Preservation of Human Epithelial-Like and Fibroblast-Like Cell Strains at Low Temperatures.*† (24067)

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The development of numerous strains of mammalian cells has created many problems with regard to their maintenance. For example human epithelial-like (Ep-L) cell strains undergo biologic changes both during their development into "stable" strains and while being maintained under conditions of rapid growth and frequent subculture(1). In addition, human fibroblast-like (Fb-L) cell strains may be difficult to maintain as continuous cell lines(1). Scherer and Hoogasian(2) studied various methods of preserving cell strains at low temperatures, and observed that either fast or slow freezing in 20-30% glycerol medium, storage at −70°C. and rapid thawing, were optimal conditions for preserving HeLa cells. Swim *et al.*(3), have found that survival of their cell strains depended on methods of freezing and glycerol concentration, as well as on methods of cultivation prior to freezing and on sources of their strains. Experience in our laboratories has shown that although rapid freezing in 20% glycerol medium could be used for preservation of various Detroit Ep-L strains for as long as one year, the technic was not a satisfactory solution to the problem because cells were damaged during the freezing and their recovery was unpredictable. Moreover, by that procedure it was not possible to recover human fibroblast-like strains at all.

In a recent review of the mechanics of freezing(4), it was pointed out that cell damage might be minimized if non-toxic concentrations of a water binder are allowed to pass through cell membranes over temperature ranges where intracellular crystallization and dehydration denaturation usually occur. This principle was applied in the present study. This communication describes a procedure of slow freezing in 5% glycerol medium, storage at −70°C, and rapid thawing, as an efficient and practical means of preserving both epithelial-like and fibroblast-like human cell strains.

Procedure. Establishment and characterization of 17 epithelial-like (Ep-L) human cell strains employed, have been described (1,5,6). The fibroblast-like (Fb-L) human cell strains were derived from a teratoma of the testes (Detroit-504 Fb-L) and from kidney (Detroit-506 Fb-L) by methods previously described(7). The cells of various strains were cultivated in 3-ounce prescription bottles with Eagle's basal medium(8) supplemented with 16% human serum. Cellular monolayers occurring in 7-day-old cultures were treated with 0.25% trypsin in calcium-free Hanks' balanced salt solution for 10 minutes at room temperature. The resulting cell suspensions were centrifuged and resuspended in a medium consisting of basal medium 75%, human serum 20%, and glycerol 5%. Following cell counts made with a hemacytometer, cell suspensions were appropriately diluted (and cells recounted) so as to contain approximately 5 x 10^5 cells per ml. One ml aliquots were transferred to ampules which were then either glass-sealed or rubber-stoppered. In earlier experiments, slow freezing was accomplished by immersing the ampules in an ice-alcohol bath at approximately −4°C and then slowly lowering the temperature to −15°C over a period of 30 minutes. The ampules were then placed in a freezer maintained at −25°C until their contents were solidly frozen, then transferred to a dry-ice chest maintained at −70°C for permanent storage. This will be referred to as procedure "A". More recently, we have used a mechanical slow-freeze apparatus‡ with

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‡ Manufactured by Canal Industrial Corp., Bethesda, Md.

TABLE I. Preservation and Recovery of Human Cell Strains by Slow Freezing in 5% Glycerol-Medium, Storage at –70°C and Rapid Thawing.

Cell strain (Detroit)	Cell type	Slow-freeze procedure	Months stored (–70°C)	Cell counts × 10^5			
				Frozen	Thawed	Harvested from 7-day culture of thawed cells	Harvested from Subcultures (inoculum 3 × 10^5, cultured 7 days)
6	Ep-L	A	6	5.1	4.9	36.8	82.5
32	"	A	6	5.4	4.5	20.5	60.2
34	"	A	6	4.7	4.7	27.0	92.0
52	"	A	6	3.6	2.9	21.0	46.3
56A	"	A	6	4.0	4.2	16.0	48.6
98	"	A	6	3.4	2.8	23.9	35.6
196 Clone V	"	A	2	5.5	4.5	72.0	
" "	"	B	2	5.5	4.8	86.0	
" III	"	B	2	5.4	5.1		
B16	"	A	6	5.5	4.4	25.2	74.0
" I	"	B	2	5.4	4.7		
" II	"	B	2	5.0	5.6		
B17	"	A	6	5.1	4.7	46.0	80.6
B78	"	A	6	4.3	4.5	21.3	71.9
" I	"	A	1	4.7	4.5	22.8	106.0
" I	"	B	1	4.7	5.0	39.6	103.0
" II	"	A	1	5.3	3.0		
" II	"	B	1	5.3	3.6		
173B	"	A	6	4.8	4.8	68.0	109.0
501	"	A	6	4.9	5.5	19.8	50.2
504	Fb-L	A	1	4.7	3.5	6.7	21.0
506	"	A	1	5.1	3.5	8.0	
"	"	B	1	5.1	3.8		

which it was possible to lower the temperature at a controlled rate of one degree per minute until it reached –25°C. At this point, the temperature was quickly lowered to –70°C, at which level the cells were permanently stored. The controlled rate method will be referred to as procedure "B". Cell suspensions were thawed within 30 seconds by placing ampules under running water at 37°C. The entire contents of an ampule were diluted to 8 ml with growth medium (basal medium + 16% human serum, pH 7.2), and after determination of the number of thawed cells present, were cultured according to the procedure described above. Replicate bottle cultures of thawed cells were prepared to determine their growth curves by cell counts.

Results. In Table I are listed examples of each of 17 epithelial-like cell strains frozen by procedures A or B, and stored at –70°C. With the single exception of Detroit-B78 Clone II, a comparison of the number of Ep-L cells frozen to the number seen immediately after thawing revealed that 80% to 100% appeared undamaged (intact) as viewed in the counting chamber. Each of the thawed cell strains was then cultured as described (the medium changed on 3rd and 6th day), and resultant cell yield was determined at end of 7-day incubation periods. In most instances these cell yields were lower than those usually obtained from weekly serial passage. This would suggest that not all the thawed cells counted were actually capable of reproduction. From the harvested cells uniform cell inocula (3 x 10^5 cells) were prepared which allowed comparisons of growth rates of recovered strains to those regularly maintained by routine subculture every 7 days. The cell yields obtained from each recovered strain after one subculture were of the same order of magnitude as unfrozen strains. No changes in the appearances of cell monolayers or individual cell morphology could be detected. Also, thawed cells of clonal strains were recovered in numbers equivalent to those recovered in the parent strain cultures and when cultured, gave yields equivalent to unfrozen strains (Table

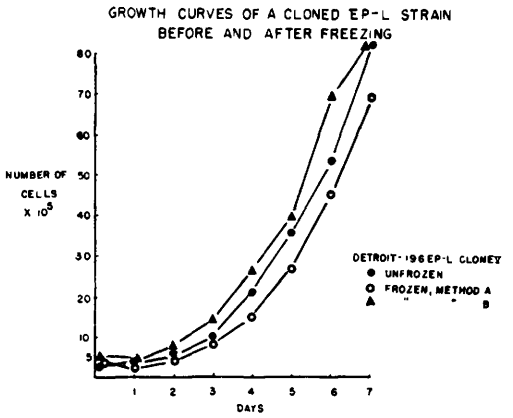

FIG. 1. Growth curves of Detroit-196 Ep-L Clone V cell strain. Curves depict rate of multiplication of unfrozen cells compared to the thawed cells which had been frozen according to procedures A and B.

I). Of such strains studied, only one, Detroit-B78, derived from normal peripheral blood (6), yielded 2 clonal strains that were affected differently when frozen simultaneously either by procedure A or B. The number of cells recovered from the original cell line and one of the clonal lines were similar. Detroit-B78 Clone II, however, yielded fewer cells after thawing.

The 2 fibroblast-like strains, Detroit-504 Fb-L and -506 Fb-L, were subjected to the freezing procedures. Although the number of cells recovered immediately after thawing was somewhat lower than from Ep-L strains (65-75% as compared to 80-100%), the thawed cells could easily be cultured with no change in morphology or growth rate. Cell yields of established Detroit Fb-L strains have always been characteristically lower than Ep-L strains. It is noteworthy that 3 months after its isolation as a stable Fb-L strain, growth rate of Detroit-504 Fb-L diminished and eventually the strain was lost. Cells frozen before this event, however, have been consistently recovered from the frozen state, as illustrated by the example in Table I. Moreover, the recovered strains have been continuously maintained by serial subculture in several laboratories in addition to our own. Because of the importance of fibroblast-like strains for certain virologic problems(7,9), the ability to preserve them in this fashion is of considerable practical significance.

Fig. 1 illustrates the growth curve of an unfrozen clonal strain of Ep-L cells as compared to the same strain frozen by method A and method B, and stored for 2 months at –70°C. The similarities of the curves indicate that most, if not all, of the thawed cells retained their ability to multiply.

Discussion. These results indicate that the controlled rate slow freezing procedures are a practical and efficient means for preserving various kinds of human cell strains. Moreover, a comparison of rapid and slow methods of freezing expose differences between human fibroblast-like (Fb-L) and epithelial-like (Ep-L) cell strains. With rapid freezing technics, Fb-L cells cannot be recovered, but with the slow freeze methods described, the Fb-L cells can be recovered successfully. The practical significance of this is that it has been shown that human Fb-L cell strains are capable of supporting the propagation of viruses not able to multiply readily in Ep-L cell strains(7,9), yet human Fb-L strains are difficult to maintain indefinitely by serial subculture. The fact that the fibroblast-like form of human cell strains cultured by present methods usually represents a temporary stage in the development of epithelial-like lines(1), and the relative rarity of long term human Fb-L cell lines, makes it important to utilize methods which will insure a supply of human cell strains in the Fb-L state. We feel that the freezing procedures described herein satisfy these requirements. A more obvious application of these procedures is the circumvention of difficulties involved in continuous maintenance of large collections of cell strains such as cost, contamination, changes in virus susceptibility, and cell variation.

Summary. Studies of cell preservation by slow freezing in 5% glycerol medium, storage at –70°C, and rapid thawing were carried out on 17 epithelial-like and 2 fibroblast-like human cell strains (Detroit). Following this procedure, each of the strains could be quickly recovered as actively proliferating cell strains with no changes in growth rate or strain characteristics.

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An Orally Active Compound with Anti-Fertility Effects in Rats.* (24068)

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Inhibition of fertility in rats can be achieved by interfering with normal mechanisms at any one of several vulnerable points in the reproductive process. These include impairment of gametogenesis by compounds having direct action on germ cells(1,2), prevention of ovulation by administering steroids which suppress pituitary production of gonadotrophins(3), withdrawal of progestational support of the endometrium(4), or destruction of developing embryos with anti-metabolites(5). The use of estrogens to terminate pregnancy in rats was reported 3 decades ago by Smith(6). Subsequently, numerous investigations established that high dosages of estrogen cause tubal blockage when administered immediately after fertilization, while later treatment interferes with maintenance of a progestational endometrium(7,8). A type of activity completely different from any of those mentioned above appears to be characteristic of a particular triphenyl ethanol derivative, 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-anisylethanol.† When female rats are treated for a brief post-copulatory period with an adequate dose of this compound, implantation never occurs. These animals enter a period of pseudopregnancy, characteristic of rats following sterile mating, and upon resumption of estrous cycles, are completely capable of bearing normal litters(9). This report presents experiments leading to these observations. We have attempted to establish the phase of the reproductive process influenced by MER-25, dosage requirements for the compound's anti-fertility effects, and site of its activity.‡

Methods. Fertile female rats of Sprague-Dawley strain were placed with proven male breeders during early evening and vaginal smears were examined for spermatozoa the following morning. The day on which sperm were found in the vaginal smear is considered day 1 of pregnancy. On this basis, days 1-3 and part of day 4 comprise the period of oviducal passage. The remainder of day 4 and day 5 follow tubal exit when blastocysts are free in the uterus and days 6, 7, 8 are the period of implantation. Formulating dosage schedules in relation to these reproductive events, MER-25 was administered by stomach tube on a body weight basis. The effectiveness of systemic administration has been reported(10). Concentrations were established so that the animals uniformly ingested 0.1 ml of olive oil solution/100 g body weight. Body weights and vaginal smears were recorded daily. Eight to 12 days post-coitus, the animals were laparotomized and both uterine horns

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