NOTE

Optimum Density of the Separating Fluid for Centrifugal Separation of Lymphocytes from Bovine Peripheral Blood

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Bovine lymphocytes were separated from peripheral blood by the method of Böyum [1], that is commonly used for separating lymphocytes from human blood. In the separation of bovine lymphocytes by density gradient centrifugation, most investigators used a density of 1.077 [4, 5, 8, 11, 13] and others: 1.075 [9, 12], 1.080 [7, 10] or 1.084 [3]. There is no report, however, on the optimum density of the fluid and the recovery rate for the separation of lymphocytes from bovine peripheral blood. If the recovery of lymphocytes from the blood is lower than 60 to 70%, there would be a problem concerning the proportions of T- and B- lymphocytes [14]. Therefore, the present study was made to determine the optimum density of the fluid for separation of lymphocytes from bovine blood to attain a high yield and purity of the lymphocytes.

Peripheral blood was collected from the jugular vein of 15 healthy Holstein cows, and placed into 20-ml syringes containing heparin (20 units/ml of blood). Total leukocyte and differential leukocyte counts were made for each sample, and then one part of the blood was diluted with two parts of phosphate-buffered saline (PBS)

[6, 14]. After the optimum density was determined, the effect of further dilution of the blood was also investigated.

Separation fluid: Ficoll- a sucrose polymer (Ficoll 400, Pharmacia Fine Chemicals, Uppsala, Sweden) and Conray- sodium iotalamate (Conray 400, Daiichi Pharmaceutical Co., Tokyo, Japan) were used. The separation fluid was prepared by mixing 9 w/v% Ficoll (24 parts) with 33.4 w/v% Conray (10 parts) to make solutions with densities ranging from 1.073 to 1.101 at intervals of 0.002 (Table 1). The density of each solution was adjusted to each value by the procedure described by Böyum [2]. Then, the resulting solutions were measured in duplicate with a 10-ml pycnometer at room temperature (approx. 20°C), and the measured values were rounded to the four decimal point. The Ficoll-Conray solutions with different densities of 3-ml quantities were placed in 15 siliconized glass tubes (100 mm×13 mm).

Separation method: Six milliliters of the diluted blood (1:2) was layered carefully over the 3 ml Ficoll-Conray solution and centrifuged at 400 g for 30 min at 20°C. After centrifugation each mononuclear cell-layer was carefully removed from the inter-

Fig. 1. Effect of varying the density (1.073 to 1.101 at intervals of 0.002) on lymphocyte separation. Arrows indicate the mononuclear cell-layer centrifuged at 400 g for 30 min at 20°C.

face with a Pasteur pipette and transferred to a 10-ml centrifuge tube. The cells were washed twice by resuspending with PBS and discarding the supernatant with an aspirator. After the final wash, the cells were suspended in 2 ml of PBS and mononuclear cells were counted three times with a hemacytometer (Thoma). After counting, the cell tubes were centrifuged at 200 g for 10 min at 20°C, and smears were made for differential counts. The yield of lymphocytes was expressed in percentage of the number of lymphocytes recovered from the total number.

Figure one shows the effects of various densities on lymphocyte separation. The higher the density, the nearer the mononuclear cell-layer to the red cell layer (Fig. 1). Between No. 10 (density 1.091) and No. 15 (density 1.101), isolation of lymphocytes was difficult and contamination with the red cells and granulocytes increased. Therefore, to obtain a good yield of lymphocytes, isolation in solution between No. 1 (density 1.073) and No. 9 (density 1.089) should be used. Table 1 shows the mean yields of lymphocytes from blood at vari-

ous densities. The averages were as follows: 60.1% in No. 1 (density 1.073) to No. 5 (density 1.081), and 70.1% in No. 6 (density 1.083) to No. 10 (density 1.091). The higher the density, the higher the yield of lymphocytes from blood. However, the isolation of lymphocytes was not satisfactory at densities from 1.091 (No. 10) to 1.101 (No. 15); contamination by other cells increased. The yield of lymphocytes was the highest in No. 8 (density 1.087), being $74.3 \pm 15.4\%$ (Table 1). There was a significant difference at $P < 0.01$ between the yield in No. 8 and that in No. 3 with a density of 1.077, that has widely been used to separate the lymphocytes. The density 1.087 was the optimum for separation of lymphocytes from bovine peripheral blood. The purity of the lymphocytes separated in No. 8 was $98.4 \pm 1.4\%$ and its monocyte contamination $1.2 \pm 0.7\%$ (Table 1). Trypan blue dye exclusion tests showed that the viability of the lymphocytes were over 96% in all the samples.

Effect of diluting blood: The effects of diluting the blood on the yield of lymphocytes is shown in Table 2. With the blood

Table 1. Effects of the density of Ficoll-Conray solution on the yield and the purity of lymphocytes separated from bovine peripheral blood

No.	Density (gm/ml)	Yield (%) of lymphocytes	Differential leukocyte counts (%)			
			Lymphocytes	Monocytes	Neutrophils	Eosinophils
1	1.073	55.2±13.5*	97.9±1.4*	2.0±1.3*	—	—
2	1.075	57.8±15.6	99.0±0.6	1.0±0.6	—	—
3	1.077	61.9±12.3	98.6±1.5	1.4±1.3	—	0.1±0.3*
4	1.079	57.0±14.7	98.8±0.6	1.1±0.6	—	0.2±0.3
5	1.081	68.5±10.8	99.0±0.4	1.1±0.5	—	—
6	1.083	66.1±13.8	98.7±0.7	1.2±0.7	0.1±0.3*	—
7	1.085	71.2±12.6	98.6±0.9	1.4±0.9	0.1±0.3	—
8	1.087	74.3±15.4	98.4±1.4	1.2±0.7	—	0.4±1.3
9	1.089	65.2±11.0	98.3±1.8	1.7±1.5	0.1±0.3	—
10	1.091	73.5±15.6	97.7±1.6	1.8±1.7	0.3±0.5	0.2±0.6
11	1.093	74.0±12.5	97.1±1.5	1.6±0.9	1.0±1.4	0.4±0.7
12	1.095	68.0±11.8	98.0±1.2	1.2±0.9	0.5±0.8	0.3±0.4
13	1.097	76.4±12.9	98.0±1.1	1.2±0.6	0.7±0.8	0.1±0.3
14	1.099	67.9±14.4	97.0±3.2	1.3±0.7	1.5±3.0	0.2±0.6
15	1.101	72.6±17.6	94.8±5.3	2.4±1.9	1.3±2.0	0.3±1.0

* values represent the mean ± SD (n=10)

Table 2. The effect of dilution of the blood on the yield of lymphocytes

Dilution of Blood	undiluted	1 : 1	1 : 2	1 : 3	1 : 4	1 : 5
Yield (%) of Lymphocytes	*	71.9±14.9**	72.1±4.9	69.8±8.6	66.6±7.8	70.7±12.5

mean ± SD (=5)

* Lymphocytes were not separable from any of the samples.

** Lymphocytes were not separable from two of the samples.

undiluted, diluted 1:1, 1:2, 1:3, 1:4 and 1:5, separations were carried out in the same way as the previously mentioned (density 1.087). It was found that a dilution of 1:2 to 1:5 was suitable for separation of lymphocytes, and that there was no significant difference on the yield in this range. With respect to the total yield of lymphocytes, dilution of blood at 1:2 would be appropriate.

In conclusion, by Ficoll-Conray gradient centrifugation, the optimum density of the separating fluid was 1.087 (20°C), and diluting blood at the ratio of 1:2 was found to be the optimum for separation of lymphocytes from bovine peripheral blood.

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要 約

成牛末梢血からのリンパ球分離における最適比重値の検討 (短報): 永幡 肇・野田 寛・工藤忠明¹⁾ (酪農大学家畜衛生学教室,¹⁾北海道大学獣医学部家畜外科学講座)——9% Ficoll (24容) + 33.4% Conray (10容) を分離液とし, 比重 1.073~1.101 (0.002 間隔) の15種類の比重液を用いて比重遠心法による成牛末梢血リンパ球の分離・回収率を検討した. 比重 1.087 (20°C) が成牛末梢血リンパ球の回収には最適 (回収率 74.3±15.4%, 純度 98.4±1.4%) であり, また本法実施時の血液希釈倍数は3倍が適当であった.