A Whole Blood Lymphocyte Proliferation Assay in Healthy Thais: Comparison of Heparinized Blood and Acid Citrate Dextrose Blood

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The lymphocyte proliferation assay (LPA) is a technique to determine T-lymphocyte functions in vitro. The standard LPA using peripheral blood mononuclear cells (PBMC) separated from heparinized blood requires a large blood sample, time consuming and expensive. It is more useful if acid citrate dextrose (ACD) blood could be used not only for LPA but also for other purposes. To determine whether whole blood composing between heparinized blood and ACD blood could be substituted for standard LPA using PBMC. Heparinized and ACD blood of 35 healthy Thai blood donors were studied herein. PBMC separated by density gradient centrifugation and diluted heparinized and ACD blood were used to test and compare for lymphoproliferative responses to phytohemagglutinin (PHA), pokeweed mitogen (PWM), and tetanus toxoid. A stimulation index (SI) for each mitogen or antigen was calculated. All Thai blood donors demonstrated positive proliferative responses to PHA and PWM by using PBMC and whole blood culture assays from both heparinized and ACD blood. However, the difference in the frequency of positive proliferative responses to tetanus toxoid by using PBMC and whole blood culture assays was significant. Nevertheless, no significant difference in frequency of positive responses to tetanus toxoid between heparinized and ACD blood was observed. This results suggested that no significant difference between using heparinized and ACD blood in standard LPA using PBMC. However, the whole blood LPA for measuring mitogen induced lymphoproliferation could be substituted for standard LPA from heparinized and ACD blood. Whole blood LPA is easy, rapid, and more cost effective than PBMC culture assay. Thus, it would be applicable in a clinical laboratory as well as in research setting.

Keywords: *Lymphocyte proliferation assay, Whole blood, Heparin, Acid citrate dextrose*

J Med Assoc Thai 2010; 93 (Suppl. 2): S21-26
Full text. e-Journal: *http://www.mat.or.th/journal*

Lymphocyte proliferation assay (LPA) is a technique to determine T-lymphocyte functions *in vitro* and has been widely used to describe the abnormalities associated with diverse congenital immunodeficiencies as well as those considered to be secondary to infectious diseases, cancer, aging, malnutrition, stress and autoimmune diseases. In HIV infection, loss of lymphocyte responsiveness has been claimed to add to the predictive value of the CD4 count in predicting progression to AIDS[1]. The standard LPA using peripheral blood mononuclear cells (PBMC) requires a large blood samples, time consuming, well-trained laboratory workers, and expensive.

Methods for measuring lymphoproliferative responsiveness using whole blood have been established by several laboratories[2-8]. Many advantages of using whole-blood cultures are small blood volume needed, many samples processed at once, easier and more rapid assay, and more cost effective than standard LPA using PBMC. Moreover, it is more useful if

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other anticoagulants than heparin which is a reagent commonly used for LPA could be used. Because we can used them not only in LPA but also multiassays. The objective of this study was to compare heparinized blood with ACD blood in both standard LPA using PBMC and whole blood LPA in healthy Thai blood donors.

Material and Method

Study subjects

Thirty five healthy adults who were routine blood donors of the Army Institute of Pathology, Bangkok, Thailand were voluntarily recruited. All subjects gave their informed consent prior to venepuncture. The mean age was 34 years (± 10.59 SD, range 18-60). Peripheral blood samples were collected in vacutainer tubes containing sodium heparin (10 ml) and tubes containing ACD (10 ml). For comparison of standard LPA and whole blood LPA, 20 ml of blood was obtained from each healthy blood donor; 1 ml was used for WB cultures and 9 ml for PBMC cultures of each anticoagulant used. Each subject's blood specimen was cultured by techniques employing both PBMC, prepared by density gradient centrifugation, and WB as the source of cells. This study has already been approved by the Institutional Review Board of Royal Thai Army.

Preparation and culturing of whole blood specimens

Heparinized blood and ACD blood samples were diluted 1:10 and 1:20 (v/v) with sterile RPMI 1640 tissue culture medium (Gibco) supplemented with 2 mM L-glutamine (Gibco), 10,000 U/ml penicillin and 10,000 µg/ml streptomycin (Gibco) and without adding serum. One hundread microlitres of each diluted blood sample were cultured in triplicate wells of round bottom both mitogen and antigen plates. Cultures were pulsed, harvested, and counted as described below.

Preparation and culturing of PBMC

PBMCs were isolated from heparinized blood and ACD blood by density gradient centrifugation over Ficoll-Hypaque (Sigma). PBMCs were washed three times, counted and adjusted to a concentration of 1 x 10^6/ml in RPMI 1640 supplemented with 2 mM L-glutamine 10,000 U/ml penicillin and 10,000 µg/ml streptomycin and 5% fetal bovine serum (Gibco). Viability was estimated by trypan blue dye exclusion. One hundread microlitres were cultured in triplicate wells of round bottom both mitogen and antigen plates and carried out as described below.

Lymphocyte proliferation assay

The ability of lymphocytes to proliferate *in vitro* in response to mitogens or antigen was assessed by both standard LPA and WB LPA. The mitogen and antigen plates were prepared using round-bottomed 96 well plates (Costar). Each well contained the appropriate amount of mitogen or antigen in a volume of 100 µl of complete medium (RPMI 1640 supplemented with L-glutamine [25 mM], penicillin [200 U/ml], streptomycin [200 µg/ml] and 10% heat-inactivated human AB serum (Gemini Bio-product) to yield the following final concentrations after PBMC were added: phytohemagglutinin-L (PHA-L; Sigma), 16 µg/ml; pokeweed mitogen (PWM; Sigma) 0.63 µg/ml; Tetanus toxoid (TT; Connaught and Staten Serum Institute) 6.5 Lfu/ml. Plates were sealed, frozen, and stored at -70°C until used.

The plates with mitogen were incubated for 3 days and the plates with antigen were incubated for 6 days at 37°C under 5% CO_2 and a 95% humidified atmosphere. Each well was pulse-labeled with 1 µCi (25 µl) of [^{3}H] thymidine in supplemented RPMI 1640 without adding serum. After 6 h, cells were harvested using an automatic cell harvester (Titertek) and the incorporated radioactivity into DNA was measured in a liquid scintillation counter (Packard Tricarb). Stimulation index (SI) for each mitogen or antigen are presented herein. The stimulation index (SI) was calculated by dividing the median counts per minute (cpm) in stimulated cultures by the median cpm in control cultures. The SI value > 3 was considered a positive response.

Statistical analysis

Chi-square test was applied to compare the frequency of positive lymphoproliferative responses to each mitogen and antigen by using different anticoagulants and different methods. Paired data (median) of SI from subjects using different anticoagulants and methods were compared by the Wilcoxon signed ranks test. The p-values < 0.05 were considered statistically significant.

Results

Optimization of culturing system

The optimal dilution of whole blood was studied for WB LPA. WB diluted 1:10 and 1:20 (v/v) of all subjects demonstrated positive responses to various mitogens (Table 1). No significant difference was shown between the frequency of positive responses to TT from WB 1:10 (v/v) and WB 1:20 (v/v) in both heparinized blood and ACD blood (Table 1). However,

the SI of positive responses from WB 1:10 (v/v) were significantly higher than the SI of positive responses from WB 1:20 (v/v) (Table 2). It suggested that WB dilution 1:10 (v/v) was optimum for this WB LPA.

Effect of anticoagulant on standard lymphocyte proliferation assay

Thirty-five healthy Thai blood donors (35/35) all demonstrated positive proliferative responses to PHA and PWM using PBMC (standard LPA) from both heparinized and ACD blood (100% positive responses; Table 1). The frequency of proliferative response to TT from both heparinized and ACD blood were 88.6% (31/35) and 82.9% (29/35) respectively (Table 1). However, no significant difference in the frequency of positive responses to TT using heparinized and ACD blood was found ($p = 0.733$; Table 1). Moreover, the SI values comparing heparinized and ACD blood in response to PHA, PWM and TT in standard LPA were not significant (Table 2).

Effect of anticoagulant on whole blood lymphocyte proliferation assay

In WB LPA, both heparinized and ACD blood of all blood donors (100%, 35/35) showed positive responses to PHA and PWM. No significant difference between the frequency of proliferative response to PHA, PWM, and TT from heparinized and ACD blood was observed in WB LPA (Table 1). The frequency of proliferative response to TT using both heparinized and ACD blood were 45.7% (16/35) and 40.0% (14/35), respectively (Table 1). However, no significant difference in the frequency of positive responses to TT using heparinized and ACD blood was found ($p = 0.809$; Table 1). The results indicated that in WB LPA, the SI values of PWM and TT except PHA using heparinized blood were significantly higher than those using ACD blood (Table 2).

Comparison of whole blood and standard lymphocyte proliferation assay

No significant difference in the frequency of proliferative response to PHA, PWM, comparing between WB LPA and standard LPA using both heparinized and ACD blood was observed. All showed positive responses to PHA and PWM (100%; Table 1). Nevertheless it was found that the frequency of positive responses to TT in standard LPA was significant by higher than that in WB LPA [88.6% (31/35) vs. 45.7% (16/35) $p < 0.001$ for heparinized blood and 82.9% (29/35) vs. 40.0% (14/35) [$p < 0.001$ for ACD blood, respectively; Table 1]. In addition, it was found that the SI values of PHA and PWM in WB LPA were significantly by lower than those of standard LPA using heparinized blood (Table 2). For ACD blood, the SI values to PWM and TT in WB LPA were significantly by lower than those of standard LPA (Table 2).

Discussion

In this study, heparinized blood with ACD

Table 1. The frequency of positive lymphoproliferative responses* (LPR) to mitogens and antigen using PBMC and WB between heparinized and ACD blood

Stimulant	Source of cells	n	Proportion of volunteers with positive LPR		
			Heparin	ACD	p-value**
PHA	PBMC	35	35/35 (100%)	35/35 (100%)	
	WB 1:10	35	35/35 (100%)	35/35 (100%)	
	WB 1:20	35	35/35 (100%)	35/35 (100%)	
PWM	PBMC	35	35/35 (100%)	35/35 (100%)	
	WB 1:10	35	35/35 (100%)	35/35 (100%)	
	WB 1:20	35	35/35 (100%)	35/35 (100%)	
TT	PBMC	35	31/35 (88.6%)	29/35 (82.9%)	0.733
	WB 1:10	35	16/35 (45.7%)	14/35 (40.0%)	0.809
	WB 1:20	35	18/35 (51.4%)	8/35 (22.9%)	0.026
	PBMC versus WB 1:10 (p**)		< 0.001	< 0.001	
	PBMC versus WB 1:10 (p**)		0.811	0.198	

* Positive LPR, SI > 3

** Comparison by Chi-square test

Table 2. The stimulation index of positive lymphoproliferative responses* to mitogens and antigen using PBMC and WB between heparinized and ACD blood

Stimulants	Source of cells	Median of Stimulation Index (25th, 75th)**		
		Heparin	ACD	p-value***
PHA	PBMC	211.3 (133.4, 771.4)	188.4 (127.4, 679.7)	0.342
	WB 1:10	397.3 (215.9, 1889.9)	221.5 (90.7, 1225.9)	0.059
	WB 1:20	185.0 (119.3, 361.9)	135.6 (96.0, 365.2)	0.830
	PBMC versus WB 1:10 (p)***	< 0.001	0.280	
	WB 1:10 versus WB 1:20 (p)***	< 0.001	< 0.001	
PWM	PBMC	88.7 (52.3, 166.1)	79.9 (53.6, 166.6)	0.523
	WB 1:10	46.0 (16.6, 118.7)	35.9 (11.1, 86.2)	0.001
	WB 1:20	17.2 (11.9, 24.6)	20.9 (10.7, 33.4)	0.049
	PBMC versus WB 1:10 (p)***	< 0.001	< 0.001	
	WB 1:10 versus WB 1:20 (p)***	< 0.001	< 0.001	
TT	PBMC	26.5 (15.9, 54.8)	23.0 (11.3, 65.3)	0.247
	WB 1:10	19.9 (12.3, 55.2)	15.2 (4.9, 23.6)	0.016
	WB 1:20	6.9 (4.2, 17.6)	4.6 (3.4, 14.3)	0.500
	PBMC versus WB 1:10 (p)***	0.121	0.002	
	WB 1:10 versus WB 1:20 (p)***	0.019	0.043	

*Positive LPR, SI > 3
** Data were calculated from positive LPR
*** Comparison by Wilcoxon-signed ranks test

blood in both standard LPA using PBMC and whole blood LPA in healthy Thai blood donors were compared. It was shown the results using heparinized and ACD blood were not significantly different from standard LPA using PBMC. This finding is consistent with the previous report which demonstrated no significantly difference in the frequency of positive responses between heparin and ACD blood for any of stimulants (PWM, Tetanus toxoid, Candida albicans, Streptokinase) in standard LPA[9].

However, the study herein demonstrated that lymphocyte proliferative responses of whole blood to recall antigentetanus toxoid using both heparinized and ACD blood yielded a significantly lower number of positive results than those of standard LPA using PBMC. It indicated that regardless of anticoagulant, stimulation with antigen (tetanus toxoid) required more cell numbers especially monocytes-antigen presenting cells than stimulation with mitogen and the red blood cell content in whole blood LPA which was probably interfered by the culture dynamic in proliferative responses. Stimulation with the mitogenic lectins PHA and PWM involves activation through several pathways including CD2, CD3 and additional cell surface receptors[10-12]. Mitogens activate a broad population of mononuclear cells and unlike antigens which do not require processing, presentation, or functional memory T cells. Moreover, studies of the proliferative ability of lymphocytes from HIV-infected patients are reported that 24 hours of storage of blood using heparin or ACD does affect antigen-specific responses, while mitogen-specific responses are better preserved[13].

The interesting result herein showed that there were variations in the SI values of PHA of healthy Thai blood donors. Thus, it may be the reason of no significant difference in the SI values of PHA between heparinized blood and ACD blood in WB LPA and between PBMC and WB 1:10 (v/v) of ACD blood.

In vitro lymphocyte proliferation assay is an important and widely used assay to assess specific immunological memory and general immune function. There are some studies simplified by adapting a well-established seperated lymphocyte method to be used with whole blood. Previous investigators had developed whole blood assays for measuring responses to mitogens and specific antigens for investigating a variety of infectious diseases in which T cell mediated immunity plays an important role[3,14-16] for intense military training with reduced caloric intake[6] and also for assessing and following the changes in immune

function which occur in asymptomatic HIV-infected subjects[5].

In conclusion, the whole blood LPA using both heparinized and ACD blood for measuring mitogen induced lymphoproliferation could be substituted for standard LPA using PBMC. The whole blood LPA is less cumbersome, less blood used, less time consuming and more cost effective than the PBMC culture assay. Moreover, the immune cells are maintained in an environment more similar to that found *in vivo*. Thus, it would be applicable for service in a clinical laboratory as well as for research uses.

Acknowledgements

This work was financially supported by Armed Forces Research Institute of Medical Sciences, Royal Thai Army and the Henry M. Jackson Foundation. We thank all the volunteers for generous donations of blood used in these studies and Dr. Chitraporn Karnasuta, Rapee Trichavasroj, Suchada Sukhumvittaya, and Surat Jongrakthaitae for excellent technical assistance.

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วิธีทดสอบ Whole blood lymphocyte proliferation ในคนไทยที่มีสุขภาพดี: เปรียบเทียบระหว่างเลือดที่ใช้ Heparin และ Acid Citrate Dextrose เป็นสารป้องกันการแข็งตัวของเลือด

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วิธี lymphocyte proliferation เป็นเทคนิคที่ใช้ทดสอบการทำงานของเม็ดเลือดขาวชนิดที ลิมโฟซัยท์ภายนอกร่างกาย การทดสอบ lymphocyte proliferation แบบมาตรฐานโดยใช้เม็ดเลือดขาวชนิดโมโนนิวเคลียสที่แยกจากเลือดที่ใช้ heparin เป็นสารป้องกันการแข็งตัว จะใช้เลือดปริมาณมาก เสียเวลา และราคาแพง ถ้าสามารถใช้เลือดที่มี acid citrate dextrose เป็นสารป้องกันการแข็งตัว ได้จะเป็นประโยชน์ไม่เพียงแต่ใช้ในการทดสอบ lymphocyte proliferation เท่านั้นแต่ใช้สำหรับการทดสอบอื่นๆได้อีก จึงศึกษาวิจัยการใช้เลือดโดยตรงแทนเม็ดเลือดขาวชนิดโมโนนิวเคลียส เปรียบเทียบ ระหว่างเลือดที่ใช้ heparin และ acid citrate dextrose โดยใช้เลือดที่มี heparin และ acid citrate dextrose ที่เจือจางแล้วโดยตรง และ เม็ดเลือดขาวชนิดโมโนนิวเคลียสจากเลือดที่ใช้ heparin และ acid citrate dextrose ซึ่งถูกแยกโดยวิธีการแยกชั้นตามความหนาแน่นหลังการปั่น (density gradient centrifugation) นำมาใช้เพื่อทดสอบ และเปรียบเทียบการตอบสนองโดยการแบ่งตัวเพิ่มจำนวนของเม็ดเลือดขาวชนิดโมโนนิวเคลียส (lymphoproliferative responses) ต่อไมโตเจน คือ phytohemagglutinin, pokeweed mitogen และแอนติเจน คือ tetanus toxoid ที่ใช้กระตุ้น ค่าดัชนีการกระตุ้น (stimulation index) สำหรับแต่ละไมโตเจนและแอนติเจนของเลือดคนไทยผู้บริจาคโลหิตทั้งหมด แสดงผลบวกในการ ตอบสนองโดย การแบ่งตัวเพิ่มจำนวนของเม็ดเลือดขาวชนิดโมโนนิวเคลียส ต่อ phytohemagglutinin, และ pokeweed mitogen เมื่อใช้การทดสอบแบบมาตรฐานโดยใช้เม็ดเลือดขาวชนิดโมโนนิวเคลียส และแบบใช้เลือดโดยตรงทั้งจากเลือดที่ใช้ heparin และ acid citrate dextrose ผลที่ได้แสดงความแตกต่าง อย่างมีนัยสำคัญทางสถิติของจำนวนผลบวกในการตอบสนองโดยการแบ่งตัวเพิ่มจำนวนของเม็ดเลือดขาว ชนิดโมโนนิวเคลียส ต่อ tetanus toxoid เมื่อใช้เม็ดเลือดขาวชนิดโมโนนิวเคลียส และแบบใช้เลือดโดยตรง แต่ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติของจำนวนผลบวก ในการตอบสนองโดยการแบ่งตัวเพิ่มจำนวนของเม็ดเลือดขาวชนิดโมโนนิวเคลียส ต่อ tetanus toxoid เมื่อใช้การทดสอบแบบมาตรฐานโดยใช้เม็ดเลือดขาวชนิดโมโนนิวเคลียสจากเลือดที่ใช้ heparin และ acid citrate dextrose ผลการศึกษานี้แสดงให้เห็นความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างการใช้เลือดที่ใช้ heparin และ acid citrate dextrose ในวิธีการทดสอบ lymphocyte proliferation แบบมาตรฐาน อย่างไรก็ตามวิธีการทดสอบ lymphocyte proliferation โดยใช้เลือดโดยตรงสำหรับการวัดการตอบสนองโดยการ แบ่งตัวเพิ่มจำนวนของเม็ดเลือดขาวชนิดโมโนนิวเคลียสต่อไมโตเจนสามารถใช้แทนวิธีแบบมาตรฐานได้ วิธีการทดสอบ lymphocyte proliferation โดยใช้เลือดโดยตรงเป็นวิธีที่ง่าย รวดเร็ว และประหยัดคุ้มค่ากว่าวิธีที่ใช้เม็ดเลือดขาว ชนิดโมโนนิวเคลียสสามารถนำวิธีนี้มาประยุกต์ใช้ในงานบริการของห้องปฏิบัติการทางคลินิกและงานวิจัยภาคสนาม