specified infected targets for a cytotoxicity assay system were obtained from human BM. BM has certain advantages

TABLE 3. Use of BM fibroblasts in a ^{51}Cr-release assay for cell-mediated cytotoxicity[a]

E/T combination and donor	BM fibroblast target	% Specific lysis[b] at an E/T ratio of:			
		50:1	20:1	10:1	2:1
Autologous					
Immune	VZV infected	34 ± 3.8	31 ± 10.0	22 ± 5.3	7 ± 8.6
	Uninfected	7 ± 7.1	4 ± 2.5	1 ± 1.5	2 ± 2.1
Nonimmune	VZV infected	17 ± 6.9	16 ± 1.5	10 ± 2.5	2 ± 3.2
	Uninfected	2 ± 2.5	2 ± 1.2	1 ± 1.5	2 ± 2.5
Partial match or HLA mismatched[c]					
Immune	Partial match, infected	27 ± 5.3	21 ± 4.6	14 ± 1.2	4 ± 2.0
	Partial match, uninfected	3 ± 1.0	2 ± 3.2	0 ± 0.6	2 ± 2.7
Immune	Unmatched, infected	24 ± 1.2	6 ± 8.3	8 ± 1.5	4 ± 3.5
	Unmatched, uninfected	3 ± 2.9	0 ± 0	0 ± 0	0 ± 0

[a] All values signify the mean of three representative experiments.
[b] Percent specific lysis is defined in the text. An 18-h ^{51}Cr-release assay was used. Three donors were studied in each group, except that only a single nonimmune donor was studied. Values represent the mean ± standard deviation.
[c] Effector and target cells came from unrelated healthy adults who shared HLA-A1, -A2, or -B7 (partial match) or shared no A or B antigen group (unmatched).

over skin: it is easier to obtain, does not require a lengthy trypsinization step (15), and leaves a smaller scar. Furthermore, BM is often obtained during a routine evaluation of patients with myeloproliferative and other malignancies for which immunosuppressive therapy is administered. Adequate fibroblasts were grown from a small amount of material, making it ideal for a pediatric population.

It has previously been demonstrated that human BM contains single-cell fibroblast CFU (4, 18). We encountered no major problems in the harvesting of or in the initial or subsequent passage of these cells, which appeared to grow in a fashion similar to fibroblasts obtained from other sources. The fibroblast lines appear to be chromosomally stable and intact through serial passages. We confirmed the suitability of D-MEM for maintaining BM fibroblasts, the superiority of FCS to human serum, and that the optimal concentration of FCS is 15% (4). We found that the BM fibroblast cultures should be started within hours after the marrow is aspirated and that the rate of growth of the cultures is not enhanced by lymphokines present in the supernatant from phytohemagglutin-stimulated mononuclear cells.

The BM fibroblasts were easily infected with VZV and expressed VZV antigens on their surface, and VZV-infected BM fibroblasts were recognized by MNC. Our preliminary experiments demonstrate that lysis of VZV-infected fibroblasts is optimal when effector and target cells are matched for HLA-A and -B locus antigens. This preference for matched targets implicates cytotoxic lymphocytes in the response, acting either through direct cytotoxicity or through the release of lymphokines and recruitment of NK cells (12). The summation of OKT8 and HNK-1 depletion of the effector cells (Table 4) suggests that both mechanisms operate in vitro.

Although partially matched targets are suitable, as shown here, for measuring virus-specific immune responses, HLA antigens are known to be important determinants of the completeness of these responses in both human and animal virus infections (17). Matched targets are necessary for optimal responses and for analysis of the immune mechanisms involved. These targets are potentially available from any patient who has a bone marrow aspirate for diagnostic purposes.

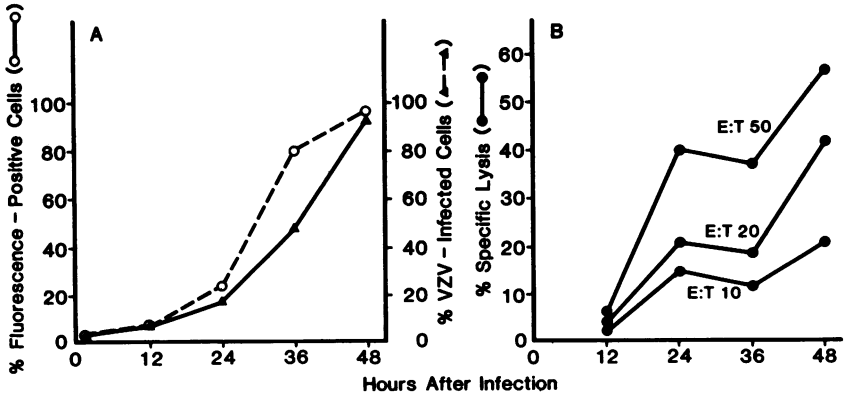

FIG. 2. Development of infectivity and appearance of VZV antigen after VZV infection. The multiplicity of infection was 0.2. (A) Symbols: O, surface antigen detected by VZV-specific fluorescent antibody; ▲, infected cells as measured by an infectious center assay. (B) Appearance of antigen as measured by susceptibility to cytotoxic cells. Percent specific lysis is defined in the text. The effector cells and target cells were autologous, and the effector cells were from a donor immune to VZV.

TABLE 4. Inhibition of lysis of VZV-infected BM fibroblasts by selective depletion of effector cells[a]

Effector population depleted	% Specific lysis	% Inhibition[b]
O	28	
OKT8	12	57
OKT4	19	32
HNK-1	20	28
OKM1	19	32
OKT8 + HNK-1	8	71
OKT4 + HNK-1	12	57

[a] Results shown are at an E/T ratio of 20:1 with percent lysis calculated as described in the text. Maximal release was 7,200 cpm, and control lysis was 2,200 cpm.

[b] Percent inhibition is the percent decrease in chromium release of depleted populations compared with the effector population not depleted by panning.

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