New Methods for Establishment of Lymphoblastoid Cell Lines

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Abstract

Background: 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 ,the lymphocyte can be cultured specifically and will outgrow the others, eventually resulting in highly enriched population.

Objective: this series of experiments were done as a trial to establish a new method for preparation of lymphocytes cell line.

Methods: the same protocols were applied to all individuals, they were separated in to two groups:-

Group 1:- one ml of heparinized blood were cultured in tissue culture flask containing 9 ml of stimulation medium and incubated in tissue culture flasks at 37ºC [(4)] . The flasks were subcultured at 37 ^{O}C in CO_2 incubator every 3 days.

Group 2:- Mononuclear cells were separated from whole blood, washed, counted, assessed for viability, then 1 ml of $2x10^6$ cells were cultured in tissue culture flask containing stimulation medium and. The flask was incubated at 37 ^{O}C in CO_2 incubator for 3 days. Another 1ml of mononuclear cells was cultured in tissue culture flask containing 1ml of growth medium, the flasks were incubated at 37 ^{O}C in CO_2 incubator for 3 days.For both groups the cell cultures were propagated and maintained.

Results: After initiation of cell culture for both groups, the mononuclear cell cultures were maintained up for 3 weeks, pure rich mononuclear cells were obtained and seen under inverted microscope.

Conclusion: a new method for establishment of lymphoblast cell line were developed by cultivation of lymphocyte from whole blood . pure rich lymphocytes culture were obtained and maintained.

Key words: whole blood lymphocyte culture, cell culture, phytohemagglutinin.

Introduction

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The Advances in the understanding of lymphocyte biology have been accompanied by improvement in the techniques of long-term culturing of human 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 are 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 outgrow 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 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% [6], 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.

This stimulation not only select 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].

Antibody interaction with a specific epitope of the HLA class I alpha1 domain triggers apoptosis of activated but not resting T and B cells by a pathway which involves neither Fas ligand nor tumor necrosis factor-alpha. They investigated at which stage of activation and proliferation T cells become sensitive to HLA class I-mediated apoptosis, using two monoclonal antibodies (mAb) which recognize the same monomorphic epitope of the HLA class I alpha1 domain (mAb9O, mouse IgG1, and YTH862, rat IgG2b) and can induce apoptosis of phytohemagglutinin (PHA)-activated peripheral blood lymphocytes [9].

This study was conducted as a trial of establishment of lymphoblast cell lines.

Materials and Methods

This series of experiments were done for five selected normal adult subjects , their ages ranged from 15 to 45 years, the same protocols were applied to all individuals, their blood were separated in to two groups. Five ml of venous blood was collected from each subject and placed in sterile heparinized tube:-

Initiation of cell culture:-

Group 1:- 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, streptomycin 100µg/ml, 10mM L-glutamine, 20% FBS, 10mM HEPES, 200µl/ml PHA) . The flasks were incubated at 37 ^{O}C in CO_2 incubator.

Group 2:- Mononuclear cells were separated from whole blood by the method described by Boyam [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 incubated at 37 ^{O}C in CO_2 incubator.

Propagation and maintenance of cell culture:

For group 1 ; every three days 1 ml of whole blood culture was subcultured into tissue culture flask containing 9 ml SM until visible suspension of mononuclear cells intervening agglutinated red blood cells were seen within two weeks of culture. Another 1ml of whole blood cells was cultured in tissue culture flask containing 1ml of growth medium (as stimulation medium without PHA), the flask was incubated at 37 ^{O}C in CO_2 incubator

For group 2 ; every three days 1 ml of purified cells was subcultured into tissue culture flask containing 9 ml SM. The flask were incubated at 37 ^{O}C in CO_2 incubator. until visible suspension of mononuclear cells within the tissue culture flaks were seen within two weeks of culture.

Both groups of cell culture were examined under inverted microscope. At that time , the cells washed twice with serum free medium, counted ,assessed for viability, then 1 ml of $2x10^6$ cells were cultured in tissue culture flask containing stimulation medium and incubated in tissue culture flasks at 37ºC. at indicated time the cells counted and assessed for viability. The percent of viable cells were determined at indicated time intervals , and $2x10^6$ cells were subcultured in tissue culture flask containing 1ml of SM every 72 hour. The cell cultures were maintained continuously by this method.

Results

Initiation of cell culture:-

For group 1 heparinized blood was cultured in SM for 72 hour, some visible suspension of mononuclear cells were seen intervening the agglutinated RBCs as seen in (figure 1a) also after 6 days (figure 1b).

For group 2 purified mononuclear cells after 72 hour culture a visible suspension of MNCs were seen under inverted microscope.

Propagation of MNCs culture:-

For group 1; whole blood culture were maintained as described in materials and methods, after two weeks of cell culture, 24 hour incubation of the cultured cells in SM, pure rich MNCs were seen under inverted microscope (figure 2), the cell stimulation is indicated by the rapid increase in the number of cells as seen in (table 1 figure 3). many cells became enlarged, and the medium turn yellow after 3 days of subculture. A clear stimulation was recognized without the aid of inverted microscope when the bottom of the tissue culture flask showed a large grayish – white central button of cells surrounded by pinpoint satellite colonies, while the cells cultured in growth medium a decrease in viable cell count as indicated in (table 1 figure 4).

For group 2 after 72 hour cell culture, 24 hour incubation of the separated lymphocyte in SM, pure MNCs were seen under inverted microscope, the cell stimulation is indicated by the increase in the number of cells as seen in (table 2 figure 5). Some cells became enlarged, and the medium turn yellow after 3 days of subculture. Another $2x10^6$ ml of cells were subcultured GM. Reductions in viable cell counts after day 5 were observed .

All the stimulated cells gave lymphoblast characteristics when stained with giemza stain.

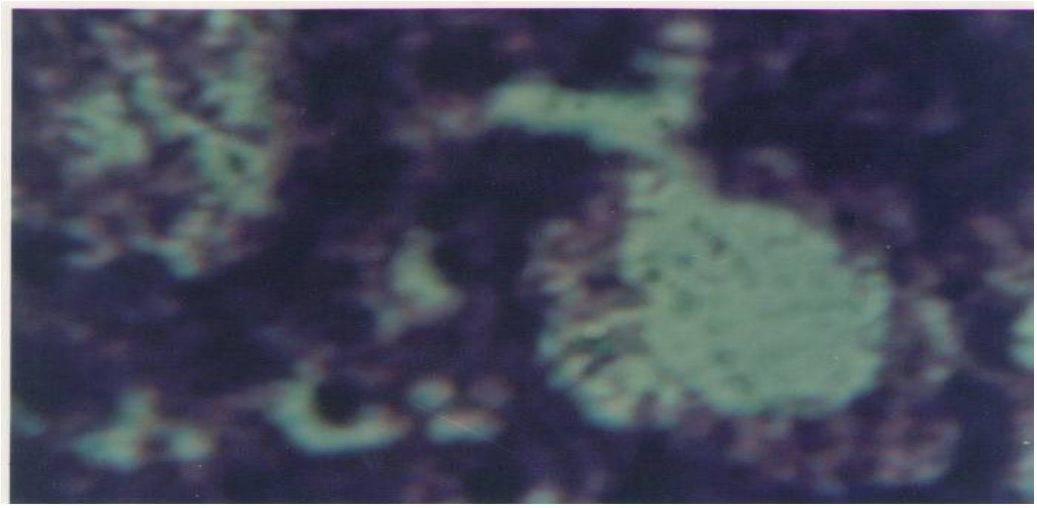

Figure (1.a): Photomicrograph shows whole blood lymphocytes culture after 3 days (x 250).

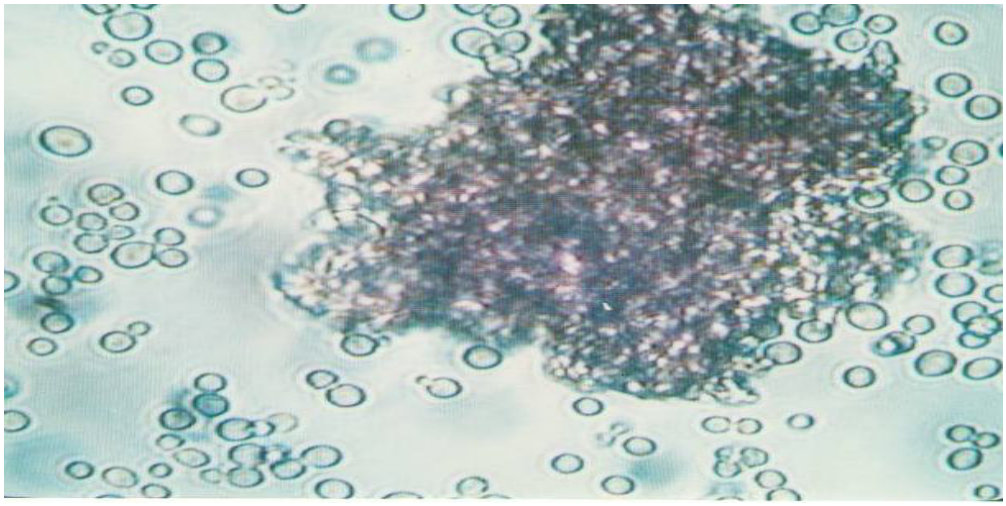

Figure (1b): Photomicrograph shows whole blood lymphocytes culture after 6 days (x 250).

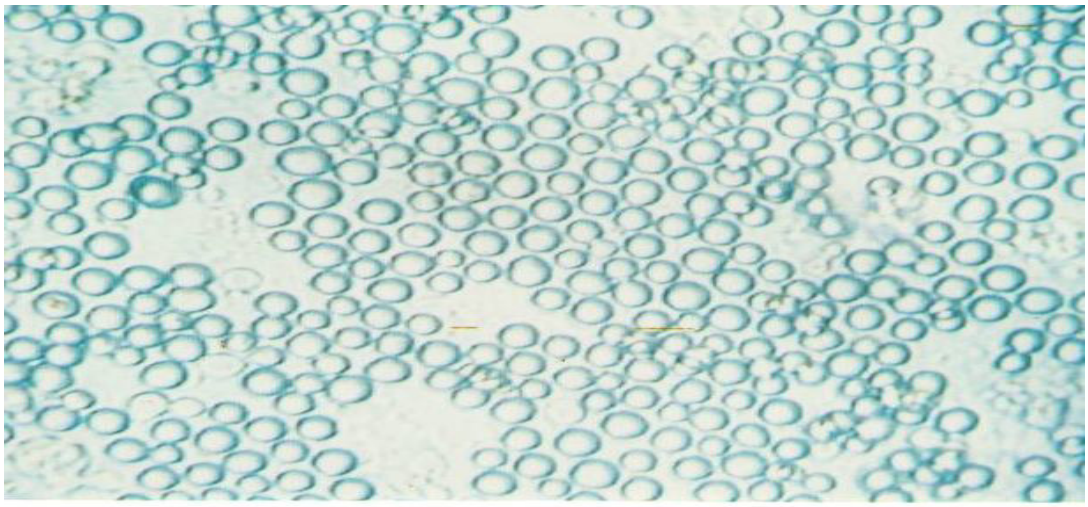

Figure (2): Photomicrograph shows stimulated lymphocytes culture after two weeks of whole blood culture initiation (x 250).

Table (1): Effect of cell proliferation and cell death on viable count over 20 days using growth or stimulation medium. The seeding density was 2x 10^5/ ml

culture media	*No.*	*Absolute Number of viable lymphocyte count / ml (x10^6)*							
		Zero time	*Days after culture initiation*						
			1	*3*	*5*	*8*	*11*	*14*	*17*	*20*
Stimulation medium	Sample1	0.2	2	20	60	110	230	400	800	1600
	Sample2	0.2	2	25	65	110	220	380	700	1450
	Sample3	0.2	2	22	75	150	280	410	810	1650

	Sample4	0.2	2.5	30	75	150	300	420	830	1680
	Sample5	0.2	1.5	18	60	100	200	350	700	1400
	Mean	***0.2***								
	SD	***0***								
Growth medium	***Sample1***	0.2	2	10	25	25	15	5	2	0
	Sample2	0.2	1.5	20	39	35	20	5	1	0
	Sample3	0.2	2.5	30	60	50	35	10	2	0
	Sample4	0.2	2	25	40	80	50	20	10	2
	Sample5	0.2	0.5	15	40	60	30	20	5	0
	Mean	***0.2***								
	SD	***0***								

Table (2): (group 2) Effect of cell proliferation and cell death on viable count over 20 days using stimulation medium. The seeding density was 2x 10^5/ ml

culture media	***No.***	***Absolute Number of viable lymphocyte count / ml (x10^6)***								
		Zero time	***Days after culture initiation***							
			1	***3***	***5***	***8***	***11***	***14***	***17***	***20***
Group 2 SM	Sample1	0.2	1	5	8	16	40	80	160	300
	Sample2	0.2	0.5	0.7	1.5	3	15	30	80	200
	Sample3	0.2	0.5	1	2.5	10	20	40	100	250
	Sample4	0.2	1.5	2.5	5	15	30	75	150	300
	Sample5	0.2	1	2	3.5	10	20	50	150	250
	Mean	***0.2***								
	SD	***0***								

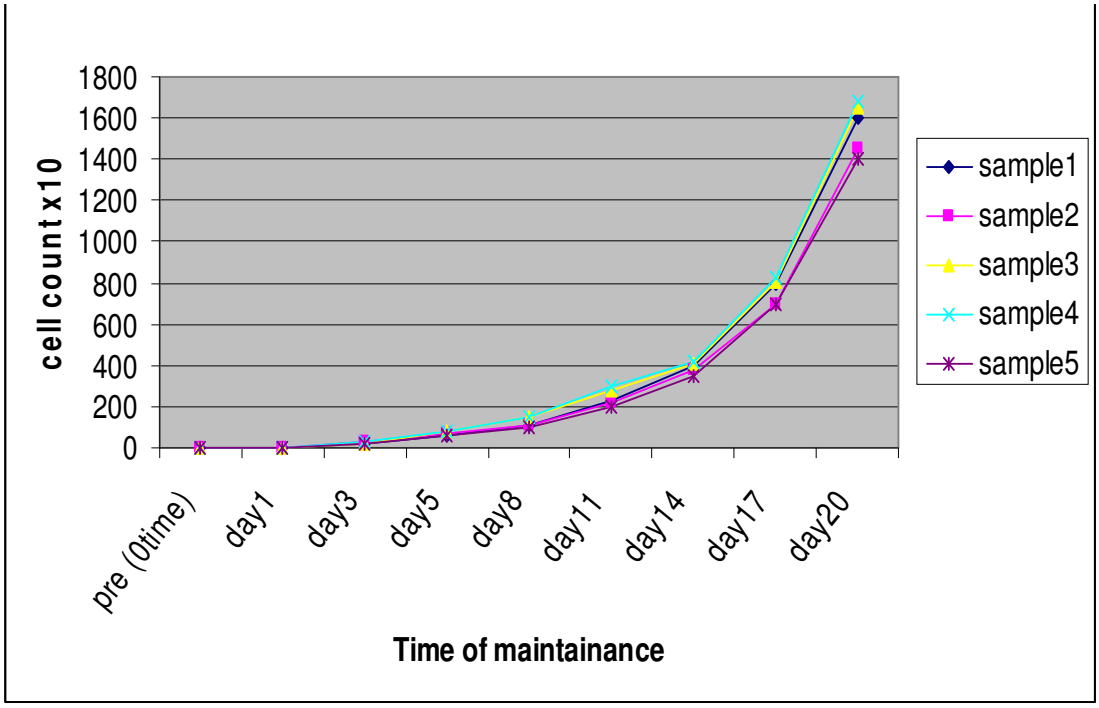

Figure (3): Effect of cell proliferation on viable cell count over 20 days period using SM. Each point represents the mean of three values, the seeding density was $2x1o^6$

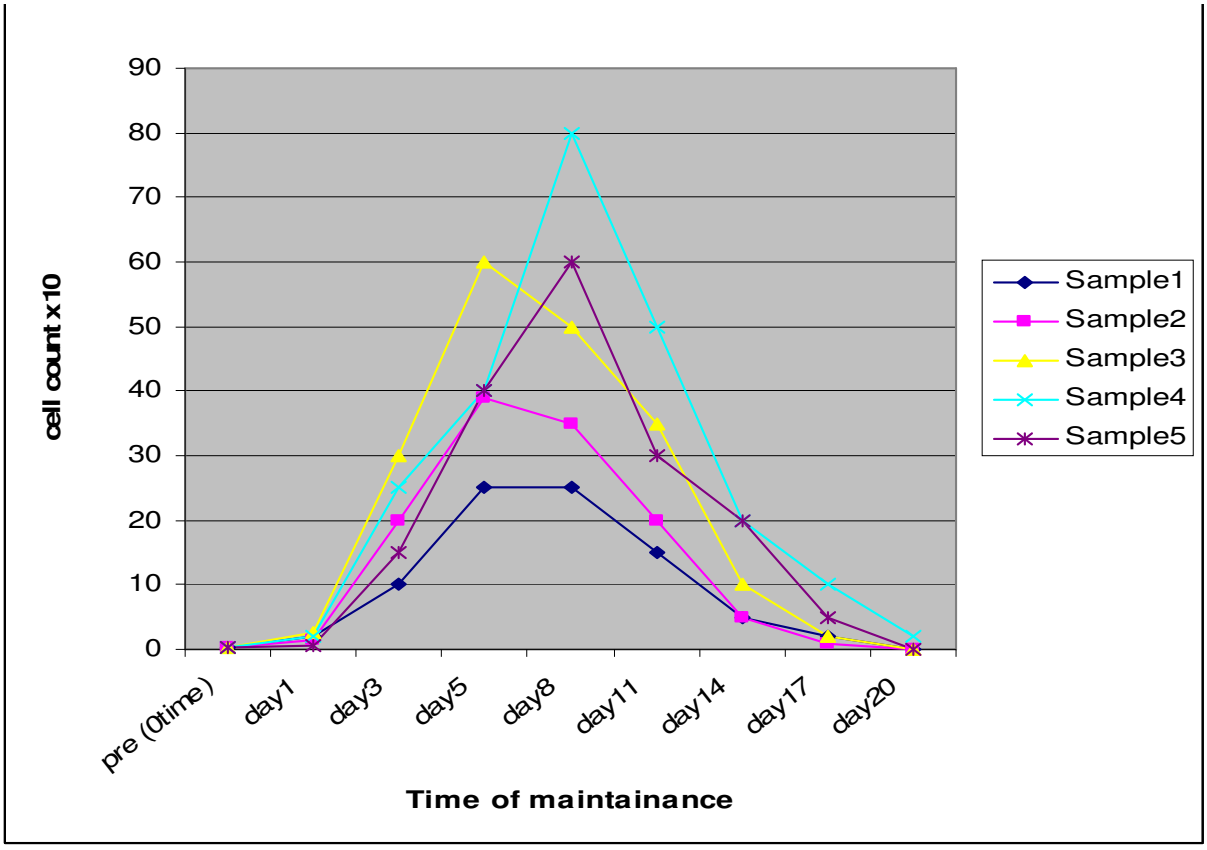

Figure (4): Effect of cell proliferation on viable cell count over 20 days period using GM. Each point represents the mean of three values, the seeding density was $2x1o^6$

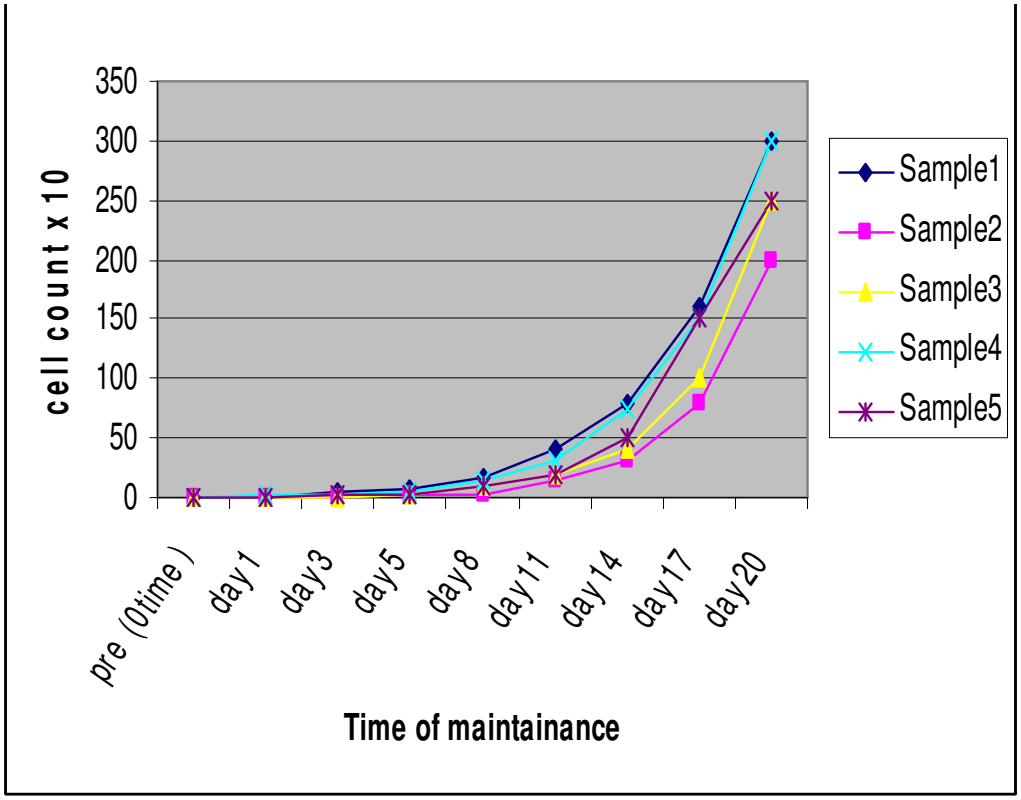

Figure (5): Group 2 Effect of cell proliferation on viable cell count over 20 days period using SM. Each point represents the mean of three values, the seeding density was $2x1o^6$

Discussion

This study compared two new invented techniques for establishment of lymphoblast cell lines, probably this is the modification of the first method of establishment of reproducible lymphoblast cell line invented by Latif *et al* [11]. As its evident in the results that the application of a technique on group I has better outcome then the protocol applied for the second group, it has higher chance for success, with least interruption of cell growth, beside that I had omitted the step of separation of lymphocyte by byoum method [10],as the step of purification of cells will depend of sequential subculture of the cells witch end in purified lymphocyte culture and disappearance of agglutinated erythrocytes and other cells with continuous subculture.

A culture started with mononuclear cell fraction would contain several different cell types. Nevertheless, the T-lymphocytes can be cultured specifically and will outgrow 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 and provide additional T-cell growth factors [12,13].

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 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 [14].

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 lab [15].

It has been suggested that the 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 [16,17].

The potentiality of human peripheral lymphocytes themselves to act as precursor has been demonstrated in vitro by cloning of established lymphoblastoid cell line [11,18].

Latif had established lymphoblastoid cell line and used whole blood culture and maintained their cell division [11]. The main differences in our protocol is omitting the purification step of lymphocyte from whole blood, which simplify the cultivation process.

Different procedures for generation of T cell lines were outlined by [5]. They used Daudi or any lymphoblastoid cell line that are different from that of donor PBMNCs. Antigen specific T- cell line 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].

Others established CKW–1 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 [19]. 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.

Hofmann *et al* (1989), made investigations on resting T cells, i.e., lymphocytes depleted of macrophages and pre-activated cells. 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 SRBC, which bind to the CD2 receptors, caused IL-2R expression, IL-2 production, and proliferation [20].

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 [21].

Great benefits can be obtained from this work. Same cell line had been used for propagation of measles virus [22]. Different cytokine production, and for different immunological techniques

Monitoring the growth of cultures by direct observation.

Although the growth of the lymphoblast cell lines was, to a large extend, synchronized by stimulation culture, some cultures will grow faster than the others. It was only by examining the cells on a routine bases with the inverted microscope that the rapid growing cultures can be identified and cultured accordingly, or by supplementation of growth media. 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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