SELECTING INTERSPECIFIC HUMAN-MOUSE AND CHINESE HAMSTER-MOUSE HYBRIDS USING A NEW HALF-SELECTION TECHNIQUE WITH A POLYENE ANTIBIOTIC

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Interspecific human-mouse and Chinese hamster-mouse hybrids were isolated from polyethylene glycol fused cells by a new half-selection technique employing a structurally modified polyene macrolide antibiotic, amphotericin B methyl ester (AME), and HAT media. Unfused parental cells were killed as a result of innate sensitivity to AME or their genetic deficiency, absence of thymidine kinase (TK^-) or hypoxanthine guanine-phosphoribosyl transferase ($HGPRT^-$). In contrast, hybrid colonies were isolated after two to three weeks growth in three or four changes of HAT-AME media and subsequent growth in HAT media alone. The ability of hybrid cells to proliferate using this selective protocol indicates that genetic complementation resulted, and polyene antibiotic resistance was expressed as a dominant phenotypic property in the hybrids. Hybrid selection was dependent on: (1) the number of cells of each parental cell type co-cultivated; (2) the level of polyene antibiotic administered; and (3) the time interval before selection was initiated.

The half-selection technique described in this report is simple to use, very effective in eliminating unfused parental cells and increases the potential types of hybrids which can be formed. Only one parental cell type need contain a biochemical defect, whereas the second parental type can be genetically normal.

The technique of somatic cell hybridization has provided scientists with a unique methodology for investigating many diverse genetic characteristics of eukaryotic cells[1]. A factor limiting the usefulness of this procedure, however, has been a lack of different types of selective systems for hybrid isolation. The most commonly used selective system called HAT selection[2,3] requires two different genetically deficient cell types, one resistant to 5-bromodeoxyuridine (lacking thymidine kinase) and one resistant to azaguanine or thioguanine (lacking hypoxanthine guanine phosphoribosyltransferase). Hybrid cells proliferate in HAT media, containing hypoxanthine, aminopterin and thymidine, as a result of genetic complementation. Other selective systems have used mutant cells resistant to fluoroadenine[4], cytosine arabinoside[5], 5-bromodeoxycytidine[6], and ouabain[7]. Additional selection methods have employed auxotrophic mutants[8] or temperature sensitive mutant mammalian cells.[9] A problem in using mutant cells for hybridization is the difficult and time-consuming process required for formation, isolation and characterization of mutants[10,11].

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Studies using the structurally modified polyene macrolide antibiotics, amphotericin B methyl ester (AME) and nystatin methyl ester (NME) have demonstrated major differences, in some cases 50 fold, in the innate sensitivity of eucaryotic cells to these agents[12~16]. In addition, investigation of the effect of AME and NME on intraspecific mouse-mouse and interspecific hamster-mouse hybrids[15,16] have indicated that polyene macrolide resistance was expressed as a dominant trait in these systems. Hybrid cells were found to exhibit the same resistance pattern as the more resistant parental cell type. These observations clearly suggested the possibility of utilizing AME or NME as a half-selection agent for isolating somatic cell hybrids. This half-selection procedure, HAT media plus AME or NME, has been utilized to isolate mouse-Syrian hamster hybrids[17,18]. In the present study, we report on the use of AME in conjunction with HAT media in the selection of human-mouse and Chinese hamster-mouse hybrids.

Materials and Methods

Cells and Culture Conditions

The genetically normal cell types used were CHO, a Chinese hamster ovary cell line, and GM 17, a normal human skin fibroblast culture (passage number 10). The genetically mutant cell lines consisted of three mouse L cell derivatives; C1 1D and B82 cells which are resistant to 5-bromodeoxyuridine (BUdR) and are TK^- (lack thymidine kinase) and A9 cells which are resistant to 8-azaguanine (AZ) and are $HGPRT^-$ (lack hypoxanthine guanine phosphoribosyltransferase).

CHO cells were grown in DULBECCO's modified EAGLE's medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin-referred to as DMEM-10. GM 17 cells were grown in DMEM containing 20% FCS (DMEM-20). C1 1D and B82 cells were maintained in DMEM-10 containing 30 μg/ml of BUdR. A9 cells were routinely cultured in DMEM-10 supplemented with 20 μg/ml of AZ. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

After selection, hybrids were maintained at 37°C in DMEM-10 containing 10^{-4} M hypoxanthine, 5×10^{-5} M aminopterin and 1.6×10^{-5} M thymidine (DMEM-HAT-10).

Polyene Macrolide Antibiotics

The structurally modified polyene macrolide antibiotic amphotericin B methyl ester (AME) was synthesized as previously described[19]. AME was dissolved in dimethyl sulfoxide (DMSO) immediately prior to use. The final concentrations of AME in DMEM-HAT-10 were 10, 25, 50, 100, 200 or 250 μg/ml in 1% DMSO.

Cell Fusion

Genetically mutant (C1 1D, B82 or A9) and normal (CHO or GM 17) cells were fused in monolayer culture using polyethylene glycol (PEG 6000) as described by PONTECORVO *et al.*[20]. Approximately $0.8\sim1.2\times10^6$ mutant and various concentrations of normal cells, 1×10^5, 5×10^4, 1×10^4 or 5×10^3, were co-cultivated in T-flasks (Microbiol. Assoc.) of 25 cm^2 area in 5 ml of DMEM-10 for 24 hours at 37°C prior to fusion. Immediately following the fusion protocol, DMEM-10 was added to the monolayers and the cells were incubated for 24, 48 or 72 hours at 37°C before initiating the selection process.

Hybrid Selection

Two procedures were used in hybrid selection: (1) selection of hybrid clones from the original T-flasks used for fusion; and (2) subculturing cells (1 into 2 T-flasks, 1: 2 split ratio) and then initiating selection. The first technique permits the isolation of different hybrid clones of presumably independent origin from the same T-flask. The second technique results in large number of hybrid colonies, but has the disadvantage that there is no assurance that the colonies obtained represent separate fusion events.

The basic selection procedure consisted of: (a) adding a fixed concentration of AME (10~250

μg/ml) in HAT media to fused cultures 24, 48, or 72 hours after fusion; (b) refeeding fused cultures every 2~3 days with HAT-polyene media (a total of 3 or 4 media changes); (c) growth of HAT-poly ene surviving cells in HAT media alone; and (d) isolating presumptive hybrid colonies, based on morphology, after 2~3 weeks growth using the steel cloning cylinder technique[21].

Karyotype Analysis

Chromosome spreads from parental (CHO, GM 17, C1 1D, B82 and A9), and HAT-AME selected human-mouse (GM 17-C1 1D, GM 17-B82, GM 17-A9) and Chinese hamster-mouse (CHO-C1 1D, CHO-B82, CHO-A9) hybrid clones were prepared by colcemid pretreatment using the method of SUMNER *et al.*[22]. A total of 50 metaphase spreads from each culture was used to determine average chromosome number and chromosome morphology.

Growth Properties

The effect of AME on the growth and survival of parental and hybrid cells was determined as previously described[12~16]. The concentrations of AME or NME resulting in a 50% reduction in viable cell number (TCD_{50}), after a 48~72 hours exposure, was determined. In addition, the lethal concentration, *i.e.* the level of polyene antibiotic resulting in <1% viable cells, was also determined.

Results

Amphotericin B methyl ester demonstrated a differential toxicity toward CHO, GM 17, C1 1D, B82 and A9 cells (Table 1). A comparison of the TCD_{50}'s (the level of polyene macrolide antibiotic resulting in approximately a 50% reduction of viable cell number after a 48~72-hour exposure) indicated that AME was approximately 3-fold more toxic toward CHO and about 1.5-fold more toxic toward GM 17 cells when compared with either C1 1D, B82 or A9 cells. Total cell death (<1% viable cells) resulted after a single exposure of CHO or GM 17 cells to 200~250 μg/ml of AME, whereas there was significant survival of C1 1D, B82, or A9 cells even after treatment with 400 μg/ml (Table 1). Additional studies, not shown here, provided further evidence for selective toxicity as revealed by the effect of AME on the growth rates of parental cells. The exponential population doubling time of CHO cells was increased (growth rate decreased) after exposure to >45 μg/ml of AME and >100 μg/ml was required to increase the population doubling time of GM 17 cells. In contrast, >200 μg/ml was required to reduce the growth rate of C1 1D, B82, or A9 cells.

HAT selective media was not toxic toward GM 17 or CHO cells, whereas C1 1D, B82 and A9 cells failed to survive in this media. The genetically normal parental cells grew when plated at $0.1 \sim 2 \times 10^6$

Table 1. Toxicity of amphotericin B methyl ester (AME) toward parental cells.

Cell type	Origin of cells	TCD_{50}* (μg/ml)	Lethal concentration (μg/ml)**
GM 17	Human skin fibroblast (passage number 8)	175	250
CHO	Chinese hamster ovary	75	200
C1 1D	TK^-, L cell derivative	250	>400***
B82	TK^-, L cell derivative	250	>400***
A9	$HGPRT^-$, L cell derivative	275	>400***

* Concentration of polyene macrolide antibiotic resulting in approximately a 50% reduction in viable cell number, after 48~72-hour exposure when compared with control (untreated or DMSO treated) cultures.

** Lowest concentration of AME resulting in death of >99% of the treated cell population after 24~72 hour exposure. Viability was determined by trypan blue dye exclusion[12].

*** Considerable numbers of these resistant cells (>10%) survived after 72-hour exposure to 400 μg/ml, the maximum AME concentration tested.

cells in 4 ml of DMEM-HAT. In contrast, all three genetically mutant cell types failed to survive in DMEM-HAT media, even when plated at $2\sim3\times10^6$ cells, as a consequence of their genetic deficiency, lack of TK (C1 1D and B82) or HGPRT (A9).

The basic procedure used to isolate hybrid clones from fused cultures of genetically normal and mutant parental cells with HAT media and a structurally modified polyene macrolide antibiotic (AME) is presented in Fig. 1. The efficiency of this new "half-selection" technique was found to be dependent on: (a) the number of each parental cell type co-cultivated prior to fusion; (b) the level of polyene macrolide antibiotic administered to fused cultures; and (c) the time interval before HAT-polyene macrolide selection was initiated. In general, HAT-polyene macrolide selection of hybrids was most efficient when $0.8\sim1.2\times10^6$ mutant L-cell parents were co-cultivated with $0.5\sim1\times10^4$ normal parental cells exhibiting an AME lethal concentration of $>125\,\mu$g/ml (Table 1). When using normal cell types with an AME lethal concentration of $>125\,\mu$g/ml, higher efficiencies of hybrid formation were

Fig. 1.

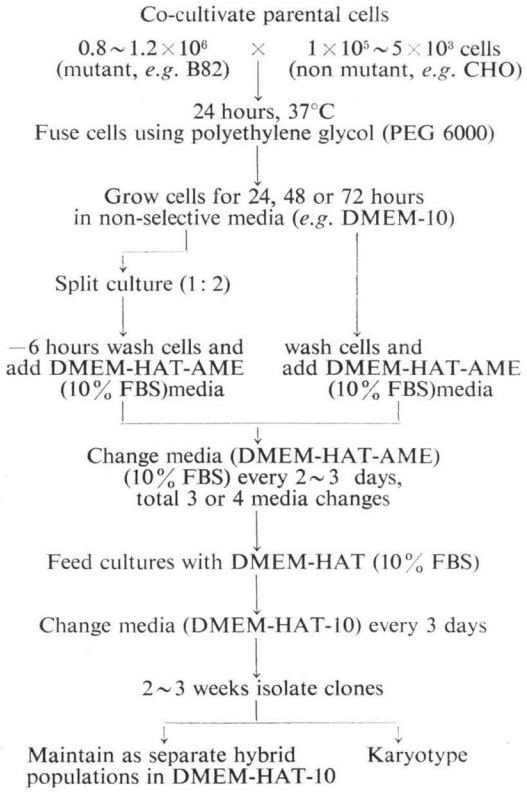

Table 2. Karyotype analysis of parental and hybrid cells selected using HAT media supplemented with amphotericin B methyl ester (AME).

Cell type	Total chromosome number*	Chromosome morphology**		
		Metacentrics	Acrocentrics with distinct shorter arms	Acrocentrics without distinct shorter arms
GM 17	46±1	10~12	26~29	5~7
CHO	20±1	9~10	7~8	2~3
C1 1D	52±4	7~12	—	40~46
B82	49±4	12~13	7~8	26~29
A9	57±6	14~17	34~40	3~6
GM 17-C1 1D***	86±6	15~22	22~27	39~44
GM 17-B82***	90±3	18~24	30~35	28~34
GM 17-A9***	104±5	24~37	58~68	5~8
CHO-C1 1D+	70±4	15~22	6~8	42~49
CHO-B82+	69±5	18~23	14~16	27~32
CHO-A9+	75±6	23~27	42~48	5~9

* Average chromosome number was determined from 50 metaphase spreads.
** Range of chromosomes of each distinct type from 50 metaphase spreads.
*** 250 μg/ml AME used in selection protocol.
\+ 200 μg/ml AME used in selection protocol.

obtained by co-cultivating $0.5 \sim 1 \times 10^5$ of these cells with $0.8 \sim 1.2 \times 10^6$ C1 1D, B82 or A9 cells. The level of polyene macrolide antibiotic which resulted in the highest selection efficiency (greatest number of hybrid clones and lowest number of parental cells) was dependent on the types of cells being fused. The innate polyene macrolide sensitivity of eucaryotic cells appears to be genetically stable[23] and must be determined for each potential parental cell type prior to fusion. In general, we have obtained the best results by using an AME concentration which is lethal for the more sensitive parental cell type. In addition, hybrid selection was most efficient when initiated 48 or 72 hours after fusion and growth in non-selective media[18].

In most cases, presumptive hybrid colonies were clearly distinguishable from parental cells by an increase in overall size and/or number of nucleoli present in their nuclei. Confirmation of the hybrid nature of HAT-AME selected colonies was clearly demonstrated by karyotype analyses (Table 2). In all cases, except certain human-mouse hybrid clones, the hybrids contained approximately the equivalent of one genome from each parental cell type and marker chromosomes characteristic of each respective parent. In the case of some early passage human-mouse hybrid clones, a decrease in the total complement of human chromosomes was apparent. However, in all hybrid systems examined, no evidence of gross qualitative karyotype abnormalities were evident.

Discussion

Polyene macrolide antibiotic toxicity toward eucaryotic cells has most often been equated with membrane permeability alterations which can result in a loss of essential metabolites and consequently cell death[24~27]. However, studies comparing membrane damage and cytotoxicity induced by amphotericin B and nystatin and the structurally modified polyene macrolides, AME and NME, suggest that the ability of cells to repair membrane damage is also an important mediator of polyene macrolide toxicity[14~16]. Further evidence indicating the importance of repair processes in the modulation of polyene macrolide antibiotic toxicity has been presented for filipin and mediocidin[27]. The innate differential toxicity of AME toward eucaryotic cells probably reflects differences in membrane structure, specifically with respect to cholesterol components. Differences in the amount, type and/or orientation of specific sterol(s) in the membranes of eucaryotic cells may be the reason for this innate differential sensitivity to AME and NME[15~19]. The expression of high level polyene macrolide resistance (characteristic of the mutant mouse L-cell lines) and complementation of the genetic defect (by the non-mutant cell lines) in hybrids has, therefore, enabled us to use HAT-media supplemented with AME in hybrid selection.

The efficiency of hybrid selection using HAT-media supplemented with AME was directly related to: (a) the initial number of co-cultivated parental cells; (b) the level of polyene macrolide antibiotic used in selection; and (c) the time interval before initiating HAT-polyene selection. The number of non-mutant polyene macrolide sensitive cells co-cultivated with mutant polyene macrolide resistant cells was dependent on the degree of sensitivity of the two parental strains. In general, lower numbers of cells and higher levels of polyene macrolides were required to eliminate the more resistant normal cells (*i.e.*, CHO or GM 17) following fusion, whereas more efficient hybrid selection has resulted when using higher numbers of cells and lower levels of polyene macrolides to eliminate more sensitive normal cells (*i.e.*, BHK/C 13) after fusion[17,18]. The time sequence before initiation HAT-polyene macrolide selection was also an important factor in determining hybrid selection efficiency. Best results were obtained when initiating selection $48 \sim 72$ hours after fusion and growth in non-selective media[18]. Apparently, some period of time (usually > 24 hours) is required for cell recovery from polyethylene glycol fusion, for membrane reorganization to express polyene macrolide resistance and/or for expression of growth in HAT, before using HAT-polyene macrolide selection.

The presently described "half-selection" technique should find wide application in hybrid selection because: (a) eucaryotic cells exhibit wide spectrums of sensitivity to AME[12~15], therefore, increas-

ing the potential types of hybrids which can be formed using this procedure; (b) polyene macrolide sensitive cells are efficiently killed using the appropriate concentrations of AME (usually after 48~72 hours) and complete resistance to polyene macrolides in general is rare[23]; (c) only one parental cell type need have a specific genetic defect, thereby avoiding the tedious process of forming and isolating two different and complementing mutant cell strains; and, (d) the basic selection procedure is fast and efficiently eliminates unfused parental cells, thus enabling scientists to form and select hybrids between cell combinations which would not yield a high percentage of hybrids by previous selection techniques.

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