ESTABLISHMENT OF REPRODUCIBLE LYMPHOBLASTOID CELL LINES

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Abstract

Background: A whole blood culture started with mononuclear cell fraction would contain several different cell types. Nevertheless, the T-lymphocytes can be cultured specifically and will out grow the others, eventually resulting in highly enriched population.

Objective: The study was conducted as a trial of establishment of lymphocytes cell lines. This series of experiments were done for five selected subjects. The same protocols were applied to all individuals.

Method: One ml of heparinized blood (HB) was cultured in tissue culture flask containing 9 ml stimulation medium. The flask was incubated at 37ºC in CO_2 incubator for 3 days. Then every three days 1ml of whole blood culture from the flasks was subcultured into other tissue culture flask containing 9 ml stimulation medium, until visible suspension of mononuclear cells intervening agglutinated red blood cells were seen within two weeks of culture.

Results: After the whole blood mononuclear cells cultures were maintained up to two weeks, the mononuclear cells were separated. Twenty four-hour incubation of these separated lymphocytes in stimulation medium, pure rich mononuclear cells were obtained and seen under inverted microscope.

Conclusion: A new method for cultivation of lymphocytes from whole blood was developed for the first time and probably no other reported comparable method conducted elsewhere until know. Pure lymphocytes cultures were established, and maintained cell division. The potentiality of human peripheral lymphocytes themselves to act as precursor has been demonstrated *in vitro* by the cloning of established lymphoblastoid cell line. Different procedures for generation of T- cell lines were outlined. No feeder cells were needed in our protocol or the addition of IL2.

Key words: lymphocyte culture, cell line, Phytohemagglutinin

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Introduction

Advances in the understanding of lymphocyte biology have been accompanied by improvement in the techniques of long-term culturing of human T- lymphocytes[1]. Long-term culturing of human T-lymphocytes can lead to establishment of stable line. These cultures respond to produce a wide range of cytokines. More importantly, T-cell lines can be generated which is antigen responsive. Many aspects of the T-cell response to antigen have been described using antigen specific cell lines[2,3,4].

Human lymphocytes can be isolated from whole blood by centrifugation using a commercially available high-density medium. This allows a single step gradient separation of blood, which yields the mononuclear cell fraction[5].

A culture started with mononuclear cell fraction would contain several different cell types. Nevertheless, the T-lymphocytes can be cultured specifically and will out grow the others, eventually resulting in highly enriched population. To achieve this, the antigen reactive cells must be first stimulated with antigen followed by expansion of cell

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numbers using the T- cell specific growth hormone interleukin 2 (IL2). It should be noted that the absolute frequency of antigen response T-cells is low, being between 0.01% and 0.001%, even when blood known to contain relevant T-cells (i.e. blood from an immune donors) is used. Thus, the majority of the cells in the starting culture will die and only the T-cell activated by antigenic stimulation will survive[6].

This stimulation not only selects out the antigen-specific T-cells but result in the cellular expression of the receptor for the IL2[7]. When exogenous source of IL2 is now added to the activated T-cells, they will undergo further round of replication. Since T-cells eventually become refractory to the effect of IL2, they must be restimulated for receptor expression[8]. A T-cell line can thus be maintained in culture by alternate stimulation with antigen and expansion with IL2[5].

Phytohemagglutinin (PHA) activated peripheral CD95+ T-cells are resistant to CD95 mediated apoptosis. After prolonged interleukin-2 treatment, these T- cells become CD95 mediated apoptosis sensitive. In T- cells activated in vitro, up-regulation of bcl-xL, has previously been correlated with general apoptosis resistance[9].

Therefore, this study was conducted as a trial of establishment of lymphocytes cell lines from whole blood.

Materials & Methods

This series of experiments were done for five selected normal adult subjects, their ages ranged from 15 to 45 years, (four males and one female). The same protocol was applied to all individuals. Two ml of venous blood was collected from each subject and placed in sterile heparinzed tube.

One ml of heparinized blood was cultured in tissue culture flask containing 9 ml of **stimulation medium** (SM) (1 x RPMI 1640, penicillin 100 unit/ml, and streptomycin 100µg/ml, 10mM L-glutamine, 20% fetal bovine serum (FBS), 10mM HEPES, 200µl/ml PHA). The flask was incubated at 37ºC in CO_2 incubator. Then every three days 1ml of whole blood culture was subcultured into other tissue culture flask containing 9 ml of SM, until visible suspension of mononuclear cells intervening agglutinated red blood cells were seen within two weeks of culture.

Then, the mononuclear cells were separated by the method described by Boyum (1968)[10], washed twice with serum free medium (1x RPMI 1640, penicillin 100 unit/ml, and streptomycin 100µg/ml, 10mM L-glutamine 10mM HEPES), counted, assessed for viability, then 1ml of 2 x 10^6 cells were cultured in tissue culture flask containing 9 ml of **SM**. Another 1 ml of cells was cultured in tissue culture flask containing 9 ml of **growth medium** (GM) (as stimulation medium without PHA). After 24-hour incubation, cell culture was examined under inverted microscope.

At that time, the cells were counted and assessed for viability. The percent of viable cells were determined at indicated time intervals in both GM and SM, and 2 x 10^6 cells were subcultured in tissue culture flask containing 9 ml of SM or GM, every 72 hour. The lymphocytes culture was maintained continuously by this method.

Results

Preparation of Primary Human Peripheral Blood Mononuclear Cells Culture:

Heparinized blood was cultured in SM, and subcultured frequently as mentioned in materials and methods. The visible suspensions of mononuclear cells intervening agglutinated red blood cells were seen within the second week of culture (figures 1 a & b).

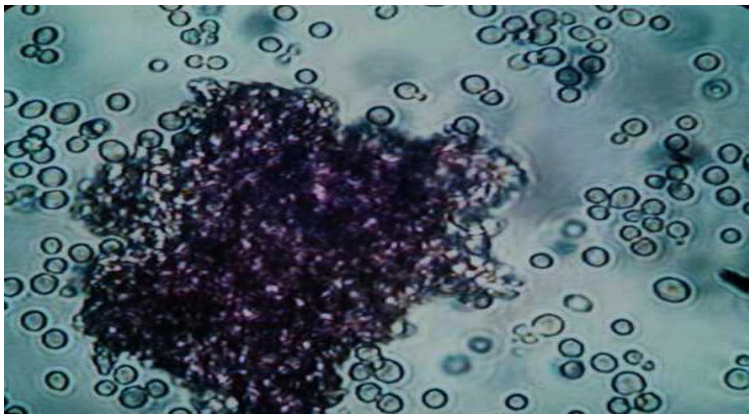

Figure 1a: Photomicrograph Shows Whole Blood Lymphocytes Culture during the first week (X 250)

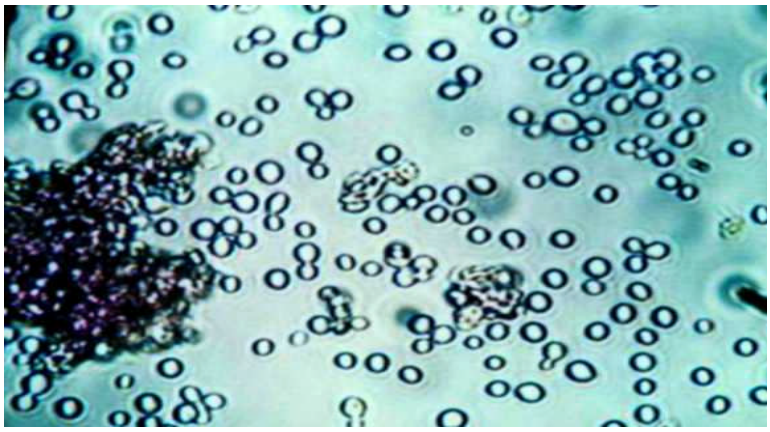

Figure 1b: Photomicrograph Shows Whole Blood Lymphocytes Culture during the second week (X 250)

Purification and propagation of PBMC culture:

After the whole blood mononuclear cell cultures were maintained up to two weeks, the mononuclear cells were separated as described in materials and methods. Twenty-four hour incubation of these separated lymphocytes in SM, pure rich mononuclear cells were obtained and seen under inverted microscope (figure 2).

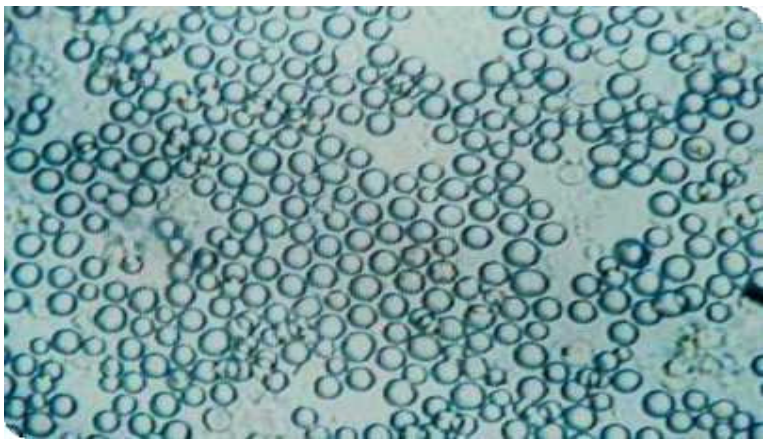

Figure 2: Photomicrograph Shows Purified Stimulated Lymphocytes Culture after the second week of whole blood culture (X 250).

Stimulation was indicated by a rapid increase in the number of cells (count were increased 200 fold within two weeks of subculture of the purified lymphocyte) (table 1 & figure 3).

Table 1: Effect of cell proliferation and cell death on viable count over two week's period, using growth or stimulation medium. The seeding density was 2x 10^5/ml

culture media	Number	Absolute Number of viable lymphocyte count /ml (x10^6)						
		Zero time	Days after culture initiation					
			1	3	5	8	11	14
Stimulation medium	Sample1	0.2	2	25	70	125	250	400
	Sample2	0.2	1.5	20	50	100	200	350
	Sample3	0.2	2.5	30	80	150	300	450
	Sample4	0.2	2	22	75	130	260	410
	Sample5	0.2	2	18	65	110	220	380
	Mean	**0.2**	**2**	**23**	**68**	**123**	**246**	**398**
	SD	**0**	**0.3**	**4.6**	**11.5**	**19.2**	**38.4**	**37**
Growth medium	Sample1	0.2	2	25	60	50	31	3
	Sample2	0.2	1.5	20	39	35	26	2.5
	Sample3	0.2	2.5	30	62	52	35	3
	Sample4	0.2	2	22	60	48	25	3
	Sample5	0.2	2	18	58	47	24	4
	Mean	**0.2**	**2**	**23**	**55.8**	**46.4**	**28.2**	**3.1**
	SD	**0**	**0.1**	**4.6**	**9.4**	**6.6**	**4.6**	**0.5**

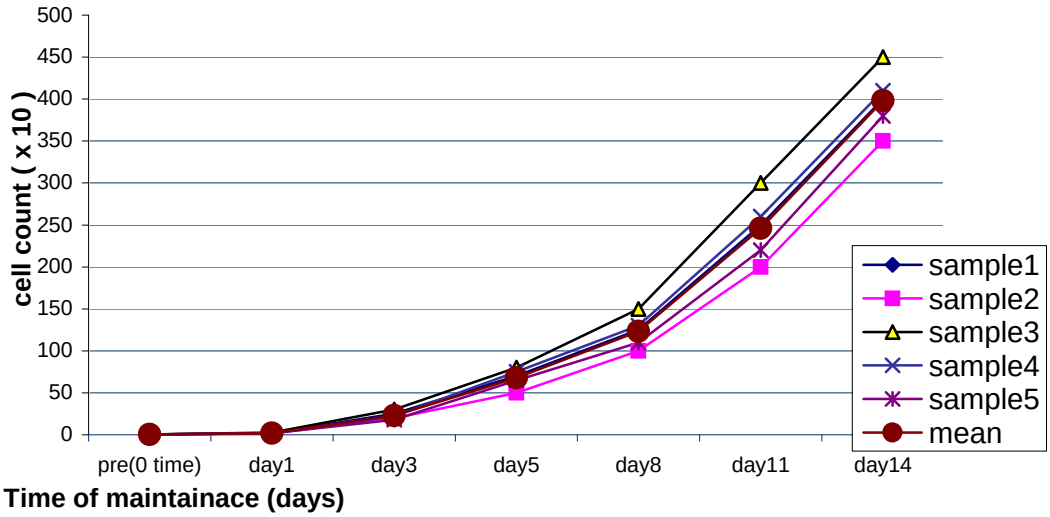

Figure 3: Effect of cell proliferation and cell death on viable count over two weeks period, using stimulation medium, each point represent the mean of 4 values. The seeding density was 2x10 /ml

Many cells became enlarged, and the medium turning yellow 2-3 days after subculture. A clear stimulation event was recognized without the aid of an inverted microscope when the bottom of the tissue culture flask showed a large grayish white central button of cells surrounded by white pinpoint satellilate colonies.

Another (2 x 10 ^{6}/ ml) of cells from day one culture were cultured in growth medium. A reduction in viable cell

counts after day 5 of culture was observed as shown in table 1 & figure 4.

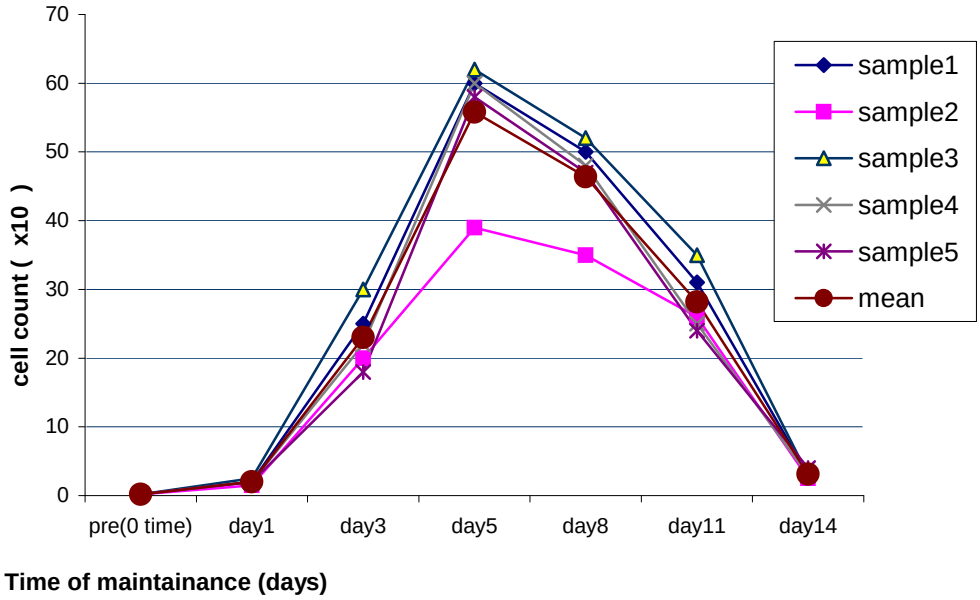

Figure 4: Effect of cell Proliferation and cell death on viable count over two weeks period, using growth medium. Each point represents the mean of 4 values. The seeding density was 2x 10 /ml

All the stimulated cells gave lymphoblast characteristics when stained with Giemsa stain, so actually we can apply the lymphoblastoid cell line as indicated from their morphological appearances.

Discussion

This work probably to be the **first** method to be reported for preparation of continuous lymphocytes culture and probably no other reported comparable method conducted elsewhere till know.

Purification and propagation of PBMC culture:

Human lymphocytes can be isolated from whole blood by centrifugation using a commercially available high-density medium, which relies on lymphocytes being less dense than erythrocytes and granulocytes. This allows a single step gradient separation of blood, which yields the mononuclear cell fraction[5].

Then pure lymphocyte cultures were established, and induced and maintained cell division.

A culture started with mononuclear cell fraction would contain several different cell types. Nevertheless, the T-lymphocytes can be cultured specifically and will out grow the others, eventually resulting in highly enriched population. Beside T-cells, other mononuclear cell types are present in the initial culture. These cells have the capacity to activate T-lymphocyte by processing and presenting antigen to the T-cell receptor (TCR) and provide additional T-cell growth factors[11,12].

T-cells can be activated by some mitogens. Most T-cells are stimulated by PHA, a lectin isolated from kidney beans, or by concavalin A extracted from castor bean. These molecules are able to bind to T- cell surface molecules including the T- cell receptors (TCR) complex and CD2, causing them to cluster on cell surface, there by mimicking the clustering caused by antigen presentation. Such mitogens however will activate the T- cells regardless their antigen specificity[13].

In this assay, PHA was used as mitogen because their stimulation percentage was higher than that of Con A, as indicated early by the results of our

study and easy to be manufactured in our laboratory[14].

Whole Blood Lymphocyte Culture:

We have developed a new method for isolation and cultivation of lymphocytes from whole blood. Heparinized venous blood drowns from normal individuals was added and incubated in SM and the subculture was regularly maintained. Lymphocytes were isolated when lymphocytes suspension was seen by inverted microscope intervening agglutinated red blood cells (figures 1 a & b).

It has been suggested that a cellular components of erythrocytes in whole blood play an important role for the proliferation of the lymphocytes with no effect on their genetic materials. The use of whole blood greatly simplifies lymphocyte culture. In addition, the use of non-separated blood will retain all blood cells, especially RBC, in natural condition thus allowing metabolic activation of environmental pollutants to take place[15,16].

The potentiality of human peripheral lymphocytes themselves to act as precursor has been demonstrated **in vitro** by the cloning of established lymphoblastoid cell line[17].

Choi and Bloom in 1970[18] established CKW–1 cell line involved a mixed lymphocyte culture, they used small inoculums of PGLC-33H cells (cell line from infectious mononucleosis patient) as feeder cells. They also used lysates of previously established lines as a second method of lymphocyte cell line establishment.

Different procedures for generation of T-cell lines were outlined[5]. They used Daudi or any lymphoblastoid cell line that are different from that of donor PBMNCs. Antigen specific T- cell lines were established using foreign antigens such as tetanus toxoid or PPD, restimulation with antigen, with the addition of IL2, and treatment of antigen presenting cells were included[5].

None of them used a whole blood culture for establishment of lymphocytes cell lines. No feeder cells were needed in our protocol or the addition of IL2.

Phytohemagglutinin-activated peripheral T- cells were found to be resistant to CD95 mediated apoptosis, and after prolonged IL-2 treatment, these T- cells became CD95 mediated apoptosis sensitive[9].

Investigations on resting T-cells, i.e., lymphocytes depleted of macrophages and pre-activated cells were made. When PHA was added to these cells resulted in activation with expression of IL-2R (CD25) but not in proliferation. In contrast, addition of PHA plus sheep RBC, which bind to the CD2 receptors, caused IL-2R expression, IL-2 production, and proliferation[19].

A trial of phenotype of the established cell lines was under work. Great benefits can be obtained from this work: this cell lines can be used for virus propagation for example measles virus, different cytokine production, and for different immunological techniques

Monitoring the growth of cultures by direct observation:

Although the growth of the Lymphoblastoid cell lines was, to a large extend, synchronized by stimulation culture, some cultures will grow faster than the others (as indicated in figure 1). It was only by examining the cells on a routine bases with the inverted microscope that the rapidly growing cultures can be identified and cultured accordingly, or by supplementation of GM. In addition, direct observation was the only way of verifying that the cells are actually growing, on day-to-day basis.

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