COMPARISONS OF GROWTH RATES OF KB AND L-CELL LINES IN L-15 MEDIA AS MEASURED BY HEMOCYTOMETER AND COULTER COUNTER TECHNIQUES

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

The L-fibroblast cell line was derived from normal connective tissue of a mouse and has been cultured in an antibiotic-free mixture of 30% horse serum, 20% chick embryo extract and 50% Earle's Balance Salt Solution (BSS) (Earle, 1943; Cell Culture Collection Committee, 1964).

The KB-epithelial cell line was derived from a human epidermoid carcinoma of the mouth. The culture medium used for the maintenance of this line was Minimum Essential Medium (Eagle) with non-essential amino acids and Earle's BSS, 95%; calf serum 5% and antibiotic-free (Eagle, 1955; Cell Culture Collection Committee, 1964).

The growth rates of these two lines growing in the corresponding medium as mentioned above, have been reported by the Cell Culture Collection Committee (1964) as follows: the clone of strain L, with an inoculum of 6-8 x 10^5 in 3 ml of medium, multiplies approximately 8 to 9 fold within 7 days. Whereas, the KB line, with an inoculum of 0.5 - 1.0 x 10^5 cells per milliliter, multiplies approximately 10 to 15 fold within 7 days.

Cultured cells may exhibit different properties, including growth rates, when they are cultured in different media. A better method in determination of growth rate of cells <u>in vitro</u>, in the sense of accuracy and simplicity, can facilitate the efficient study of responses of cultured cells in any given condition.

This study includes two parts: (1) a comparison of growth rates of L-fibroblast cells and KB-epithelial cells cultured in medium L-15 for 72 hours at 37.5°C; and (2) a comparison of using the hemocytometer and the Coulter counter in counting L-fibroblast cells.

CHAPTER II

LITERATURE REVIEW

The KB cell line was established from a human epidermoid carcinoma occurring in the floor of the mouth of a caucasian male in December, 1954 by H. Eagle (1955a). This cell line was cultivated directly onto a glass surface in a medium consisting of 13 essential amino acids (Eagle, 1955b), 7 vitamins (Eagle, 1955c), glucose, salts, and 10% human serum. In the course of 350 subsequent passages, the line has been adapted to 5% calf serum. The morphology of this cell line was epithelial-like. In the original medium, the KB cells had a generation time in the logarithmic phase of growth of approximately 30 hours (Eagle, 1955a). Recently, Gartler (1968) suggested that the KB cell line is a derivative of the HeLa cell line through cell contamination.

In 1940, W. R. Earle established the fibroblast strain L from normal subcutaneous areolar and adipose tissue of a 100-day-old male mouse of the C3H strain, Andervont substrain (Earle, 1943). A clone culture of strain L (clone 929) was isolated by K. K. Sanford, et al. (1948). The particular technique for single L cell isolation included restriction of the volume of culture medium to which the single cell can adjust, and conditioning the medium by growing a large culture of strain L cells in normal culture medium. The pure culture was maintained in a medium consisting of 30% of horse serum, 20% chick embryo extract and 50% Earle's Balanced Salt Solution.

The strain L cells were fibroblast-like, with characteristic spindle, flattened-spindle, or less frequently triangular shapes, and with frequent, long terminal, thread-like processes. The diameter of the rounded cell of cultures of this strain averaged 20μ (Earle, 1943; Sanford, 1948). Earle (1943) compared the growth rate of the cells with that of earlier strains and found that the rate of growth varied from flask to flask. The variations could be due to the minor differences in handling.

Growth curves of cell population in tissue culture are characterized by three distinct growth phases: the latent (or lag) phase before growth begins, the exponential phase in which cell number increase rapidly, and stationary (or plateau) phase in which the rapid increase in cell number gradually slows. Frequently, the exponential growth of tissue culture cells was interpreted to predict the behavior of some cell populations in vivo. However, most of the tumor cell populations appeared to be in the latent phase (Watanabe and Okada, 1967).

The cell cycle of adult cells has been divided into four stages (Howard and Pelg, 1953): G_1 stage, the postmitosis pre-DNA synthesis phase; S stage, the DNA synthesis phase; G_2 stage, the post-DNA synthesis pre-mitosis phase; and M stage, the mitosis phase.

When the L5178Y leukemic cells are in the exponential growth phase, the fraction of cells in the G_1, S, G_2, and M stages and the durations of each stage appear to be constant. The ΔH (change in enthalpy) and ΔS (change in entropy) are the highest in the G_1 stage and decrease in the order of S, G_2 and M stages. This indicates that the cells in the G_1 stage are carrying out reactions needed in preparation for the new life

cycle (Watanabe and Okada, 1967a; 1967b).

The limited observations of the effect of temperature on cultured mammalian cells are contradictory (Watanabe and Okada, 1967a; Rao and Engelberg, 1965). However, Watanabe and Okada (1967a) thought that because of the short duration of the M stage, the change in generation time of the cells by temperature variation resulted mainly from the alteration of the duration of the G_1 and the S stages.

Moscona (1961) studied the effect of temperature upon the behavior of adhesion and cohesion of the cells and found that endogenous thermosensitive processes may have a highly important role in cellular activity. Whereas the exogenous molecules prerequisite for normal adhesion of the cells provided only parts of the actual mechanism.

The alteration of the generation time of some mammalian cell lines by pH change was also found to be the result of alteration of the duration of the G_1 stage (Sisken, 1963). Todaro, et al (1962) found that the mouse fibroblast cells entered the S stage before division, that was, ceased growth at the G_1 stage.

Another factor which altered the life cycle was the genetic characteristics. Defendi and Manson (1963) thought that the variations of generation time were attributable to difference in the durations of the G_1 stage. While Puck and Petersen (1964) compared HeLa and Chinese hamster cells and found that the variation of the generation time was attributable to the variation in duration of all stages in the same proportion.

Eagle and Piez (1962) found that at high cell population densities some metabolites could be synthesized in amounts sufficient for sus-

tained growth, but at low population densities these metabolites were lost from the cell to the environment. The critical population density was that which was able to build up a balance in the medium of the effective intracellular influences.

Eagle (1955a) found that twelve amino acids essential for the survival and multiplication of strain L *in vitro* were histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, arginine, cysteine (or cystine) and tyrosine. The last three were not essential for the growth of the whole animal. None of the D-isomers of these essential amino acids could support growth. Absence of any one of these twelve amino acids would result in no multiplication and the cell would degenerate and eventually die.

The minimum vitamins required for growth and multiplication of strain L in tissue culture were proved as choline, folic acid, nicotinamide, panthethenic acid, pyridoxal, riboflavin, and thiamine (Eagle, 1955c).

Later, Eagle also confirmed that the same specific amino acids and vitamin requirements of strain L were necessary for the optimal medium used for isolation and cultivation of the strain KB (Eagle, 1955a).

Myo-Inosital was found to be an essential growth factor for survival and multiplication of both strain KB and strain L (Eagle, 1957).

Arginine was studied by W. J. Thomas, *et al* (1958). They found that the use of arginine could eliminate the necessary renewal of medium due to the depletion of some amino acids. The possible mechanisms were suggested to be the protection by arginine against ammonia toxicity, and also probably the direct incorporation of arginine into the tissue protein.

H. Eagle (1956) demonstrated that in a medium containing the twelve essential amino acids, seven essential vitamins, glucose, electrolytes and serum protein, the culture cells would still degenerate and die unless the medium was supplemented with glutamine and therefore suggested that glutamine was an essential metabolite for cultured mammalian cells.

Later, Levintow and Eagle (1957) studied the role of glutamine in tissue culture and found that glutamine was an essential building block for the synthesis of protein.

A. Leibovitz (1963) devised the L-15 medium to permit the growth and maintenance of normal and malignant tissue cell cultures in free gas exchanges with the atmosphere. Other than the twelve essential amino acids and 7 essential vitamins and some other essential requirements which were suggested by H. Eagle (1955b; 1955c) and Eagle, et al (1956; 1957), free base amino acids of L-arginine, L-histidine and L-cysteine were used to substitute for bicarbonate as the buffer, and D (+) galatose, sodium pyruvate, and DL-alpha alanine were used to substitute for glucose in the medium L-15. Because of the use of free base amino acids, the medium L-15 is able to maintain the final pH about 7.6, in free gas exchange with the atmosphere.

The estimation of cell number can be done directly or indirectly. The direct determination is done by the counting of cell number with hemocytometer or electronic (Coulter) counter. The indirect determination can be obtained by measurement of the packed cell volume, dry or wet weight or some cellular constituent (Paul, 1965).

The Coulter counter (electronic) was first used to count the number of red cells (Mattern, Brackett, Olson, 1957) and then extended to

counting and sizing bacteria (Kubitschek, 1958). Harris (1959) employed the Coulter counter to determine the number of cultured cells. He pointed out that the Coulter counter method is a simple, accurate, reliable and quick method for cell counting. He also said: "When carried out with suitable checks by an independent index of population changes, electronic counts (Coulter counter counts) should be broadly useful in work with isolated cell system." Brecher, Schneiderman, and Williams (1956) did an evaluation of the Coulter counter in red blood cell counts and found not only that the Coulter counter provided reproducible counts of red blood cells on the order of 2% as judged from duplicate counts and dilution curves, but also proved that the multiple counts with the hemocytometer gave very accurate results.

Berkson, Magath, Hurn (1940) reported that there are predictable errors in the hemocytometer counting. They demonstrated that the basic error sources are the error of the field and the error of the pipettes.

CHAPTER III

MATERIALS AND METHODS

Two mammalian cell lines used in this study were the L-fibroblast strain, originally derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H mouse (Earle, 1943) and the KB-epithelial strain, isolated from carcinoma of the human mouth (Eagle, 1955a). Both cell lines were obtained from the Biochemistry Department of Oklahoma State University.

The growth medium for the cell cultures used in this study was medium L-15 (Leibovitz, 1963) plus 10% calf serum, L-glutamine (0.2 millimoles) and penicillin-streptomycin mixture (100 units/ml). All reagents were purchased from the Microbiological Associates, Bethesda, Maryland.

The cell cultures were very sensitive to inorganic as well as organic unclearliness. Cleaning cell-culture glassware and utensils was found to be very important in maintaining healthy cell cultures. The cleaning procedures were as follows (Wolf, personal communication). All used, cell-culture glassware and bottle caps were soaked in Calgon-Metasilicate (C & M) solution which was prepared in the following way: 26.5 g of calgon (Calgon Water Conditioning Co., Pittsburgh, Pa.) and 238.0 g of sodium metasilicate (Fisher Scientific Co.) were dissolved in 2500 ml of deionized water. This was stored as a stock solution and was diluted 1:100 with deionized water just before use. Glassware and caps

were then transferred into Clorox solution and soaked for 24 hours. This was followed by brushing twice under warm tap water and then boiling in fresh dilute C & M solution for 20 minutes. After a thorough rinsing with hot tap water each piece was rinsed with double-deionized water three times and then air dried.

Pipettes were soaked in C & M solution after use, and thoroughly washed in hot tap water in a Nalgene automatic washer (Nalge Co., Rochester, N. Y.). This was followed by 20 minutes of boiling in fresh C & M solution, thorough rinsing in hot water, and rinsing three times in double-deionized water. After air dried, pipettes were cotton-plugged, put in pipette cans and sterilized.

The double-deionized water was prepared by running the primary deionized water, obtained from the passage of city tap water through a resin column in the building system, through a Barnstead Bantum deionizer (Barnstead Still and Sterilizer Co., Boston, Mass.).

The dried glasswares, bottle caps, pipettes, etc., were sterilized in a Castle autoclave (Model 999-C, Wilmont Castle Co., Rochester, N.Y.) at 16 lbs pressure, 250^{o}F for 30 minutes.

The cells of the order of 10^{5} per milliliter were inoculated in the milk dilution bottle, with a surface area of approximately 10 x 3.5 sq. cm. containing 10 ml of growth medium. Cell cultures were incubated in a Precision incubator (Model 2, Precision Scientific Co.) at 37.5^{o}C ± 1^{o}C for 72 hours. Cells were then dispersed with 5 ml of warmed versene (2×10^{-4} g/ml).

Preliminary tests revealed that prolonged action of versene will influence the growth characteristics of the cells. In order to shorten the time required for dispersion, the versene was warmed to 37.5^{o}C,

the culture bottles were shaken every two minutes and the culture were kept warm (37.5°C) while the versene was acting. After the cells were dispersed the suspension was transferred to a sterile centrifuge tube and centrifuged at 850 rpm for 15 minutes. The supernatant solution was discarded, the cells were washed with used medium and centrifuged again. After discarding the old medium, the cells were suspended in fresh growth medium by pipetting the cells and medium up and down for at least 20 times. Cell counts were then made with a hemocytometer (Spencer AO Bright-Line Hemocytometer, American Optical Corporation, Scientific Instrument Division, Buffalo, N.Y.) and Coulter counter (Model "B", Coulter Electronics, Inc., Hialeah, Florida), (Coulter Electronic Co., 1966).

All processes with open cultures were carried out in a hood equipped with an ultraviolet light, a vent and a Touch-O-Matic burner. The ultraviolet light was on several hours and then off about 30 minutes before the hood was used. The interior of the hood was washed with Clorox solution and rinsed with water once every week and was wiped with 70% isopropyl alcohol immediately before use.

Both KB and L cells were counted by the hemocytometer white-cell-count method, and the growth rates were determined. This was done as follows: the counting chamber was carefully filled with cell suspension without overflow. Any overflow will draw the cells over into the moat and may cause error. The cells in the corner (four) one-millimeter squares and the central (ruled) square were counted. The number counted was divided by five and multiplied by the cell suspension dilution. This value was multiplied by 10 to give the number of cells per cubic millimeter, since the ruled surface was 0.1 mm below the cover glass.

The product of the number of cells per milliliter and the number of milliliters of cell suspension gave the total number of cells in the bottle. The initial number of cells inoculated into the bottle was determined in the same way. The final number of cells in a bottle was divided by the initial number of cells of the same bottle to obtain the growth rate in terms of fold increase within 72 hours. Two counts (10 millimeter squares in total) were taken for each culture.

The electronic counting method (Coulter counter) was employed to compare with the hemocytometer method for L-cell count. The subjected bottles of the cultured L-cell for this purpose were incubated for different time periods. Both methods of counting were used for each bottle for comparison of counting methods.

The assembled Coulter counter (Model "B") includes three parts: (1) an aspirator pump; (2) the sample stand assembly, equipped with the mercury manometer, the aperture tube, the platinum electrodes, the sample platform and microscope; (3) the electronic counter, containing the pulse-amplifying system, the oscillioscope and the glow-type decade counters. The mercury manometer was already calibrated to regulate the sampling of 0.5 milliliter of the suspension. Two-tenth milliliter of the same L-cell suspension (versene dispersed) counted with the hemocytometer was suspended in 19.8 ml of counting fluid (0.9% sodium chloride, filter sterile). The cells were counted while flowing through an aperture which was immersed in the suspension. An aperture tube of 100μ in diameter was used (Coulter Electronic Co., 1966).

The background count, count number of 20 ml of counting fluid, was substracted from the count number of the L-cell sample and then multiplied by the value of the sample dilution to give the number of cells

per milliliter of that sample. At least three counts were taken for each sample.

The data obtained with the two methods were compared.

The experiment of comparison of growth rate of L-line and KB line was carried out as a completely randomized design experiment. Each bottle of either type of cell culture (the term 'bottle' will be used to indicate the bottle of cell culture in the rest of this chapter) was considered as an experimental unit. Two samples were taken from each bottle. Since the growth conditions, which includes the amounts of growth medium, size of culture area, incubation temperature, time period, and cell count method were controlled so as to be essentially homogeneous, the variation among bottles is assumed to be very small. The responses of different types of cells, in terms of growth rate, to these homogeneous condition, were considered as treatments which are the variables in the experiment.

Analysis of variance for the data was followed by the F test for the treatment means, assuming a common variance which was justified by the two-tailed F test.

The experimental design used for the comparision of the hemocytometer count and Coulter counter count was randomized complete block design. Each bottle was considered as a block. Comparison of the cell counts with hemocytometer and with Coulter counter were made only from the same bottle, but not from different bottles (blocks). Clearly variability among bottles does not affect the differences between the two methods since each method has been employed in every bottle. Variation among bottles was arithmetically removed from experimental error to give the variation between the two methods. F test was performed for testing

the variation between the two methods and for the variation among bottles.

CHAPTER IV

DATA AND RESULTS

KB cells remained epithelial-like and L cells fibroblast-like in the growth medium L-15 (see p. 9). KB cells grow in colonies. Most of the L cells appeared to be spindle shaped and in contact with each other at their rather long processes. A few of them were of triangular shape. The sizes of L cells measured, ranged from 5μ to 18μ.

The L cells were not as easily removed from the surface of the bottle as the KB cells were. But the KB cells were found to have a high tendency to form clumps and thus not be easily separated from each other. The longer the time period the cells grew on the glass, the harder it was to take them off the glass surface.

The KB line showed more sensitivity to the change of temperature, the prolonged action of versene and the mechanical effect, than the L line did. The complete breaking up of the clumps of KB cells into single cells by versene (2×10^{-4} g/ml) associated with mechanical treatment (see p. 11) seemed to slow down the growth of the KB cells.

The data obtained with hemocytometer in comparison of growth rates of KB and L cells cultured in the same amount of growth medium at 37 C^{o} $\pm$ 1^{o} C for 72 hours are shown in Table I. Thirteen bottles of KB and eighteen of L were counted. Two samples were taken from each bottle giving total counts of 26 for KB and 36 for L cells. The comparison of the two growth rates was based on the number of fold increase within 72

hours of incubation.

In order to test the hypothesis that a difference in growth rate between KB and L cells grown in L-15 medium does not exist, an analysis of the variance (AOV) was performed (Table III). This was followed by the F test for the growth rate means, assuming that these two growth rates have a common variance, which was justified by a two-tailed F test (Appendix A). The calculated F (2.7) is smaller than the tabular F (4.18) at the 0.05 level. This indicates that there was no significant difference between the growth rates of the two cell lines studied.

The second hypothesis tested was that there was no difference between using the hemocytometer and the Coulter counter to make cell counts in L-cell cultures.

Twenty separate comparisons were made. The statistical layout for the data of this part of the experiment was a randomized complete-block design, with 20 blocks (bottles) and two treatments (methods) as shown in Table II. An analysis of variance was performed to determine if there is any difference between the two counting methods (Table IV). The null hypothesis of no difference between bottles was also tested, even though bottles (blocks) are an acknowledged source of variation but this variation is arithmetically removed from the experimental error in the determination of the variation between the two methods (Steel and Torrie, 1960).

F test made on these data showed that there is no significant difference between the hemocytometer counts and the Coulter counter counts at 0.05 level. The mean difference among bottles was found to be significant at the 0.025 level. However, it is an evident that this variation did not affect the treatment error in this experimental design.

TABLE I

GROWTH RATE DATA FOR KB AND L CELLS CULTURED IN L-15 MEDIUM COUNTED BY HEMOCYTOMETER

KB			L		
Bottle	Sample	G.R.*	Bottle	Sample	G.R.*
1	1	2.01	1	1	1.92
	2	1.87		2	2.96
2	1	2.35	2	1	2.43
	2	1.87		2	2.00
3	1	1.11	3	1	1.55
	2	1.09		2	2.97
4	1	1.57	4	1	1.38
	2	1.09		2	1.60
5	1	1.03	5	1	4.21
	2	1.16		2	1.70
6	1	1.20	6	1	1.66
	2	1.22		2	1.73
7	1	3.14	7	1	1.12
	2	3.69		2	1.24
8	1	2.55	8	1	1.25
	2	3.16		2	1.30
9	1	1.53	9	1	0.94
	2	1.56		2	1.03
10	1	1.21	10	1	1.13
	2	1.25		2	1.04
11	1	1.76	11	1	2.06
	2	1.69		2	1.98
12	1	1.06	12	1	1.89
	2	1.14		2	2.21
13	1	1.53	13	1	1.51
	2	1.46		2	2.16

TABLE I (Continued)

KB			L		
Bottle	Sample	G.R.*	Bottle	Sample	G.R.*
			14	1	3.03
				2	2.42
			15	1	3.03
				2	2.81
			16	1	4.26
				2	4.80
			17	1	2.50
				2	2.33
			18	1	4.37
				2	3.97

*G.R. = Growth rate in terms of fold increase within 72 hours.

#Bottles were cultured at different times.

TABLE II

DATA COMPARING HEMOCYTOMETER COUNTS AND COULTER COUNTER COUNTS ON L-CELLS

Bottle	Hemocytometer* Count ($\times 10^5$ cells/bottle)	Coulter Counter# Count ($\times 10^5$ cells/bottle)
1	29.80	24.55
2	36.00	38.40
3	12.24	10.40
4	16.32	9.85
5	11.36	20.10
6	7.51	6.08
7	6.04	8.16
8	13.75	17.58
9	9.52	15.06
10	16.80	15.12
11	18.56	19.95
12	16.24	18.22
13	6.72	12.56
14	19.75	17.50
15	17.12	27.52
16	16.72	21.20
17	9.92	10.60
18	20.16	12.80
19	27.60	17.50
20	17.76	20.40

*Means of two samples.

#Means of three counts of one sample.

TABLE III

SUMMARY OF AOV FOR COMPARISON OF GROWTH RATES OF KB AND L CELLS CULTURED IN L-15 MEDIUM

Source	df.	SS	MS	F
Total	61	55.56		
Between Types of Cells (Treatment)	1	4.27	4.27	2.7
Among Bottles Within Types of Cells (Exp. Unit)	29	45.14	1.56	7.8*
Error	31	6.15	0.198	

*Significant at .05 level. As a result 1.56 was used to test treatment MS (4.27).

TABLE IV

SUMMARY OF AOV FOR COMPARISON OF HEMOCYTOMETER COUNT AND COULTER COUNTER COUNT ON L-CELLS

Source	df.	SS	MS	F
Total	39	2194.88		
Between Methods (Treatment)	1	4.75	4.75	0.3366
Among Bottles (Block)	19	1922.23	101.17	7.16*
Error	19	267.90	14.10	

*Significant at 0.025 level.

CHAPTER V

DISCUSSION

The use of free base amino acids of L-arginine, L-histidine and L-cysteine enables the medium L-15 to maintain pH at 7.6 in free gas exchange with the atmosphere (Leibovitz, 1963). In this study the medium, in which the studied cells were grown, maintains an almost constant pH, measured by pH paper, for 10 to 12 days. The author felt that it is convenient to use medium L-15 in cell culture study, because the pH need not be adjusted by CO_2.

The growth rate means of KB and L cells, in terms of fold increase within 72 hours, are not comparable with those reported by the Cell Culture Collection Committee (1964). The subjected cells were grown under different conditions such as: (1) growth medium used (pp. 1,9); (2) the inoculum size (pp. 1,10); and (3) the time period for which cells were incubated (pp. 1,10). These are some of the factors which can affect the cell growth rate (Leibovitz, 1963; Todaeo, 1962; Eagle and Piez, 1962). In addition, the author obtained the culture of these two cell lines from the Biochemistry Department of Oklahoma State University. The cells might have changed some of their growth characteristics due to their adaptation to the growth conditions provided in Biochemistry Department of Oklahoma State University.

The F test failed to show a significant difference in the growth rates of the two cell lines in the L-15 medium. From these data it is

inferred that the L-15 medium was equally favorable to each of the cell lines studied. The slight differences between the means may be due to unique properties of the cell type or to uncontrolled variables in the experiment.

If, the number of viable cells will be less than the inoculated number of cell recorded due to the versene action or/and mechanical effect, KB cells would have been affected more than L cells. Because as it has been mentioned in the previous chapter the KB cells appeared to be more sensitive to the prolonged action of versene and mechanical effect, this would, in turn, cause more variation in calculating the growth rate of the KB line.

Completely breaking up the KB clumps for cell count slows down their growth rate. Perhaps, this is because some of the cells were injured by either chemical or mechanical effect. Another possibility is because the separation of the clumps conflict with their colonial growth character. As a result the normal relationship which enables them to synthesize the necessary metabolites was destroyed (Eagle and Piez, 1962).

The F test for the comparison of hemocytometer counts and Coulter counter counts show no significant differences at the 0.05 level. This result agrees with Mattern's 1957 finding: "Instrumental (Coulter counter) and hemocytometer white cell counts were found to be in reasonably good agreement." It is necessary to report that discrepancies between the counts with the hemocytometer and with the Coulter counter were not absolutely absent in this study. Errors of the hemocytometer count observed obviously arose from the non-uniform cell suspension and the speed of filling the counting chamber (Brecher, 1956; Mattern, 1957).

By slowly filling the chamber the count number could be increased. Efforts were, therefore, made to eliminate these two effects as much as possible.

The "error of the field" of the hemocytometer count estimated by Berkson (1940) was not controllable.

The variations in counts with the Coulter counter in the experiments arose mainly from the relatively high number of cells per ml in the counting solution. This phenomenon was also observed by other people (Brecher, 1956; Kubitsched, 1958; Mattern, 1957). The explanation given by these people was that there were more than two cells entering the aperture together. In fact, this was considered as an inevitable phenomenon (Coulter Electronic Co., 1966). Since the size of L-cells used in this study were found to be in the range of 5μ to 18μ, it is very probable that there were frequently 2 or more small size L-cells passing through the aperture simultaneously.

Kubitschek (1958) suggested that the particles counted should be at least as large as 30% of the diameter of the aperture (see also Coulter Electronic Co., 1966).

The correction for coincidence loss was found to be necessary (Manual; Mattern, 1957; Brecher, 1956), and this can be done by reducing the size of aperture in the tube or by using a calibration chart provided in the manual or made by the investigator himself.

Therefore, it is better to make a preliminary test for the dilution range, in which the number of the studied cells counted is independent of the dilution, before the experiment. Determination of the distribution of the studied cell size will be helpful in selecting the suitable size of the aperture tube.

Swanton, et al, (1962) found that "some viable bacteria do not react independently of current flow, as do inert particles and killed bacteria" and so the bacteria count was found lower than it should be. Perhaps, this can also be true in cultured cell counting with the Coulter counter.

One of the advantages of using completely random design in studying the comparison of two cell lines growth rate is that the statistical analysis is simple. Also, with this design, the number of degrees of freedom for estimating experimental error is maximum and thus improves the precision of the experiment.

In the comparison of the two cell count methods, F test of bottles (blocks) is valid. Because, the sources of variation among bottles were acknowledged, if F test for block effects show them to be significant, it indicates that the precision of the experiment has been increased by use of this design. This is found true in this study. Also, the scope of the experiment may be increased when bottles are significantly different since the two methods will have been tested over a wider range of experimental conditions.

CHAPTER VI

SUMMARY

The purposes of this study were to compare the growth rates of KB and L cells cultured in medium L-15 and to compare L-cell counts made with the hemocytometer and the Coulter counter techniques.

The KB and L cell lines, obtained from the Biochemistry Department of Oklahoma State University, were cultured in medium L-15 with 10% calf serum, L-glutamine (0.2 millimoles) penicillin-streptomycin mixture (100 units/ml).

In general observation throughout the experiment, it was found that both cell lines were capable of growth in the L-15 medium.

The growth rates of KB and L lines were determined and compared after 72 hours incubation at 37.5°C. Both lines of cells were counted with the hemocytometer.

Analysis of variance for the data obtained from 13 bottles of KB cells and 18 bottles of L cells was followed by the F test. The result showed that there was no significant difference between the growth rates of KB and L cells when they were grown in the above medium.

In the comparison of the two cell count methods, 20 bottles of L cells were grown in the L-15 medium at 37.5°C for various time periods. The number of cells in each bottle was determined by both hemocytometer and Coulter counter techniques. An F test of the method means showed that there was no significant difference between the hemocytometer and Coulter counter counts of the L-cells.

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APPENDIX A

TESTING THE EQUALITY OF KB AND L-CELL GROWTH RATE VARIANCES

	KB	L
df.	25	35
SS	88.57	218.1
MS	3.57	6.23

$$H_o: \sigma^2_{KB} = \sigma^2_L$$

$$H_A: \sigma^2_{KB} \neq \sigma^2_L$$

$$F_{cal} = \frac{\sigma^2_L}{\sigma^2_{KB}} = \frac{6.23}{3.54} = 1.759$$

For two-tailed F test at 10% of significant level:

$$F_{0.05\ (35,25)} = 1.895$$

Therefore: Accept $H_o: \sigma^2_{KB} = \sigma^2_L = \sigma^2$

Hence the populations of KB and L-cell-growth rates have the same variance.

APPENDIX B

In addition to the studies on the L and KB cell lines herein reported, the author has tried to develop a fibroblast cell line from the gonads of the fathead minnow (Primephales promelas) since February of 1968, and has not been successful.

After spending a few months learning the fish cell culture techniques, the first primary culture was attempted in May 1968. Others were attempted on October 12, 19, November 7, 11, 23, December 2, 12 of 1968 and January 24, 28 of 1969. None of the primary cultures attempted were successful until the one done on January 24, 1969.

The author believes that the contamination which occurred in the primary culture was mainly due to the failure of the disection of the fish and removal of the gonads asceptically. Fifty percent isopropyl alcohol was first used to kill the fishes, and also served as a primary disinfectant. The killed fishes were then washed with commercial Clorox to complete the external sterilization. After a few tries the author inferred that the cells of the gonads might be killed by the excess Clorox. Thereafter the fish were killed by severing the brain and then placed in clorox for one minute. The fish was then rinsed with sterile water, and then with 70% isopropyl alcohol immediately before dissection.

The dissecting instruments were sterilized in boiling water followed by immersion in 70% isopropyl alcohol, flaming and then cooling in sterile deionized water immediately before use. Special care was

taken not to puncture or even touch the intestine while removing the gonads.

Instead of using sterile deionized water and PBS[1] to wash the gonads, cold Hanks' BSS was found to be more reliable in keeping the gonad cells alive. Throughout the manipulation, the gonads were kept on an ice tray.

Two methods of dispersion were tried. One used a digestion mixture[2], after the gonads were minced. A magnetic stirrer was also used to help break down the tissue mechanically. Another method tried was that of squeezing the gonads through the needle of a syringe at least twice. The latter one was found to be more effective.

A needle of size no. 20 was found to be more practical than that of no. 24, because the no. 24 was very easily plugged by the tissue, while no. 18 seemed not to break the tissue down into small enough clumps.

The incubation was tried at 25°C and 20°C. The final attempt began at 16°C, and gradually increased to 20°C, was made on January 24, 1969.

Using the following experimental technique some fish fibroblast cells grown in the primary culture made on January 24, were obtained.

The procedures of the primary culture done on January 24, 1969, are summarized as follows: fourteen fathead minnows were killed by

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[1]PBS: Dissolve NaCl 8g, $CaCl_2$-$2H_2O$ 0.13g, KCl 0.2g, Na_2HPO_4 1.06g, KH_2PO_4 0.249g, $MgCl_2 \cdot 6H_2O$ 0.1g in 1 liter sterile water, add 1 vial Phenol Red (1 ml of 1% solution) then filter sterile.

[2]Digestion Mixture: PBS 51 ml, Fetal Bovine Serum 1.5 ml, penicillin-streptomycin mixture 1.2 ml--60 units, Trypsin 6 ml--0.15 g.

severing their brains and immersing them in 50% clorox for at least one minute. After rinsing with sterile water, they were wiped with cotton swabs saturated with 70% isopropyl alcohol. The abdomen was then opened with sterile scissors. The gonads were removed aseptically, and immediately submerged in cold Hanks' BSS. After being washed with cold Hanks' BSS three times, the gonads were transferred into a sterile syringe to which was added 10 ml of the L-15 growing medium previously described containing fetal bovine serum, L-glutamine and penicillin-streptomycin mixture. The contents of the syringe were forced through a No. 20 needle twice, then divided equally and placed in two milk dilution bottles, each with 10 ml of fresh growth medium. The bottles were incubated at 16°C for 20 hours. The old medium with some of the larger residues was poured off and 10 ml fresh growth medium was added. The incubator temperature was gradually increased to 20°C within a week. The culture was not moved from January 26, 1969 until April 18, 1969. At this time a very healthy cell layer was found. Pictures taken on April 21, 1969, showed that most of the cells were fibroblast-like. The growing cells covered about 60-70% of the bottle surface. It was decided to change the medium on April 25, 1969. The composition of the new growth medium was the same as the one used on January 24, 1969. Two hours after the fresh growth medium was added, the medium color changed to a yellowish pink (acidic side). After 24 hours, the cells appeared to be very pale, the cell boundaries were gradually lost. Another 24 hours later, the cells had all disappeared.

In comparison with the growth rates of the other fish cell lines (Morgans, 1968; Bourne, 1964), the author inferred that the slow growth of the fibroblast cell of the studied fish was probably due

partly to the relatively low incubation temperature used and that the percentage of antibiotic used was too high, so high that the cell growth was inhibited and the cells killed.

It is, therefore, suggested that in further study, the preliminary testing should determine the optimal incubation temperature and the percentage of antibiotic in which not only the bacteria can be well controlled but also the cells can grow maximally.

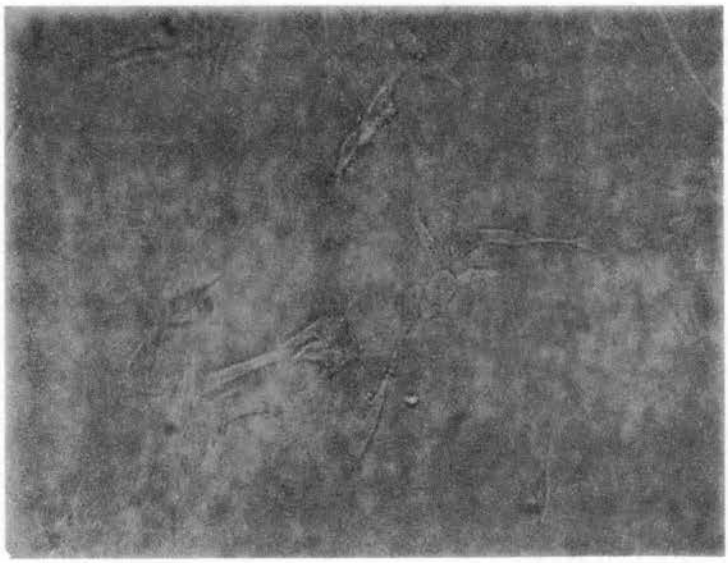

Figure 1. Fibroblast Cells From the Gonads of Fathead Minnows Growing in the Primary Culture Made on January 24, 1969.

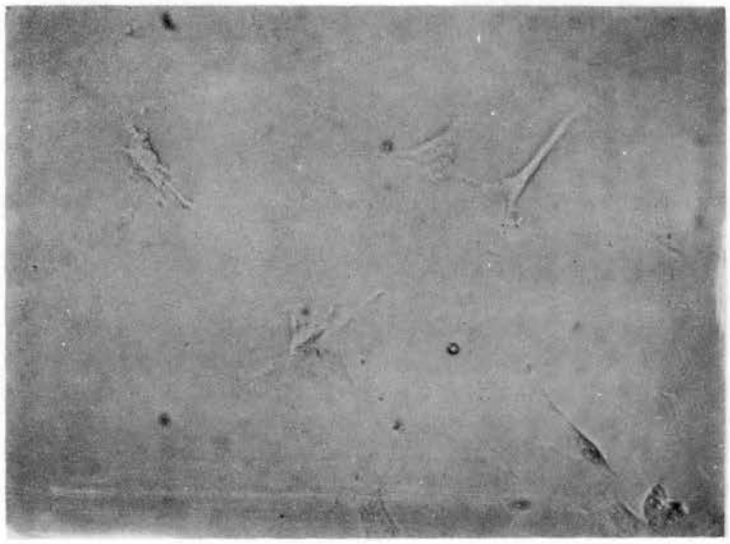

Figure 2. Fibroblast Cells From the Gonads of Fathead Minnows Growing in the Primary Culture Made on January 24, 1969.

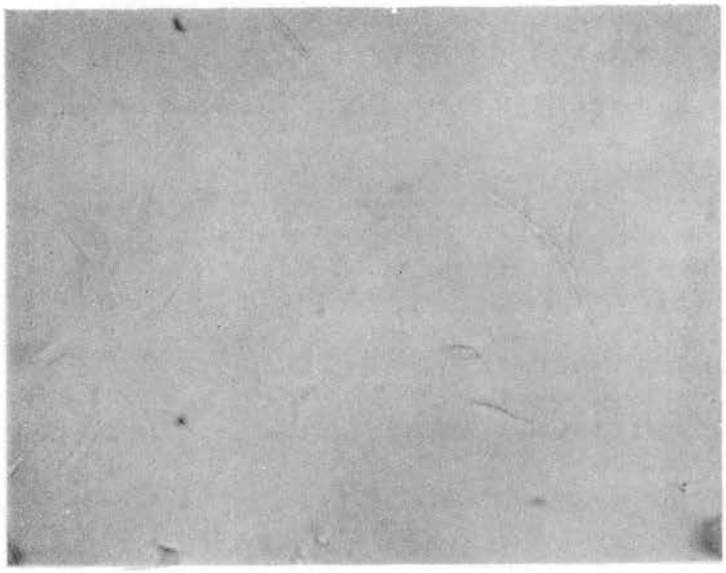

Figure 3. Fibroblast Cells From the Gonads of Fathead Minnows Growing in the Primary Culture Made on January 24, 1969.

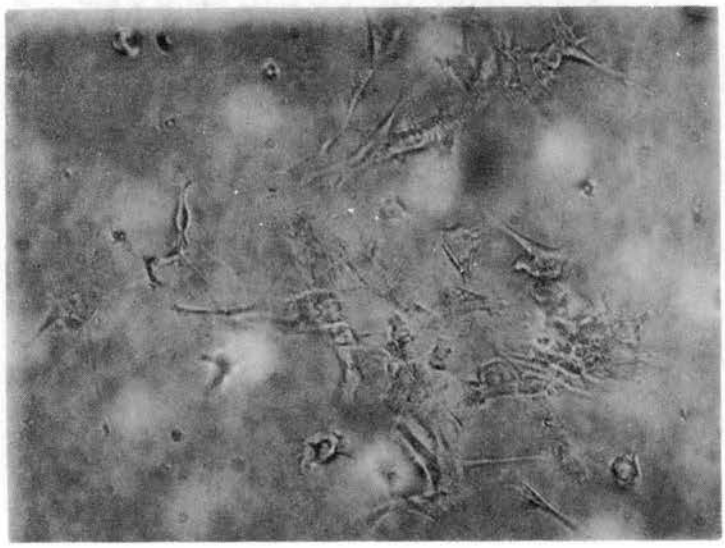

Figure 4. Some Epithelial Cells Also Found in the Primary Culture Made on January 24, 1969.

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VITA 2

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