วารสารเภสัชกรรมเชียงใหม่ เชียงใหม่
Bulletin of Chiang Mai Associated Medical Sciences

Vol 12 No. 1
January 1979

COMPARISON OF PERCENTAGE OF T-LYMPHOCYTE WITH NUMBER OF LYMPHOCYTE IN HUMAN PERIPHERAL BLOOD OBTAINED BY TWO DIFFERENT METHODS; DEXTRAN FLOATATION AND FICOLL-HYPAQUE SEPARATION TECHNIQUE

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ABSTRACT

The present paper described a technique for isolation of lymphocyte from 12 ml whole blood by the methods of dextran floatation and Ficoll-Hypaque separation. When dextran was used as a separating agent the whole blood cells were separated into two parts, the lower part was almost red cells and some leukocytes and platelets. The upper part was white blood cell-rich plasma. The total white blood cell count were $28x10^6 \pm 6.06x10^6$ cells/ml which were $16.25x10^6 \pm 6.43x10^6$ cells/ml of mononuclear cells and contained $64.21 \pm 10.40\%$ T-rosettes. With Ficoll-Hypaque separation, the blood was separated into two fractions. The botto n fraction contained erythrocyte and granulocyte. The white band, upper fraction, contained total mononuclear cells of $11.25x10^6 \pm 2.09x10^6$ cells/ml from total white blood cell count of $12.0x10^6 \pm 2.21x10^6$ cells/ml and $65.06 \pm 8.83\%$ T-rosettes.

The number of lymphocyte obtained from dextran floatation was more higher than from Ficoll-Hypaque separation. However, the percentage of T-lymphocyte per white blood cell count by dextran floatation was lower and more leukocyte, platelet contamination when compared with Ficoll-Hypaque separation.

The ratio of T-lymphocyte and B-lymphocyte has been reported but varying in the results depned on the methods used to separate or identify them. There were many methods used for separating lymphocyte from the peripheral blood such as 3% gelatin, glass wool filtration, bouyanted

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Received for Publication; August 15, 1978

density gradient centrifugation and Ficoll - Hypaque [1,2,3,4,6,7]. This study is therefore initiated for the comparison of yield human peripheral blood lymphocyte separated by dextran floatation and Ficoll-Hypaque separation technique. The percentage of the T-lymphocyte prepared by those two technique was identified by rosette-formation.

MATERIALS AND METHODS

Twelve mililiters of blood was drawn from normal volunteers. The blood was divided into two 6 ml portions; one portion was mixed well with 0.6 ml of 6% dextran solution with 0.9 ml of 500 IU heparin and kept vertically in 37°C incubator for 40-50 minutes to allow red cell sedimentation. The leukocyte-rich plasma, upper layer, was collected. The other portion was mixed well with 18 ml of normal saline with 0.6 ml of 500 IU heparin and overlayered on Ficoll-Hypaque mixture (4.8 ml of 9% Ficoll, 2 ml of 34% Hypaque). The mixture was centrifuged at 1000 rpm (400 g) for 40 minutes. The white opaque layer between the plasma and Ficoll-Hypaque layers was collected. The cells obtained from those two methods were washed three times with buffered saline solution (BSS), pH 7.4. and both were resuspended with 1 ml of Hank's solution. The cells were then counted, so called total white blood cell count. The cell suspension was diluted to $5x10^6$ cells/ml with Hank's solution.

The T-rosette was determined by mixing 0.2 ml of the adjusted lymphocyte suspension with 0.2 ml of 5% sheep red blood cell suspension in Hank's solution. The mixture was incubated at 37°C in a waterbath for 15 minutes and then centrifuged at 200g for 5 minutes and reincubated overnight at 4°C. The supernatant fluid was removed and the cell pellet was resuspended gently with 0.2 ml of Hank's solution. One drop of the cell suspension was placed on slide and mixed with one drop of fetal calf serum (or AB serum), as a fixing agent. Using a second slide smeared forward until nearly to the terminal end of the first slide, tilted the smear slide backward to allow the mixture to flow backward and kept the smear dry at room temperature. The smear was fixed by flaming for 2-3 seconds before staining with Wright's stain. Five hundred lymphocytes was counted, those cells bound more than three sheep red cells was considered to be rosette forming cells or T-lymhocytes. The percentage of rosette forming T-lymphocyte was then calculated. A differential leukocyte counting was also performed on each specimen.

RESULTS

The separation lymphocyte from red blood cell by dextran floatation, the blood cells were separated into two portions after standing for 40-50 minutes. The upper portion was granulocyte-rich plasma containing mainly lymphocyte, granulocytes, platelets, and lower portion was mainly red blood cells with some leukocytes and platelets.

By Ficoll-Hypaque separation, the blood cells were separated into two fractions after centrifugation. A white layer appeared at the interface region containing of mononuclear cells and a small number of platelets and a bottom fraction containing erythrocytes and granulocytes. The plasma layer, upper layer, was clear and containd no cells.

In this study of 10 normal adult volunteers with the age of 20-30 years, the yield of lymphocytes when separated by dextran floatation were $16.25 \times 10^6 \pm 6.43 \times 10^6$ cells/ml (range 8.6×10^6-25.6×10^6 cells/ml) from the total white blood cell count of $28.0 \times 10^6 \pm 6.06 \times 10^6$ cells/ml (range 15.3×10^6-34.2×10^6 cells/ml), the differential counts were 57.8 ± 16.78% mononuclear cells, 41.1 ± 17.62% neutrophils and 2.7 ± 2.16% eosinophils and 64.2 ± 10.04% T-rosettes (range 40.1-74.2). The lymphocytes obtained by Ficoll-Hypaque separation were $11.25 \times 10^6 \pm 2.09 \times 10^6$ cells/ml (range 7.5×10^6-13.8×10^6 cells/ml) from the total white blood cell count of $12.0 \times 10^6 \pm 2.21 \times 10^6$ cells/ml (range 7.7×10^6 - 14.3×10^6 cells/ml), differential counts were 95.8 ± 2.25% mononuclear cell (range 93-100), 4.1 ± 2.28% neutrophils (range 0-7%), 0.1 ± 0.31% eosinophils (range 0-1%), and 65.06 ± 8.83% T-rosettes (range 49.8 - 74.6%). The total white blood cells and mononuclear cells counts by dextran were more than by Ficoll Hypaque, a significant difference, $p < 0.05$, respectively. The percentage of the mononuclear cells differential count from both methods were also significantly different $p < 0.001$, but the percentages of T-rosette were not different.

Table: Comparison of the yield of white blood cell and %T-lymphocyte by two methods

No.	Dextran							Ficoll-Hypaque						
	Total WBC count ($x10^6$/ml)	Differential count M	N	E	B	Total M ($x10^6$/ml)	% T-cell	Total WBC count ($x10^6$/ml)	Differential count M	N	E	B	Total M ($x10^6$/ml)	% T-cell
1	21.8	79	21	-	-	17.2	40.1	10.4	95	5	-	-	9.9	49.8
2	25.8	45	52	3	-	11.6	69.6	10.9	93	7	-	-	10.1	69.0
3	34.2	82	15	3	-	28.0	74.2	14.1	98	2	-	-	13.8	74.6
4	28.0	61	33	6	-	17.1	72.0	10.2	96	3	1	-	9.8	67.6
5	25.9	35	63	2	-	9.1	69.6	11.4	100	-	-	-	11.4	70.4
6	15.3	57	43	-	-	8.7	64.6	7.7	97	3	-	-	7.5	65.0
7	33.2	77	23	-	-	25.6	66.6	14.0	97	3	-	-	13.6	74.6
8	33.1	39	57	4	-	12.9	68.0	13.3	94	6	-	-	12.5	67.0
9	29.4	50	46	4	-	14.7	62.4	13.7	93	7	-	-	12.7	62.6
10	33.3	53	42	5	-	17.6	55.0	14.3	95	5	-	-	13.6	50.0
Mean	28.0	58	41	3	-	16.3	64.2	12.0	96	4	0.1	-	11.3	65.1
S.D.	6.1	17	18	2	-	6.4	10.0	2.2	2.3	2	0.3	-	2.1	8.8
S.E.	1.9	5	6	1	-	2.0	3.2	0.7	1	1	0.1	-	0.7	2.8

M = Mononuclear cell N = neutrophil B = basophil E = eosinophil

Discussion

In this experiment the rosette-forming lymphocyte value assumed to be T-cells (5,8), of 10 normal adults had the mean of 64% by dextran and 65% by Ficoll-Hypaque separation which was in agreement with other reports. It was reported that in normal 18-45 years old male and female had 65% T-cells in blood (5). In addition, by using sandwich radio-immuno-labelling method for separating lymphocyte from blood, it is reported that normal persons had 66% T-cells (8).

The processes of lymphocyte separation by Ficoll-Hypaque base on the difference of density and relative viscosity. Small and large lymphocytes were different in the density and relative viscosity (6). This reason could answer the question that why do total lymphocyte count which was separated by dextran solution was higher increased than by Ficoll-Hypaque mixture. By Ficoll-Hypaque mixture there was small lymphocyte only. The phenomenon of rosetting between rosette-forming cells and uncoated sheep erythrocyte was temperature-dependent in that it occured maximally between 4-25°C and failed to occur at 37°C (1). The nature of human T-lymphocyte receptor was unknown but it was highly unlikely that the binding was antigenically specific. The species of origin of the erythrocyte was critical, however, in that rosette did not occur with uncoated human, rabbit, pigeon, mouse, rat, monkey, cow, cat, chicken or guinea pig erythrocytes and thus far been seem only with sheep, goat, horse and pig erythrocytes. The rosette cell examined by dry smear method. Because the pseudorosette cells with occurred from granulocyte, could be differentiate by examining its difference of nucleus. In addition, the smear slide by kept for record or for reevaluation for a long time.

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ย่อเรื่องที่ผ่านมาแล้วข้างต้น

จากการเปรียบเทียบระหว่างวิธี dextran floatation กับ Ficoll-Hypaque separation เพื่อแยก lymphocyte ออกจากเลือดนั้น ปรากฏว่าสำหรับวิธี dextran floatation เลือดจะถูกแยกเป็น 2 ส่วน ส่วนกลางได้แก่ เม็ดเลือดแดง, เม็ดเลือดขาวบางส่วนและ platelets. สำหรับส่วนบนเป็น plasma ที่เต็มไปด้วยเม็ดเลือดขาว จำนวนเม็ดเลือดขาวที่นับได้ทั้งหมดมี $28.0 \times 10^6 \pm 6.06 \times 10^6$ cells/ml ซึ่งเป็นพวก mononuclear cells เสีย $16.25 \times 10^6 \pm 6.43 \times 10^6$ cells/ml และประกอบด้วย T-rosettes $64.21 \pm 10.54\%$ สำหรับวิธี Ficoll-Hypaque separation นั้นปรากฏว่าเลือดที่แยกได้เป็น 2 ส่วนเช่นเดียวกัน ส่วนล่างเป็นพวกเม็ดเลือดแดงและ granulocyte ส่วนแถบขาวด้านบนประกอบด้วย mononuclear cell $11.25 \times 10^6 \pm 2.09 \times 10^6$ cells/ml เมื่อคิดจากเม็ดเลือดขาวที่นับได้ทั้งหมด $12.0 \times 10^6 \pm 2.21 \times 10^6$ cells/ml และประกอบด้วย T-rosettes $65.06 \pm 8.83\%$

ถึงแม้ว่าจำนวน lymphocyte ที่นับได้จากวิธี dextran floatation สูงแต่ว่าเปอร์เซ็นต์ของ T-lymphocyte ต่อการนับจำนวนเม็ดเลือดขาวทั้งหมดจะมีค่าต่ำกว่าวิธีของ Ficoll-Hypaque separation อีกทั้งยังมีการปะปนของเม็ดเลือดขาวและ platelet เป็นจำนวนมาก