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A METHOD TO GENERATE MICROCELLS FROM HUMAN LYMPHOBLASTS FOR USE IN MICROCELL MEDIATED CHROMOSOME TRANSFER

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SUMMARY

A method is described to generate microcells from human lymphobalsts for use in microcell-mediated chromosome transfer (MMCT). Micronuclei were induced in cells from a human lymphoblastic cell line by prolonged colcemid treatment, and were separated from these lymphoblasts by: (a) attaching the cells to Concanavalin A coated plastic slides designed for enucleation, and (b) centrifuging the slides in medium containing cytochalasin B. Microcells of less than 3 μm in diameter were fused with thymidine kinase negative mouse fibroblasts (LMTK$^-$). HAT medium (hypoxanthine, aminopterin, and thymidine) was used to select microcell hybrids expressing thymidine kinase activity. Positive clones were isolated and Q-banded for chromosome analysis. Unlike previous methods, this procedure permits microcells to be easily generated from lymphoid cells. This methodology of enucleation of microcells may be extended to a variety of other donor cell types which can be micronucleated but which do not adhere tightly to enucleation slides and do not exhibit extrusion subdivision. This feature makes our methodology particularly useful for constructing a library of hybrid clones containing one or a few human chromosomes.

Key words: microcells; gene mapping; chromosomes.

INTRODUCTION

It has been well established that microcell-mediated chromosome transfer (MMCT) is an effective technique for transferring a limited number of chromosomes from donor to recipient cells. Earlier work by Stubblefield (16) and Phillips and Phillips (14) suggested that under optimal conditions the mitotic spindle inhibitor colcemid could be used to generate micronuclei containing a single chromosome. Ege and Ringertz (2) produced the first microcells by centrifuging micronucleate rodent cells in medium containing cytochalasin B (Cyt B). The resulting microcells consisted of a single micronucleus enveloped by a thin sheath of cytoplasm and a plasma membrane. Fournier and Ruddle (4) have used this technique to transfer as few as one to five murine chromosomes to mouse, Chinese hamster, and human recipient cells. Using normal human fibroblasts, McNeill and Brown (9) have transferred a single human chromosome to mouse cells, a procedure that required only 6 wk from fusion to karyotype analysis.

Johnson et al. (6) have found that human HeLa cells can be induced to form small minisegregant cells containing reduced amounts of chromatin by subjecting the cells to hyperbaric nitrous oxide, followed by prolonged cold shock, and then return to 37° C. This process, known as extrusion subdivision, has produced HeLa minisegregants that are effective vehicles for transferring human chromosomes to recipient mouse cells (10,17).

An advantage of transferring a limited number of chromosomes is that such hybrid clones are simple and karyotypically stable, unlike whole cell hybrids which characteristically experience extensive chromosome elimination before stability is achieved (19). Consequently, microcell hybrids expedite gene mapping by permitting prompt assignment of the few donor chromosomes.

Although both methods of microcell (minisegregant) production have been successfully used on MMCT, each has limitations. The choice of donor cells used in the Ege and Ringertz (2) procedure has been limited to cells that readily form monolayers on plastic enucleation slides and that remain firmly attached during centrifugation. These limitations are imposed by the physical requirements that large numbers of micronucleate cells maintain a fixed position during centrifugation such that numerous microcells are drawn off. To circumvent this restriction, other investigators have enucleated cells by centrifugation in discontinuous Ficoll density gradients (20). This procedure, however, has proven impractical because large numbers of cells could not be easily processed at once. Johnson's method of generating minisegregants by extrusion subdivision is also limited to certain cell types. Although subdivision is easily induced in heteroploid cultures, it is difficult to induce in fibroblasts (12).

This communication reports a technique for deriving microcells from human lymphoblasts which cannot be

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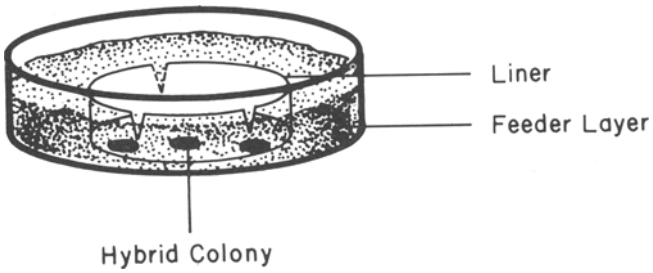

FIG. 1. *Selection dishes.* Vertical slits are made in a culture dish liner which is placed on a layer of feeder cells grown in a 100-mm culture dish. A suspension of fusion cells is placed in the liner followed by addition of selective medium.

enucleated by the method of Ege and Ringertz (2) nor induced to extrude minisegregants. Lymphoblastic cells, grown in suspension, are ideal for micronucleation because of the simplicity in handling the cultures and the ease of obtaining numerous cells at one time. A method to physically link these cells to plastic slides would allow for efficient enucleation of microcells. Gopalakrishnan and Thompson (5) have enucleated lymphoid cells by linking them to slides coated with Concanavalin A (Con A) before centrifugation in the presence of cytochalasin B (Cyt B). We have modified this method and used it to enucleate the micronuclei induced in human lymphoblasts. Microcells, derived in this manner, have been recovered, purified, and fused with recipient LMTK$^-$ mouse fibroblasts. Microcell hybrids surviving hypoxanthine, aminopterin, and thymidine (HAT) selection have been Q-banded to identify the human chromosomes transferred.

MATERIALS AND METHODS

Cells. The GM 130 lymphoblastic cell line was obtained from the Human Genetic Mutant Cell Repository, Camden, NJ. LMTK$^-$ mouse fibroblasts and BG-9 human foreskin cells were donated by Dr. Thomas B. Shows and Dr. Judith O'Malley of Roswell Park Memorial Institute.

Chemicals. Concanavalin A, Cyt B, polyethylene glycol (PEG), thymidine, hypoxanthine, aminopterin, and the water-soluble carbodiimide (WSC) [1-cyclohexyl-3-(2-morpho-linoethyl) carbodiimide metho-*p*-toluenesulfonate] were purchased from Sigma Chemical Co., St. Louis, MO. Phytohemagglutinin P (PHA-P) was obtained from Difco, Detroit, MI.

Micronucleation. GM 130 human lymphoblasts were used as donor cells for micronucleation. Optimal conditions for micronucleation were ascertained by exposing 3.35×10^5 cells to 5, 10, 15, and 20 μg/ml of colcemid for 48 and 72 h.

Production of microcells. Enucleation slides were prepared by modifying the method of Gopalakrishnan and Thompson (5). The WSC and Con A were concomitantly solubilized in 0.15 *M* NaCl at concentrations of 55 and 2.7 mg/ml, respectively. This solution was pipetted onto plastic slides (7 × 3.7 cm) cut from the bottom of T flasks. Slides were kept at room temperature for 30 min before removing the WSC-Con A solution and washing three times with phosphate buffered saline (PBS).

Lymphoblasts treated for 48 h with 10 μg/ml colcemid were concentrated in 20 ml of PBS at room temperature. This suspension, containing approximately 1.5×10^8 viable lymphoblasts, as determined by trypan blue exclusion, was applied in equal volumes to six Con A-coated slides. Fifteen minutes later the cell suspensions were removed, pooled, and counted using trypan blue exclusion. More than 50% of the cells remained attached to the slides. Slides were subsequently washed twice with PBS and positioned back to back in ultracentrifuge tubes containing 5 μg/ml of Cyt B in minimum essential medium (MEM), 5% fetal bovine serum (FBS), followed by 3.5 h of incubation at 37° C. The slides were centrifuged in the Cyt B medium for 30 min at 12 000 *g*.

Purification of microcells. The procedure used to purify the crude microcell preparation was comparable to that of Fournier (3). The microcell pellet was washed twice with Hanks' balanced salt solution (HBSS) and resuspended in three 5-ml aliquots. Each aliquot was successively filtered through an 8, 5, and 3 μm Nucleopore filter. Filtrates were pooled and concentrated in 0.5 ml of 200 μg/ml PHA-P in HBSS (3).

Microcell fusion. Purified microcells were fused with LMTK$^-$ fibroblasts by a modification of Mercer and Schlegel's procedure (11). Fifteen minutes before fusion, a monolayer (25 cm^2) of LMTK$^-$ cells was washed free of medium with HBSS and incubated in an HBSS solution of 200 μg/ml PHA-P. This medium was discarded before adding the microcell suspension to the monolayer. The fibroblasts and microcells were incubated together for 10 min at 37° C.

To induce fusion, 3 ml of 30% PEG (molecular weight 1000) was introduced into the flask and incubated at 37° C for

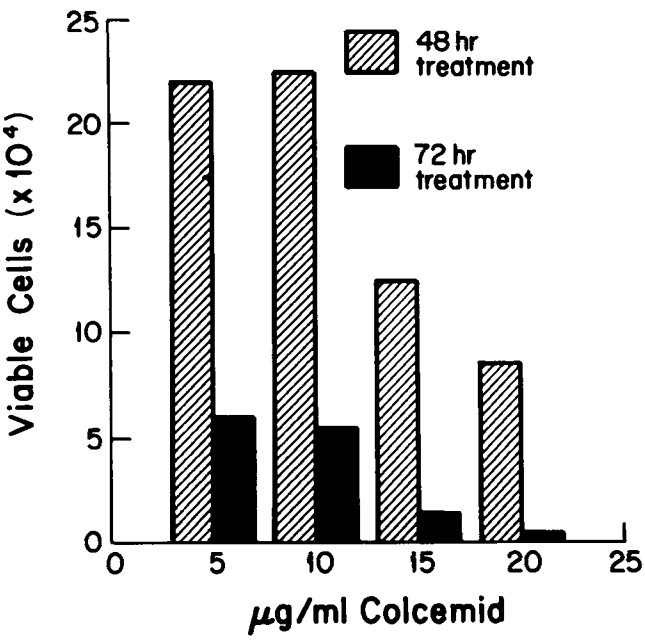

FIG. 2. *Cell viability.* This histogram depicts the number of viable GM 130 lymphoblasts after exposure of 3.35×10^5 cells to either 5, 10, 15, or 20 μg/ml colcemid for either 48 or 72 h.

TABLE 1

AVERAGE MICRONUCLEI PER CELL AFTER 48 H COLCEMID TREATMENT

Colcemid Conc	Micronuclei/Cell[a]
5 μg/ml	6.9 ± 0.4
10 μg/ml	10.1 ± 0.8
15 μg/ml	ND[b]
20 μg/ml	ND

[a] $n = 50$ cells at each concentration.
[b] ND = not determined due to poor morphology.

1 min. The flask was immediately washed four times with 5 ml MEM containing 10% FBS and incubated for 18 h in a 5% CO_2 incubator at 37° C. Cells were trypsinized and replated in five 25-cm² flasks. Fusion cells were grown to confluency in MEM plus 10% FBS.

Feeder cells. Five days before the microcell fusion, feeder cells were made by irradiating 70% confluent monolayers of BG-9 fibroblasts with 1740 rads from a cesium 137 source. These feeder cells were used to "condition" the medium such that individual hybrid cells would reproduce and become self-sustaining (15).

Hybrid selection. Thymidine kinase positive hybrids were propagated in the following manner. A Falcon 35-mm tissue culture dish liner into which vertical slits had been cut (Fig. 1) was placed inside each of four 100-mm tissue culture dishes containing BG-9 feeder cells. Two milliliters of resuspended fusion cells were added to each liner. When the cells had adhered to the liner, 35 ml of HAT medium (7) was introduced such that growth factors could circulate freely between culture dish and liner. The medium was replenished regularly over a 6-wk period to remove dead cells. Alternately, fusion cells in 35 ml HAT medium were added directly to culture plates with and without feeder cells.

Isolation of hybrids. After 6 wk of selection, hybrid colonies grown on the liners were easily isolated under an inverted microscope by aspirating each colony with a Pasteur pipette. Cells from a single colony, isolated from the liner or feeder cells, were transferred to one well of a 24-well microtiter plate and grown to confluency in HAT medium.

Stabilization of hybrid cells. To determine whether the human thymidine kinase gene had become a stable component of the hybrid genome, cells from each HAT-resistant colony were removed from HAT selection and passaged at least 12 times in MEM before returning them to HAT selection.

Q-banding. Chromosomes were Q-banded by a method derived from Yoshida et al. (21). Slides were sequentially stained in 0.5 μg/ml 33258 Hoechst in McIlvan's buffer (pH 4.4) and then in 50 μg/ml quinacrine mustard in the same buffer. The bright fluorescence of the mouse centromeres could be easily distinguished from the dull human centromeres.

RESULTS

Micronucleation. Optimal micronucleation conditions for the GM 130 cell line were ascertained by measuring (a) cell viability, (b) percent of cells micronucleated, and (c) average micronuclei per cell after treatment with various concentrations of colcemid for two time periods. GM 130 lymphoblasts were treated with either 5, 10, 15, or 20 μg/ml of colcemid for 48 and 72 h. Fig. 2 is a histogram of the cell viabilities obtained under these conditions. Approximately 67% of the initial 3.35×10^5 cells were viable after exposure to either 5 or 10 μg/ml of colcemid for 48 h. Viability fell off sharply at 15 μg/ml of colcemid and was extremely low for all colcemid concentrations at 72 h. Cell necrosis accompanied by chromatin degradation would limit the formation of microcells containing intact, functional gene sequences. Therefore, only cells treated with 5 and 10 μg/ml of colcemid for 48 h were examined for micronuclei. Table 1 indicates the average micronuclei per cell for these treatments. Fourteen percent of the cells treated with 5 μg/ml colcemid had micronuclei, and 26% of the cells receiving 10 μg/ml colcemid contained micronuclei. A Student's *t*-test indicates that the average micronuclei per cell obtained with 10 μg/ml of colcemid is significantly greater than that obtained with 5 μg/ml of colcemid at the $P = 0.01$ level. Therefore, optimal micronucleation of GM 130 cells is obtained using 10 μg/ml of colcemid for 48 h. Fig. 3 is a representative cell containing micronuclei. Lymphoblasts subjected to either 5, 10, 15, or 20 μg/ml colcemid and allowed to recover in colcemid-free medium did not exhibit extrusion subdivision.

Modal chromosome number of GM 130 and LMTK⁻ cells. The modal chromosome number in GM 130 cells was determined by counting the chromosomes in 170 metaphase spreads. More than 50% of the GM 130 cells counted had 46 chromosomes. Two percent of the cells contained a small metacentric marker chromosome. Fifty metaphase spreads of the LMTK⁻ cells counted had chromosome numbers ranging from 23 to 59, with a mode of 47. Multiple rearrangements were also observed.

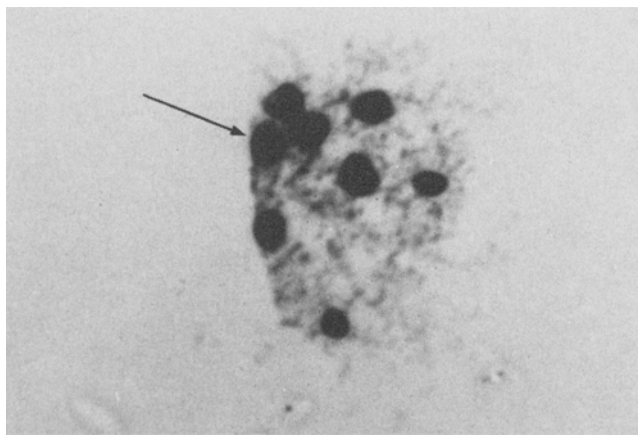

FIG. 3. *Micronuclei.* A human GM 130 lymphoblast containing eight micronuclei.

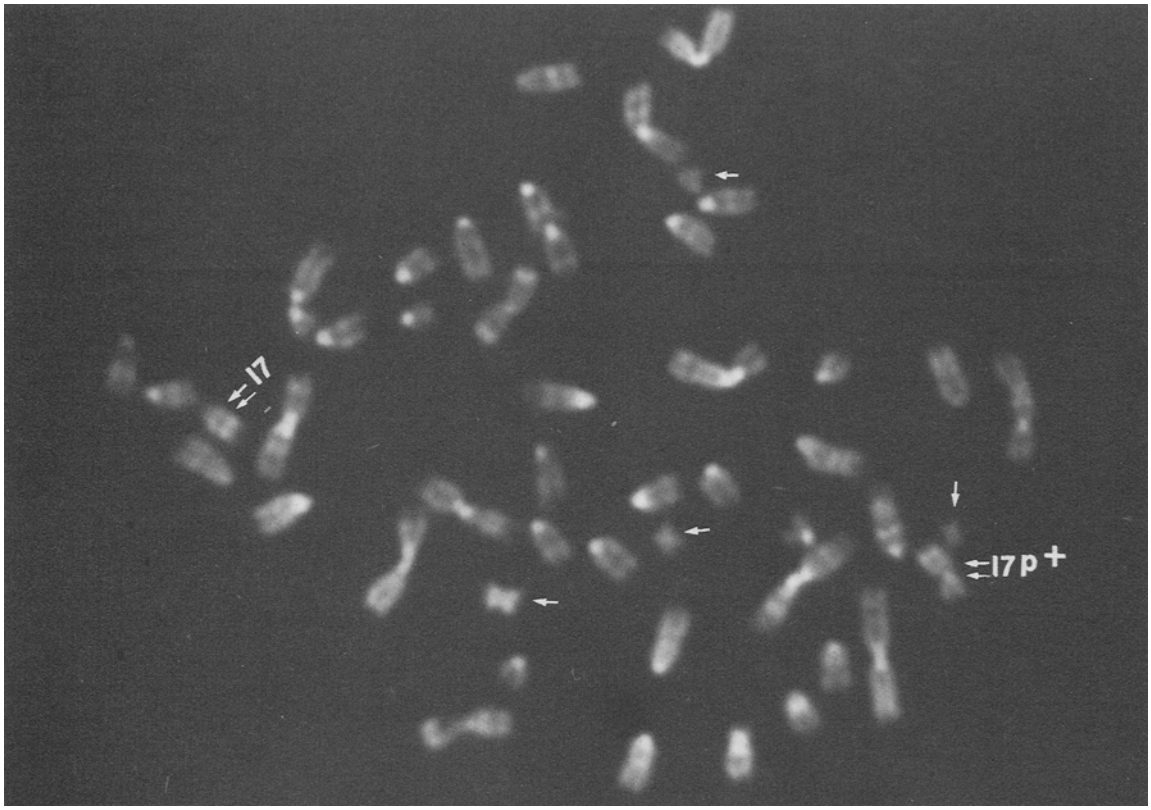

FIG. 4. *Hybrid H105. White arrows* indicate Q-banded human chromosomes in microcell hybrid H105; *double arrows* indicate 17 and 17p+ chromosomes.

Isolation and analysis of microcell hybrids. Fusion cells directly plated onto 100-mm dishes did not yield HAT-resistant colonies. However, two to five isolated cells with L-cell morphology were observed on dishes after several weeks of selection. These isolated cells did not divide but remained viable until the cultures were terminated at 10 wk of HAT selection. Fusion cells plated onto very confluent feeder layers and subjected to HAT medium gave rise to a few small colonies. Limited proliferation of the colonies, due to crowding by feeder cells, prevented their isolation.

Recovery of hybrid colonies was achieved when culture dish liners were used in conjunction with feeder cells. After 6 wk of selection, four HAT-resistant colonies were isolated from the liner and six from among the feeder cells of a culture dish. Two clones obtained from the liner, H105 and H108, had duplication times of 48 and 72 h, respectively, Sufficient metaphase spreads could be obtained for karyotype analysis by the 6th passage. The other eight clones could not be analyzed due to their extremely slow growth characteristics. Differential growth rate characteristics of each colony may be attributed to different chromosome complements of both mouse and human chromosomes in each colony. Such phenomena are well documented in cancer cells when the presence of certain chromosomes provide growth advantages to certain clones (13).

Both clones H105 and H108 contain five to seven human chromosomes. Besides chromosome #17, other human chromosomes belong to groups F or G. Chromosome #17, which carries the human thymidine kinase gene, was present in both clones. Specifically, clone H105 contains two chromosome #17, one of them is a 17p+ (Fig. 4). The 17p+ chromosome might have arisen due to long-term culture conditions of the cell line.

The stability of the human chromosome #17 in clones H105 and H108 was assessed by removing the cells from HAT medium and cultivating them in nonselective medium for more than 12 passages. The cell duplication patterns of these clones were not altered in nonselective MEM. When returned to HAT medium, the clones continued to proliferate, indicating that the clones had retained the human thymidine kinase gene.

DISCUSSION

Mapping of human genes became feasible with the advent of somatic cell hybridization in the early 1960s. Interspecific hybrids experience chromosome segregation that results in preferential loss of chromosomes from one of the parental complements. This facilitates the mapping of human genes because rodent-human hybrids typically lose human chromosomes during hybrid proliferation (19). Despite the more rapid loss of chromosomes in interspecific hybrids than in intraspecific somatic cell hybrids, the reduction in chromosome number may be insufficient for practical karyotypic

analysis. Incomplete chromosome segregation delays gene mapping studies because extensive cultivation and recloning of hybrids are often necessary to achieve the karyotypic stability from which positive correlations can be made between gene expression and the presence of a specific chromosome (8,18,19). Microcell-mediated chromosome transfer is a more direct approach to gene mapping because in a single step it produces hybrids containing one or a few human chromosomes. Nonetheless, application of this technique has been restricted by the type of donor cell that may be used for microcell production. Extrusion subdivision, although not restricted to HeLa cells, is difficult to induce in other human cell types (12). Microcell production by the method of Ege and Ringertz (2) is applicable only to cells that remain attached to slides during enucleation. It is also limited by the numerous enucleation slides required to grow enough cells to obtain an optimal number of micronuclei. Although suspension enucleation of cells in a discontinuous Ficoll gradient containing Cyt B (20) is an alternative, it is certainly more complicated, time consuming, and traumatic to the cells than the enucleation method of Gopalakrishnan and Thompson (5).

In this report we have presented a method for producing microcells which circumvents these limitations on donor cell type. We chose a lymphoblastic cell line as a model for this production method for several reasons. GM 130 lymphoblasts do not exhibit extrusion subdivision after recovery from colcemid treatment but do contain numerous micronuclei per cell. These lymphoblasts do not adhere to plastic but are easy to cultivate in suspension so that large numbers of cells can be obtained in a short time. Finally, it has been demonstrated by Gopalakrishnan and Thompson (5) that the percent enucleation of lymphoid cells approaches 100% using their Con A method. In addition, this method has proven effective for enucleating fibroblastic and epitheloid cell lines, suggesting that our microcell production method may be applied to these donor cells as well. Cells limited in Con A receptors may be linked to slides using other ligands. This would allow microcells to be easily prepared from any cell that can be micronucleated.

The major difficulty encountered using lymphoblasts as donor cells for MMCT has been the recovery of hybrid colonies. Hybrid colonies could not be recovered in culture dishes unless feeder cells were added to provide "conditioning factors" necessary for the growth of individual microcell hybrid clones. Perhaps the use of early passage donor and recipient cells would overcome this problem as suggested by McNeil and Brown (9).

The microcell hybrids we analyzed contained five and seven human chromsomes at 7 wk after fusion. Although the size of a microcell used for fusion may be reduced by filtration through a Nucleopore filter, the chromosome content of that microcell cannot be controlled. A microcell of less than 3 μm may contain a single human chromosome or several of the smallest group F and G human chromosomes as in our H105 and H108 microcell hybrids. Assuming that the process of micronucleation is random in the GM 130 cell line it is conceivable that a microcell hybrid containing a single human chromosome could be generated using our method.

A limitation of MMCT, in general, has been that it cannot be used, at this point, to generate a library of hybrids containing one of each of the 24 human chromosomes. This has been due to the lack of selection systems specific for a gene on each chromosome. However, as suggested by McNeill and Brown (9), it may be possible to randomly integrate the viral TK gene into each human chromosome using DNA-mediated gene transfer. This would allow different human chromosomes to be selected by the HAT system.

Microcell-mediated chromsome transfer, however, is no longer limited by the type of donor cell from which chromosomes may be transferred. We have defined a technique for generating microcells from donors which do not readily form minisegregants or cannot be easily enucleated by conventional methods, or both.

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