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Effects of Ficoll Concentration and Blood Storage on the Separation of Lymphocytes From Bovine Peripheral Blood

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Introduction

Bovine lymphocytes were separated from peripheral blood by using density gradient centrifugation which is commonly used for separating lymphocytes from human blood. One of the separating methods, the Ficoll-Conray method[11] a modified Böyum method,[3,4] has been widely used for separating lymphocytes from peripheral blood and various lymphoid organs. When separating lymphocytes for studies obtaining a good yield, functional integrity and high cell purity is required. In addition, such a method should be simple, reproducible and require only a short period of time.

The optimum density for obtaining good yields of lymphocytes in the separation fluid has already been determined[8]. However, the effect of the concentration of Ficoll in the separation fluid on the separation of lymphocytes has not yet been examined. One of the problems in clinical testing of blood samples is that the samples may be examined many hours after the blood has been withdrawn from animals. The effect of blood storage on the efficiency of separation is a problematic.

This experiment was attempted to investigate these effects on separating lymphocytes from bovine peripheral blood.

Materials and Methods

Blood samples

Peripheral blood was collected from the jugular vien of 15 healthy Holstein-Friesian cows, and placed in 20 ml syringes containing heparin (20 units/ml of blood). White cell counts were performed using standard blood diluting pipettes and a hemacytometer (Thoma). The blood films were stained with Giemsa stain, and a minimum of 200 white cells were examined for the differential leucoyte count for each sample.

Before use, in all the samples one part of the blood was diluted with two parts of phosphate buffered saline (PBS)[9].

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Varying the Ficoll concentration

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 the separation fluids used. A total of 7 samples of Ficoll-Conray solution with varying concentrations of Ficoll were made. They were prepared by mixing 6, 7, 8, 9, 10, 12 and 14 w/v% Ficoll (24 parts) with 33.4 w/v% Conray (10 parts) by stirring for 20 minutes, respectively. The density was measured with the 10 ml pycnometer at room temperature (approx. 20°C) and the osmolarity was determined by using a 5100 C Vapor pressure osmometer (Wescor, Inc. USA). Each 3 ml Ficoll-Conray solution was placed in siliconized glass tubes (100 mm × 13 mm).

Separation method

Six ml of the diluted blood (1 : 2) was layered carefully over the 3 ml of Ficoll-Conray solution and centrifuged at 400 g for 30 minutes at 20°C. After centrifugation each mononuclear cell-layer was removed carefully with a Pasteur pipette and transferred to a 10 ml centrifuge tube. The cells were washed twice with PBS to reduce the number of platelets and were suspended in 2 ml of PBS, and the mononuclear cells were counted twice by using a hemacytometer (Thoma). After counting, the cell tubes were centrifuged at 200 g for 10 minutes at 20°C, and smears with Giemsa staining were made for differential counts. The yield of lymphocytes was expressed as a percentage of the number of lymphocytes recovered from the total number of lymphocytes.

Viability test

The trypan blue dye exclusion test was employed[9]. Briefly, two droplets of the cell suspension were mixed with one droplet of 0.5% trypan blue solution. A number of unstained cells, among the 400 to 600 cells, were counted as viable.

Blood storage

Blood was collected and stored at both 4°C and room temperature (approx. 22°C) until used, and was used at different times as follows: 30 minutes, 1, 4, 8, 12 and 24 hours.

Separation fluid:

9 w/v% Ficoll (24 parts) and 33.4 w/v% Conray (10 parts) were mixed, and the density was adjusted to 1.087 g/ml with Conray as previously described[8]. Other preparations were made in the same way mentioned above.

Results and Discussion

Varying the concentration of Ficoll

The effect of varying the concentration of Ficoll in the Ficoll-Conray solution (F, FCS) on the figures of mononuclear cells separated is shown in Fig. 1. When the concentration of F, FCS ranged from 6 to 9%, the mononuclear cell-layer separated well, and was easy to isolate. As the concentration of F, FCS increased, the mononuclear cell-layer approached the red cells layer, and finally at the concentration of F, FCS ranging from 10 to 14% it could not be separated at all.

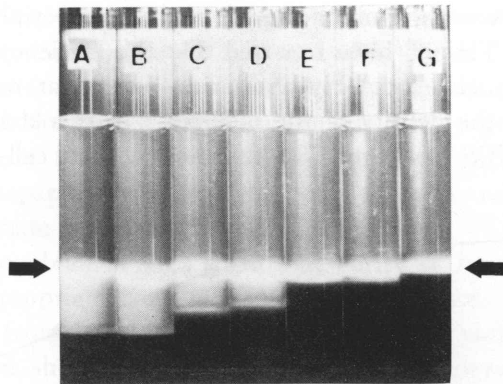

Fig. 1. The effect of variation of the Ficoll concentration on the separation of lymphocytes from bovine peripheral blood. The letters A, B, C, ··· G represent Ficoll concentrations of 6, 7, 8, 9, 10, 12 and 14% respectively. Arrows indicate the mononuclear cell-layer. (400 g, 30 minutes, 20°C)

Table 1. Effect of varying the Ficoll concentration on the quality of separation fluid and the yield of lymphocytes

Tube number	Ficoll concentration (%)	Density (g/ml)	Osmolarity (mmOs/kg)	Separation of Mononuclear cell-layer	Yield (%) of Lymphocytes
A	6	1.075±0.001	284±3	S	26.3± 8.0*
B	7	1.077±0.001	285±3	S	44.6± 6.8 ┐
C	8	1.078±0.001	283±3	S	61.3±11.7 **
D	9	1.081±0.001	284±3	S	68.2± 7.1 ┘
E	10	1.082±0.001	282±3	S—US	62.6±10.1
F	12	1.086±0.001	285±3	US	57.2±10.8
G	14	1.089±0.001	281±3	US	69.3±26.6

S—separated, US—unseparated
* Values represent the mean±SD (n=7)
** Significant at P<0.01

As shown in Table 1, varying the concentration of F, FCS effects the quality of the separation fluid and the yield of lymphocytes separated. The osmolarity of a solution has a considerable effect on the volume of suspended cells, and even small changes cause shrinkage or swelling[12]. Consequently, in spite of increasing the concentration of Ficoll each separation fluid has a consistent osmolarity. Therefore, it can be considered that osmolarity has hardly any effect on the separation. By increasing the concentration of F, FCS, the yield of lymphocytes increased gradually from 26.3±8.0 to 68.2±7.1%, and a significant difference at $P<0.01$ between 7 and 9% of F, FCS was seen. The best yield was obtained at the concentration of 9% of F, FCS. The value of 9% in this study was the same as the value reported by Böyum[3] in separating lymphocytes from human peripheral blood.

Ferrante and Thong[5] have reported that the efficiency of the separation of lymphocytes by the Ficoll-Hypaque was dependent on both the Ficoll concentration and the density of the medium. In this study, when the concentration of F, FCS was 6 to 7%, the mononuclear cell-layer was diffused

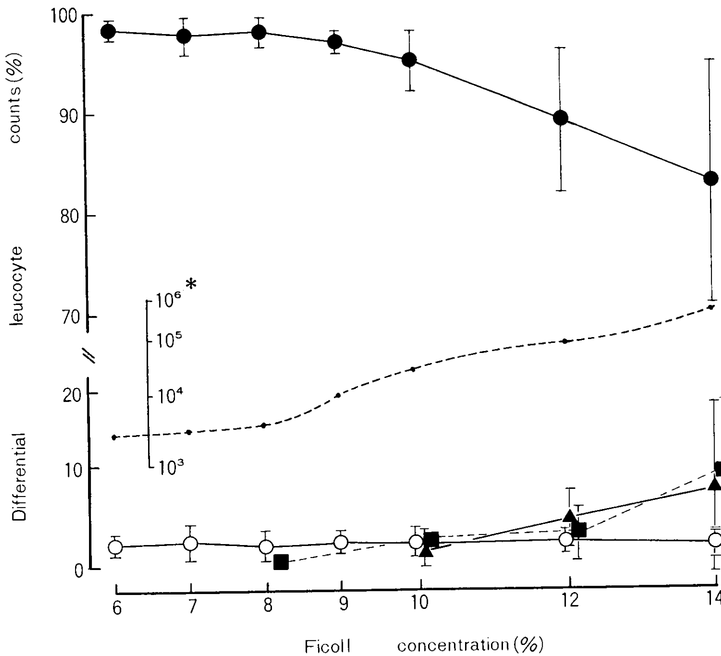

Fig. 2. Effect of varying the Ficoll concentration in the Ficoll-Conray solution on the differential leucocyte counts and red blood cells contamination of recovered cells suspension. ●—● Lymphocytes; ○—○ Monocytes; ■—■ Eosinophils; ▲—▲ Neutrophils.
* Red blood cells contamination/mm^3 in recovered fluid (2 ml).
Vertical bars represent the mean±SD (n=7).

(Fig. 1), and only a low yield of lymphocytes was recovered (Table 1). For this reason, when the separation fluid was cloudy, it can be considered that there was excessive loss of lymphocytes in the separation fluid. On the other hand, when the contamination of F, FCS ranged from 10 to 14%, the mononuclear cell-layer was overlaid on the red cell layer, and thus it was difficult to isolate them without reducing the red blood cell contamination (Fig. 2). When the concentration of F, FCS ranged from 10 to 14%, the purity of lymphocytes decreased, and the contamination of other cells, i. e., neutrophils and eosinophils increased, particularly red blood cell contamination increased markedly (Fig. 2). The monocyte contamination ranged from 1.8±0.9 to 2.4±1.9% throughout the 6 to 14% range, and it was shown that this was not related to the Ficoll concentration.

Aguad et al[1] have reported that a 14.6% Ficoll (24 parts) and 34% Urografin (10 parts) mixture was used for isolating the granulocytes from human peripheral blood by using discontinuous density gradient. Therefore, the concentration of 14% can be considered to be an excessive concentration of F, FCS for separating lymphocytes, and also the range of 10 to 12% was not an adequate concentration.

Therefore, to obtain a high yield and purity of lymphocytes, it can be said that the appropriate concentration of F, FCS was 9% for separating the lymphocytes from bovine peripheral blood. The viability test by trypan blue dye exclusion showed in all samples that lymphocytes over 98 to 99% were viable.

Effect of storage time and temperature on mononuclear cell recovery

To study the effect of blood storage on the separation of lymphocytes, separation was carried out while storing the blood from 30 minutes to 24 hours at 4°C and room temperature. In all cases separation was more successful. As shown in Fig. 3, blood stored for 30 minutes at room temperature gave a higher cell yield than did blood stored at room temperature for 4 to 24 hours. Lymphocyte recovery from stored blood was markedly less than that from freshly drawn blood samples. These experimental results agree with those previously reported[3,5,6,10]. The yield of lymphocytes decreased gradually following the elapse of time and after 4 hours it decreased considerably. The yield was also affected by the storage temperature. With a storage time of 4, 8, 12 hours, there was a significant decrease ($P<0.05$, <0.01) in the yield of lymphocytes in blood stored at 4°C, as compared with those stored at room temperature. The reduced yield at a lower temperature and/or long-term storage of the blood may be explained in relation to the spontaneous aggregation of red blood cells as mentioned earlier[2]. In particular, regarding a remarkably lower yield under the condition of lower temperature (below 15°C), we consider that when the cold

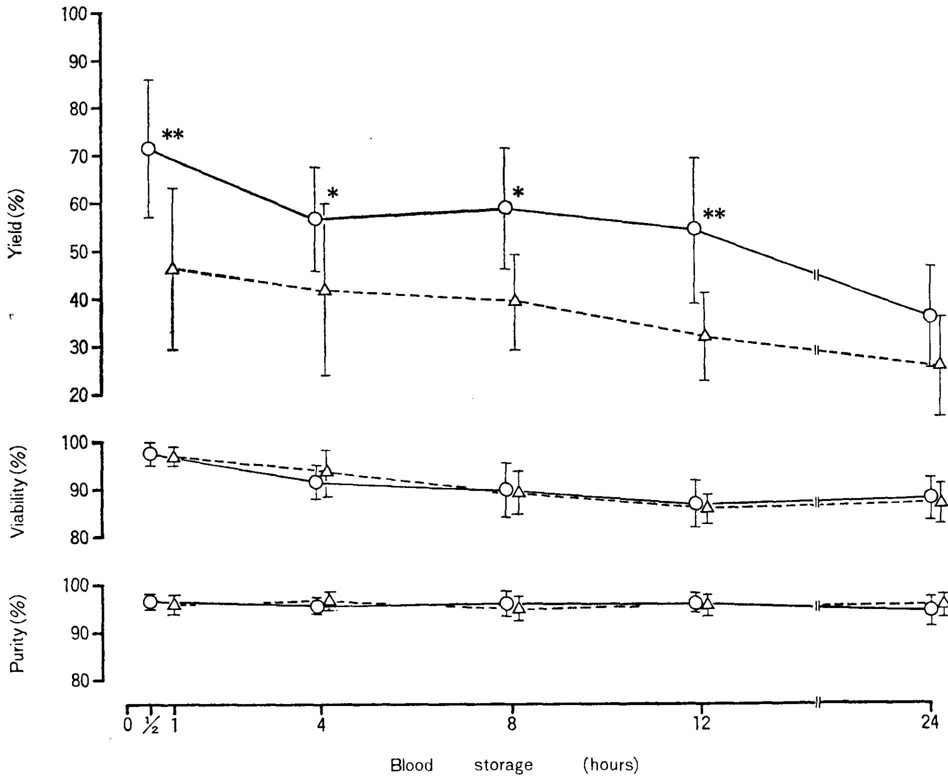

Fig. 3. Effects of blood storage time and temperature (○—○ 22°C, △···△ 4°C) on the yield, viability and purity of lymphocytes from bovine peripheral blood.
Vertical bars represent the mean±SD (n=7).
Significantly different from 4°C values: *P<0.01 **P<0.05

hemagglutinin exists in the blood, cold hemagglutination[13] occurs rapidly. This reaction enhances the spontaneous red blood cell aggregation, the red blood cells adhering firmly to each other. Therefore, a considerable number of lymphocytes were trapped in red cell clumps, and during separation the lymphocytes were drawn to the bottom of tube accompanied with the red blood cells.

In the subpopulations of lymphocytes, if the yield of lymphocytes from blood was under 60 to 70%, there was a problem concerning the proportions of T- and B- lymphocytes[9]. It is for this reason that the present study was undertaken. As mentioned above, in the separation of lymphocytes for subpopulations from blood, it is advisable to use them within 12 hours after collection, and to store them at room temperature. The recovered lymphocytes were 97.4±2.2% viable after the blood was stored for 30 minutes, and thereafter the viability decreased slightly. However, after 24

hours of storage about 88% were still viable, as determined by the trypan blue exclusion test. The effect of blood storage on the differential cell counts of recovered cells was observed to be less affected.

This study concerns only the separation problem, therefore, further study is needed to investigate the functions of lymphocytes. As for the lymphocyte blastogenesis, Senogles et al[10] reported that the optimal lymphocyte blastogenic responses to Concanavalin A can be obtained from bovine blood stored for 24 or even 48 hours at 22°C. In another recent paper on long-term storage of blood, Kaneene et al.[7] reported that bovine blood for whole-blood lymphocyte stimulation tests was good even after having been kept for 4 days at room temperature prior to culturing. It would seem that the lymphocyte transformation test would be advisable up to 24 hours with regard to the cell viability. Further study, however, is required to investigate the blastogenic responses.

Summary

Studies were conducted to investigate the effect of varying the concentration of Ficoll in the Ficoll-Conray solution and the effect of storage time and temperature of the blood on the separation of lymphocytes from bovine peripheral blood.

For obtaining a high yield and purity of lymphocytes, the concentration of 9 w/v% Ficoll in the Ficoll-Conray solution would be appropriate for separating lymphocytes (yield; 68.2±7.1%, 97.7±0.8%).

The rate of the yield of lymphocytes was affected by the storage time and temperature. It was most efficient when carried out using the freshly drawn blood samples (yield; 71.4±14.4%) at room temperature (approx. 22°C). In the case of blood stored at 4°C the yield significantly ($P<.05$, $<.01$) decreased (yield; 46.5±17.0% (1 h)··· 31.7±9.2% (12 h), as compared with that stored at room temperature (yield; 71.4±14.4% (30 min.)··· 53.8±15.2% (12 h). The viability varied from 97.4±2.2% (30 min.) to 88.8±4.7% (24 h), at room temperature; 97.0±2.0% (1 h) to 87.2±4.1% (24 h)), at 4°C; and was hardly affected by the storage temperature. The purity ranged from 94.6±3.2% to 96.8±1.4% in all cases, and again it was observed that it was hardly affected by storage. As for the yield of lymphocytes, it would be appropriate to use the blood within 12 hours after collection, and to store the blood at room temperature.

References

1) Aguad, M. T., N. Pujol, E. Rubiol, M. Tura and A. Celada, 1980. Separation of granulocytes from peripheral blood in a single step using discontinuous density gradients of Ficoll-Urografin. A comparative study with separation by Dextran.

J. Immunol. Methods., **32**: 41–50.

2) Böyum, A., 1968 a. Isolation of leucocytes from human blood. Scand. J. Clin. Lab. Invest., 21 (suppl.) **97**: 9–29.
3) Böyum, A., 1968 b. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest., 21 (suppl.) **97**: 77–89.
4) Böyum, A., 1974. Separation of blood leucocytes, granulocytes and lymphocytes. Tissue Antigens., **4**: 269–274.
5) Ferrante, A. and Y. H. Thong, 1980. Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque—Ficoll method. J. Immunol. Methods., **36**: 109–117.
6) Itoh, Y., T. Kawai and T. Kasahara, 1981. Lymphocyte transformation test, in "The japanese journal of clinical pathology" (Japanese society of clinical pathology, ed.), **45**: 209–218, Kanahara pub. Co. Ltd., Tokyo.
7) Kaneene, J. M. B., F. Soper, D. W. Johnson and R. K. Anderson, 1981. Long-term storage of blood prior to whole-blood lymphocyte culturing. Vet. Immunol. Immunopathol., **2**: 555–560.
8) Nagahata, H., H. Noda and T. Kudo, 1982. Optimum density of the separating fluid for centrifugal separation of lymphocytes from bovine peripheral blood. Jpn. J. Vet. Sci. (in press).
9) Nakagawa, T., 1980. The methods of preparations for human lymphocytes, in "Laboratory methods in lymphocytes studies" (Yata Y. and Fujiwara, M. ed.), 15–24, Chugai Igaku Co. Ltd., Tokyo.
10) Senogles, D. R., C. C. Muscoplat, J. M. Kaneene, C. O. Thoen and D. W. Johnson, 1978. In vitro stimulation of bovine peripheral blood lymphocytes: Effect of short-term storage of blood prior to lymphocyte culture. Am. J. Vet. Res., **39** (2): 341–344.
11) Tsuji, H. 1971. Separation of lymphocytes by using a differential centrifugation-Conray 400-Ficoll method, in "Experimental immunological methods" (Japanese society of immunology ed.), 443–446. Tokyo.
12) Wells, J. R., G. Opelz and M. J. Cline, 1977. Characterization of functionally distinct lymphoid and myeloid cells from human blood and bone marrow. 1. Separation by a buoyant density gradient technique. J. Immunol. Methods. **18**: 63–67.
13) Yamaguchi, Y., 1978. Immunological diagnosis, in "Medical Immunology", pp. 444–452, Kanahara pub. Co. Ltd. Tokyo.

要　約

ウシ末梢血液からのリンパ球回収に用いる Ficoll-Conray 液の Ficoll 濃度の影響および採血からリンパ球分離操作までの保存時間と温度がリンパ球回収率に与える影響について検討した。

リンパ球の分離において，リンパ球の回収率と純度から Ficoll-Conray 液中の Ficoll 濃度は 9 w/v % が適当であった (回収率；68.2±7.1%，純度；97.7±0.8%)。

リンパ球の回収率は血液の保存時間および保存温度の影響を受け，室温下 (≒22°C) で，

新鮮血を用いた場合が最も良好であった (回収率；71.4±14.4%)。低温保存下 (4°C) での回収率は，46.5±17.0% (1 h)…31.7±9.2% (12 h) であり，室温下の回収率 71.4±14.4% (30 min)…53.8±15.2% (12 h) に比較して有意に ($P<0.05$, <0.01) 低くかった。生存率は室温下で 97.4±2.2% (30 min) から 88.8±4.7% (24 h)；低温下で 97.0±2.0% (1 h) から 87.2±4.1% (24 h) であり，保存温度には殆んど影響を受けなかった。純度は，いずれの場合にも 94.6±3.2% から 96.8±1.4% の範囲にあり，保存の影響はみられなかった。リンパ球の回収に関しては，採血後，室温下に保存し 12 時間以内に用いることが適当と思われる。