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2 January 1969

Spin Filter Culture: The Propagation of Mammalian Cells in Suspension

Abstract. *A spin filter device has been used for the propagation in vitro of cells of mouse leukemia L1210 to densities approaching 10^8 cells per milliliter. By manipulation of flow rates, cells may be exposed to exponentially declining drug concentrations with half-times of 1.5 hours or less, providing a more realistic parallel to in vivo drug treatment than is obtained by other culture methods.*

We have developed a new system for cell propagation and chemotherapy studies based on a spin filter device (*1*) (Fig. 1) which allows the growth of murine leukemia cells to very high levels, and permits in vitro drug exposures under conditions paralleling the situation in vivo. Basically the unit (*2*) incorporates a spinning filter which because of boundary effects has the property of allowing passage of very large volumes of filtrate without becoming clogged by retained cells.

When mice are inoculated intraperitoneally with the murine leukemia L1210, the population density of leukemic cells in the ascitic fluid at the time of death reaches approximately 2.5×10^8 cells/ml (*3*). However, L1210 cells have been grown in vitro in suspension by conventional means only to population densities of approximately 2 to 3×10^6 cells/ml. The upper limit which such in vitro populations attain may be related to both nutrient depletion and the production of toxic materials. The standard in vitro culture techniques, including chemostats (*4*), have another serious drawback related to chemotherapy studies, namely, that it is difficult to achieve short drug exposures with exponential decrease of drug concentration. In vivo, the drug half-time can be as short as 30 minutes, and seldom exceeds 2 hours, because of alteration or excretion of the drug by the host system. Certain workers (*5*) have devised schemes for perfusing mammalian cell cultures with nutrient solutions in order to achieve higher cell densities than could be reached by conventional culture methods, and these could presumably be adapted to drug studies.

The spin filter device in one of its embodiments comprises a hollow nylon core surrounded by a filter cartridge held in place by a slip-seal over "O" rings. The filter is supported in the culture vessel within a three-legged framework, turning on a sapphire bearing and held vertical by a 21-gauge needle through the upper Teflon bearing. The needle also serves the function of providing for removal of filtrate by suction. The cartridge is made of a stainless steel mesh covered by a membrane filter with a 3-μ porosity (*6*). The filter is rotated by a magnetic stirrer at approximately 300 rev/min. The culture fluid is gassed at a rate of 10 cm^3/min with carbon dioxide and air (5:95) from an entry point below the surface of the culture. Nutrient outflow is controlled with a metering pump (*7*), and the level of culture fluid within the vessel is regulated by a constant level controller (*8*) which actuates a solenoid valve in the gravity nutrient feed line.

To date, our cell culture studies with the spin filter device have been confined to the mouse leukemia line L1210 (*9*), although there is no a priori reason why other suspension-growing cell lines could not be similarly cultured. Approximately 300 to 400 ml of a spinner culture containing 1 to 2×10^6 cells/ml is poured into the spin filter vessel and, after dilution to desired volume, the outflow is started through the spinning filter. Lower inocula can

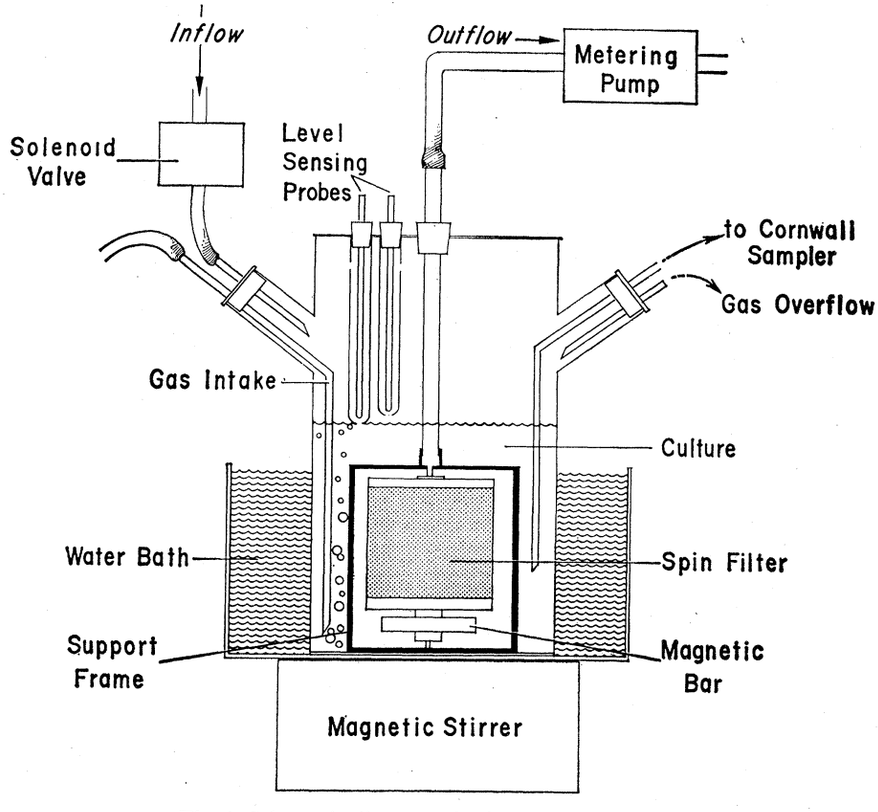

Fig. 1. Schematic diagram of spin filter culture system.

Table 1. Effect of fast perfusion on various biochemical parameters of L1210 cells in spin filter culture.

Duration (hr)*	Thymidine uptake†	Deoxyuridine uptake†	DHFR§	T.S.¶
0	34.1	21.3	970.1	5563
3.5	44.4	23.5	918.2	5070
6.5	48.3	30.0	824.1	3685
24.0	38.8	19.9	1038.0	3336

* Flow rate equivalent to half-time of 0.74 hour through 6.5 hours; 2.75 hours half-time from 6.5 to 24 hours. † Millimicromoles per gram (wet weight) per hour. § Dihydrofolate reductase, millimicromoles per gram of protein per hour. ¶ Thymidylate synthetase, millimicromoles per gram of protein per hour.

be used at some loss of efficiency and time. The nutrient consists of Fischer's medium (*10*) with 10 percent horse serum and antibiotics. Samples for cell counts or other purposes are taken at regular intervals, and nutrient flow rates are increased as the cell population rises. An initial flow rate of approximately 30 to 40 ml/hour is sufficient to support the growth of the population up to approximately 5×10^6 cells/ml at which level the flow rate is increased to ~ 50 ml/hour. At densities of ~ 1×10^7 cells/ml or higher we have used nutrient flow rates of 75 to 100 ml/hour, although higher flow rates might be required to maximize growth rates.

With the spin filter device, we have grown L1210 cells to densities which are extremely high for in vitro suspension cultures, at times reaching 10^8 cells/ml. Since centrifugation of L1210 cells shows a packed cell volume of 1 ml per about 10^9 cells, this culture density represents a 10 percent cell suspension. Figure 2 illustrates the growth curve of a culture which reached a final cell level of 6.5×10^7 per milliliter, showing a progressive decline in growth rate above 10^7 cells/ml. In a number of similar runs, doubling time has begun to increase at ~10^7 cells/ml, and even further once 4×10^7 cells/ml is reached.

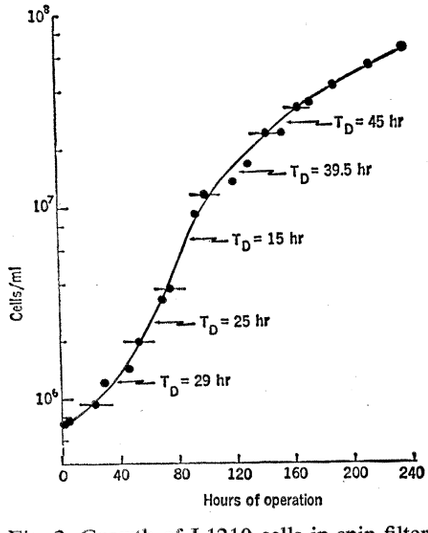

Fig. 2. Growth of L1210 cells in spin filter culture: Fischer's medium at flow rates of 30 to 70 ml/hour. T_D = doubling time calculated for period between horizontal bars.

The efficiency of the spin filter device for the propagation of cells can be expressed in two ways. First, it does not yet provide for the more efficient utilization of nutrient, the volume of medium required for the growth of a given mass of cells being quite comparable (at high cell levels) to the volume of total culture required for the same number of cells grown by other means. Second, however, it is considerably more efficient in terms of culture vessel volume and final harvesting volume by a factor of as much as 50 : 1 (for example, 300 ml at 10^8 cells/ml versus 15 liters at 2×10^6 cells/ml).

We have assayed the cloning efficiency of L1210 cells at high densities in the spin filter system by our soft agar cloning technique (*9*) and find it to be within the range of 40 to 60 percent which we customarily observe for actively growing suspension cultures in standard spinner flasks.

As for other types of cultures of this L1210 cell line, a gas atmosphere enriched with CO_2 is essential for successful growth in the spin filter system. Furthermore, the manner in which gas (5 percent CO_2, 95 percent air) is supplied to the culture vessel appears to be important. The cells will grow to densities higher than 10 to 20×10^6 only when the gas is introduced under the surface of the culture.

To use this device in chemotherapy studies for controlled drug removal, assurance is needed that concentrations of drug will decline as predicted from flow rates, that is, that the spin filter vessel is a "homogeneously stirred reactor." Furthermore, the conditions used to control drug efflux should not adversely affect the cells. Chemotherapy studies with L1210 cells have been done by the repeated addition of single doses of amethopterin (NSC 740) to the culture, followed by accelerated perfusion at drug half-times ($T_{1/2}$) of 0.5 to 1.5 hours, for 9 to 10 hours (*11*), that is, at flow rates of 550 to 185 ml/hour, through a culture volume of 400 ml. We have measured the course of drug concentration following two successive daily doses of tritiated amethopterin added at a level of 10,000 count min^{-1} ml^{-1}. The drug concentration was calculated from flow rates and was measured at several times by radioactivity. There was no distinguishable difference between the actual and calculated drug concentrations. For both days the drug half-time was 1.15 hours by calculation and 1.14 by measurement. For an exposure such as this, the integrated concentration × time ($C \times T$) can be calculated from the equation

$$C \times T = Co \left(\frac{t}{\ln 2}\right) [1 - e^{(-\ln 2 \times T)/t)}]$$

where t = half-time, T = elapsed time, and Co = initial concentration. For this case, with a half-time of 1.15 hours for a period of 10 hours, the $C \times T$ was 1.66 μg-hr. At this flow rate, the $C \times T$ does not increase significantly beyond the first 6 hours of perfusion.

The accelerated perfusion by itself does not appear to have any adverse effects upon either cloning efficiency or certain enzymatic activities of the cells. In two cases involving an accelerated perfusion of 9 hours duration without drug, there was no loss in cloning efficiency. In addition, Table 1 illustrates the effect of a 6.5-hour perfusion at a $T_{1/2}$ of 0.74 hour (followed by an overnight perfusion at a $T_{1/2}$ of 2.75 hours) on thymidine uptake, deoxyuridine uptake, dihydrofolate reductase, and thymidylate synthetase (*11*). It can be seen that there was little, if any, effect upon these parameters during the period of the accelerated perfusion or through the following day.

Although we have been primarily concerned with chemotherapy studies, there are several obvious possibilities as to how to improve the growth rate at high population densities. It is probable that certain components of the nutrient become growth-limiting before others and these could be increased. We have found, for example, that at high population densities ($> 10^7$ cells/ml) the glucose level in the culture fluid is reduced to nearly zero from the initial concentration of 1 mg/ml. At high densities there may be insufficient transfer of oxygen (or carbon dioxide). Two procedural modifications might also increase the efficiency of nutrient utilization: (i) operation of the spin filter as a modified continuous-flow system in which a portion of the cells (that is, of the total culture) would be contin-

uously harvested; (ii) recycle of partially spent nutrient. In a similar system in which we have grown *Lactobacillus casei* we have found that partially spent nutrient could be recycled, thereby improving the efficiency of nutrient utilization.

For chemotherapy studies it is of course possible to add or remove drug by other modes. To provide a model of in vivo perfusion, drug could be added with the nutrient so that the concentration in the culture would asymptotically approach the incoming level. For continuous perfusion at a constant drug level in such an experiment an initial dose of drug may be added directly to the culture. By control of flow rates, the decline of drug concentration could follow other kinetics besides the simple exponential model used so far. Further work is necessary to determine whether initial drug concentration or integrated $C \times T$ is more closely related to degree of cell kill, since for a series of initial concentrations a constant $C \times T$ can be produced by appropriate regulation of the flow rate.

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Gene Dosage at the Lactate Dehydrogenase b Locus in Triploid and Diploid Teiid Lizards

Abstract. *Triploid parthenogenetic lizards which are heterozygous at the lactate dehydrogenase* b *locus show approximately two doses of the* b *allele and approximately one dose of the* b′ *allele. This finding agrees with the triploid karyotype composed of two haploid complements of chromosomes indistinguishable from those of species carrying the* b *allele and one complement indistinguishable from that of the species which carries the* b′ *allele. Densitometry of electrophoretic patterns of lactate dehydrogenase from heart muscle and from kidney indicates approximately equal expression of each of the* b *alleles in the triploid lizards. Heterozygous diploid lizards from the same parthenogenetic species, in agreement with their karyotype, show equal doses of* b *and* b′ *alleles.*

An electrophoretic study of lactate dehydrogenase (LDH, E.C. 1.1.1.27) isozymes from seven *Cnemidophorus* species of the American southwest has revealed heterozygosity at the LDH *b* locus in two diploid parthenogenetic forms, *C. neomexicanus* and *C. tesselatus* class E (*1*). The distribution of isozyme activity in diploid heterozygotes corresponded to the presence of approximately equal amounts of two different B-type subunits. We now report quantitative corroboration of our earlier impression that each of the two *b*-type alleles is equally active in the diploid heterozygote, and more significantly, the demonstration of approximately equal activity of each of three *b*-type alleles in triploid heterozygotes also belonging to the species *C. tesselatus*.

Cnemidophorus tesselatus is a unisexual, all-female species composed of at least six defined morphotypes designated by the letters A through F (*2*). Individuals from four of these morphotypes (C through F) are diploid and possess two distinct haploid chromosome complements, one resembling that found in the *C. sexlineatus* group and the other resembling that of *C. tigris*. Individuals from the other two morphotypes (A and B) are triploid and possess two *sexlineatus*-like complements and one *tigris*-like complement (*3*). Species of the *C. sexlineatus* group, such as *C. inornatus* and *C. gularis*, carry one allele at the LDH *b* locus whereas *C. tigris* carries a different allele (*1*). These two alleles have been designated as *b* and *b′*, respectively, and their products as B and B′. There is thus a definite correlation between karyotype and the type of LDH *b* allele in these lizards. One might predict on the basis of the karyotype that triploid *C. tesselatus* would carry two *b* alleles and only one *b′* allele and would thus produce twice as many B subunits as B′ subunits, if each allele were equally active. We have tested this prediction by studying the distribution of LDH isozyme activity in both triploid and diploid *C. tesselatus*.

Electrophoresis of homogenates of heart muscle and kidney, either freshly prepared or after storage in a freezer, was analyzed. Homogenates, ground in glass, containing either 25 mg of heart or 100 mg of kidney per milliliter of gel buffer, were centrifuged at 40,000*g* for 1 hour. Samples of supernatant, either full strength or diluted with buffer, were subjected to electrophoresis on horizontal agarose gel (*4*) or vertical acrylamide gel (*5*). Gels were stained for LDH according to reported techniques (*6*). Quantitation of the dis-

Table 1. Values in the $\bar{B}$ column represent the mean proportion for each animal of electrophoretic pattern density attributable to the B subunit. The probability that diploid and triploid values have been drawn from the same population with regard to $\bar{B}$ is less than .001. The probability that diploid values have been drawn from a population where $\bar{B}$ equals 50.00 percent is between .80 and .70. The probability that triploid values have been drawn from a population where $\bar{B} = 66.67$ percent is between .10 and .05. Animals listed in the table are all *C. tesselatus* and are assigned to the following morphotypes and localities: Diploids—MCZ 101992, class E, Socorro County, New Mexico; MCZ 111861 and MCZ 111862, class C, San Miguel County, New Mexico; MCZ 111866, class F, Hidalgo County, New Mexico. Triploids—MCZ 101990, MCZ 111864, and MCZ 111865, class A, Pueblo County, Colorado; MCZ 111863, class B, Otero County, Colorado.

Animal	Analyses (No.)		Total subunit production	
	Heart	Kidney	$\bar{B}$ (%)	S.D.
	Diploid			
MCZ 101992	4		50.65	1.99
MCZ 111861		3	49.05	0.21
MCZ 111862	7	4	50.49	0.99
MCZ 111866	6	2	50.30	1.52
		Mean	50.12	0.63
	Triploid			
MCZ 101990	4		66.55	2.26
MCZ 111864		3	64.82	0.73
MCZ 111865	8		61.53	1.18
MCZ 111863		3	62.95	1.16
		Mean	63.96	1.90