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A simple method for the simultaneous separation of peripheral blood mononuclear and polymorphonuclear cells in the dog

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Abstract

A simple method for the simultaneous separation and purification of peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophil cells (PMNC) was developed for comparative and functional studies in the immune system of the dog. Purity and cell viability were $>95\%$, yields were similar to those obtained by other techniques but without red blood cell contamination. Differential blood cell count studies of the isolated cells in blood samples of beagle dogs and German shepherd dogs demonstrated that the 1.077/1.119 double density centrifugation is an effective method of acquiring both highly purified blood mononuclear cells and polymorphonuclear cells as separate entities from the same sample. The interface between plasma and 1.077 contained an average 97% blood mononuclear cells vs. 3% polymorphonuclear cells, and the interface between 1.077 and 1.119 an average 96% polymorphonuclear cells vs. 4% blood mononuclear cells. These data indicate that Histopaque 1.077/1.119 double density gradient allows the purification and physical separation of lymphocytes and phagocytes from a blood sample in the dog, enabling the investigator to examine both cell types from the same sample simultaneously. © 1998 Elsevier Science B.V.

Keywords: Dogs; Separation; Polymorphonuclear; Mononuclear; Blood; Cells

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1. Introduction

Peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMNC) play important roles in both the physiological and the pathological processes of the immune system. Dogs develop spontaneously many of the same immune-mediated diseases as humans, including autoimmune disease (Halliwell, 1978) and primary immune deficiency disease (Felsburg et al., 1985; Felsburg, 1994). However, the study of many functional immunological problems in the dog is still in its infancy (Felsburg, 1994), which can be attributed partly to the difficulty in obtaining the necessary pure polymorphonuclear cells and blood mononuclear cell preparations for in-vitro tests. For our parallel functional investigations (Strasser et al., 1996) into acquired and innate immunity in the dog (i.e. natural killer-cell activity and proliferation by blood mononuclear cells as well as phagocytotic and bacteriocidic activity by polymorphonuclear cells), it was essential to obtain these cells as separate entities from the same sample of the blood of the animal; separated and highly purified blood mononuclear cells as well as polymorphonuclear cells were necessary. The various methods used by numerous investigators to obtain blood mononuclear cells and/or polymorphonuclear cells such as Ficoll–Hypaque, Histopaque or Percoll gradients, aimed at obtaining one or other of the cell population did not yield separated populations of pure polymorphonuclear cells or blood mononuclear cells. Although the technique of Boyum (1964) is commonly used to isolate blood mononuclear cells from canine blood, the resultant cell preparations are contaminated with polymorphonuclear cells. However, highly purified populations were necessary to study age-associated changes simultaneously in both cell populations. Therefore, we analysed some technical parameters for the simultaneous purification and separation of blood mononuclear cells and polymorphonuclear cells in the peripheral blood of dogs, adapting a technique used by Toth et al. (1992) on feline blood and established a simple method of obtaining, separating and purifying these cells simultaneously in canine blood samples.

2. Materials and methods

2.1. Animals

Blood samples from male and female beagle dogs ($n = 20$) from the colony of the Inst. of Nutrition and shepherd dogs in police service ($n = 80$) at the Police dog branch in Floridsdorf, undergoing a regular health checkup and blood drawing for hematological and chemical blood investigations, were used in this study. The animals were clinically sound as regarding both hematology and blood examination. All dogs were routinely vaccinated against the usual viruses (Vanguard DA_2Pi-CPV Lepto® and Enduracell® T) and regularly treated for parasites. Eight milliliters of blood was drawn from the cephalic vein of each dog by venipuncture into EDTA vacutainer tubes. It was then used in the various separation and purification experiments and for a further diagnosis of functional activity in white blood cells.

2.2. Isolation

Discontinuous Hypaque–Ficoll gradients were prepared, with a few modifications, according to the technique of Toth et al. (1992). The isolation procedure was performed by Histopaque®-1119 and Histopaque®-1077 (Sigma Chemical, St. Louis, MO) polysucrose density gradient centrifugation at room temperature and was completed in about 2 h. Only polypropylene tubes and/or siliconized instruments were used throughout the procedure. Histopaque®-1119 was used as high specific density gradient. Two milliliters Histopaque®-1077 was carefully layered onto 2 ml Histopaque®-1119, and both solutions were stored at 4°C until immediately before use. These columns were kept in an ice bath in four separate conical 12-ml centrifugal polypropylene tubes (Greiner). Two milliliters blood at room temperature was layered carefully onto the low gradient solution. The tubes were then centrifuged at $340 \times g$ for 30 min at room temperature with a swing-out rotor and the process was terminated without applying brakes. After centrifugation the supernatant was discarded, and the two opaque bands at the interfaces between plasma and Histopaque®-1077 and Histopaque®-1077 and Histopaque®-1119 containing cells were collected carefully by separate aspiration with a siliconized Pasteur pipet and transferred to separate conical centrifugal tubes.

Lymphocytes and polymorphonuclear cells were washed separately with 9 ml Hank's balanced salt solution (HBSS). Then the lymphocytes were washed another three times in HBSS (10 min at room temperature, $300 \times g$) and used for further investigations.

Polymorphonuclear cells were treated with lysis buffer to free them of residual red blood cells. Two milliliters lysis buffer (8.3 g/l ammonium chloride and 20.6 g/l Tris buffer pH 7.65; 9 volumes ammonium chloride solution mixed with 1 volume Tris buffer, adjusted at Ph 7.2 and stored at 4°C) was added to the polymorphonuclear cells cell pellet for 10 min and shaken gently by hand. Afterwards the polymorphonuclear cells were saved with 8 ml of HBSS and centrifuged at $300 \times g$, then washed three times in HBSS.

Both cell types were re-suspended separately in 1 ml PBS and investigated for cell purity, cell count and cell viability. Cell purity was determined by the standard technique of stained cell smear and subsequent differential blood cell count. Smears prepared from cell suspensions as well as blood smears were stained by a conventional Diff-Quick® method, and 2×200 cells were counted in a microscope. White blood cell count was measured by Coulter Counter ZBI®, and isolated cells were diluted in Türk's solution and counted in a Bürker–Türk chamber. Cell viability was analysed by trypan blue exclusion. Percent yield was calculated with reference to white blood cell count, differential blood cell count and applied blood volume.

3. Results (see Table 1)

With the above-mentioned technique, we were able to isolate highly purified and functionally intact cells from canine blood. The interface between plasma and 1.077 contained $96.33 \pm 3.33\%$ mononuclear cells vs. less than 4% polymorphonuclear cells, and the interface between 1.077 and 1.119 contained $96.72 \pm 2.62\%$ polymorphonuclear cells vs. less than 4% mononuclear cells. Viability determined by trypan blue exclusion

Table 1
Data ($n = 80$; means $\pm$ std) of total and differential white blood cell count as well as of polymorphonuclear cells (PMNC) and peripheral blood mononuclear cells (PBMC) at the two interfaces, isolated out of peripheral canine blood by double density centrifugation

Total white blood cell count ($\times 10^9$/l)	10.13 ± 2.48
Differential white blood cell count:	
Neutrophils, adults ($\times 10^9$/l)	6.46 ± 2.01
Neutrophils, band forms ($\times 10^9$/l)	0.19 ± 0.16
Lymphocytes ($\times 10^9$/l)	2.76 ± 1.02
Monocytes ($\times 10^9$/l)	0.19 ± 0.29
Eosinophils ($\times 10^9$/l)	0.53 ± 0.46
Basophils ($\times 10^9$/l)	0
Polymorphonuclear cells (PMNC):	
Cell purity (%)	97.07 ± 1.87 PMNC vs. 2.93 ± 1.87 PBMC
Viability (%)	98.32 ± 1.18
Isolated cell count ($\times 10^9$/l)	17.07 ± 6.42
Total isolated cells ($\times 10^6$; out of 8 ml)	52.80 ± 16.7
Yield (%)	33.17 ± 11.66
Peripheral blood mononuclear cells (PBMC):	
Cell purity (%)	96.33 ± 3.33 PBMC vs. 3.67 ± 3.33 PMNC
Viability (%)	97.00 ± 1.83
Isolated cell count ($\times 10^9$/l)	12.97 ± 7.7
Total isolated cells ($\times 10^6$; out of 8 ml)	22.21 ± 8.29
Yield (%)	62.83 ± 33.02

was more than 97 ± 1.83 for mononuclear cells and $98.32 \pm 1.18\%$ for polymorphonuclear cells, and the total isolated cells were 22.21 ± 8.29 mononuclear cells and 52.8 ± 16.7 polymorphonuclear cells out of 8 ml blood. Our results show that Histopaque 1.077/1.119 double-density gradient allows the purification and physical separation of blood mononuclear and polymorphonuclear cells from the same blood sample in the dog. It is an effective method of acquiring both highly purified mononuclear and polymorphonuclear cells as separate entities from the same sample. Only with our improved method with adaptations of the centrifugal speed ($340 \times g$), Histopaque temperature (4°C), dilution (whole blood) and RBC lysis (10 min; lysis buffer at 4°C) we were able to get a good separation and purification of blood mononuclear cells and polymorphonuclear cells in canine blood with a purity of $> 95\%$ and cell viability of $> 95\%$ with yields similar or greater than those obtained by other techniques, and without contamination with RBC.

4. Discussion

Most in vitro assays to evaluate the T- and B-cell system as well as neutrophil granulocytes and their activities require purified blood mononuclear cells and polymorphonuclear cells. These assays include enumeration and separation of B- and T-cells, lymphocyte transformation, polyclonal B-cell activation assays and assays to evaluate

T-helper and T-suppressor cell function as well as phagocytotic and bactericidal activity. However, techniques used for other species to obtain pure blood mononuclear cells and polymorphonuclear cells populations have been unsatisfactory for the dog (Wunderli and Felsburg, 1989). Most of the reported functional studies of canine blood mononuclear cells and polymorphonuclear cells have used the conventional one-step density-gradient centrifugation technique of Boyum (1964), which results in cell preparations with cell purities between 49 and 85%. The vast amount of contamination in these preparations may help to explain the tremendous variations reported in many of the assays used. Several attempts have been made to improve the isolation of canine blood mononuclear cells and/or polymorphonuclear cells. Buurman et al. (1982) attempted isolation on discontinuous Percoll gradients. They were unable to separate the mononuclear cells from the granulocytes with any acceptable yield. Muscoplat et al. (1977) and Chandler and Yang (1981) reported a purity of 98% for blood mononuclear cells, but the yield in terms of total blood mononuclear cells was 20% or less. It is difficult to compare these yields and other studies using conventional methods since most studies do not report yields, but only the purity of the final cell preparations. Furthermore, some studies separate blood mononuclear cells (Wunderli and Felsburg, 1989; Schreuer and Hammerberg, 1996) or polymorphonuclear cells (Gosset et al., 1983/1984; Hallen-Sandgren and Björk, 1988) only and/or require too much blood volume (Hallen-Sandgren and Björk, 1988) and/or additional separation steps (Gosset et al., 1983/1984). Buurman et al. (1982) even claimed that the buoyant densities of polymorphonuclear cells and lymphocytes are so similar in canine blood that separation with high purity and yield is not possible with the use of a density gradient in the dog. On the other hand, the results of Weiss et al. (1989) demonstrated a very good separation with high purity and high yield for the dog, too. However, polymorphonuclear cells were significantly contaminated with RBC, disturbing subsequent functional assays.

Initial attempts to separate blood cells and collect polymorphonuclear cells employed a sedimentation and centrifugation technique described by Gosset et al. (1983/1984) for the assay of in vitro function of canine neutrophils. However, this technique failed to yield enough purified polymorphonuclear cells, necessary for the assay of neutrophil function out of smaller samples of blood (< 8 ml) and also did not supply purified blood mononuclear cells. Other techniques required an even higher volume of blood (Hallen-Sandgren and Björk, 1988) and/or did not yield enough purified polymorphonuclear cells. Furthermore, these techniques did not supply purified blood mononuclear cells from the same small volume of blood, which is what we required for our parallel investigations into acquired and innate immunity in the dog. Therefore, we employed the technique described by Toth et al. (1992) for the simultaneous separation and purification of mononuclear and polymorphonuclear cells from the peripheral blood of cats and adapted it to the requirements of canine blood.

The centrifugal speed set at $400 \times g$ (Toth et al., 1992) did not yield a good separation of polymorphonuclear cells in canine blood, the polymorphonuclear cell band failed to separate into a distinct band. We tested centrifugation at different speeds (300, 340, 385, 430, 530, 645, 765 and $900 \times g$). Only centrifugation at $340 \times g$ produced a good separation of polymorphonuclear cells and blood mononuclear cells, whereas separation at lower or higher speed failed to separate leukocytes into fine distinct bands.

Separation at a speed higher than $530 \times g$ strongly reduced cell viability (60% and below) and centrifugation at a speed set higher than $765 \times g$ destroyed the cells. The separation technique with the centrifuge set at $340 \times g$ without applying brakes produced distinct bands of polymorphonuclear cells and blood mononuclear cells with a good cell viability of $> 95\%$ and cell purity $> 95\%$ in the polymorphonuclear cells as well as in the blood mononuclear cells band. Both cell types were functionally intact for the following functional tests, i.e. phagocytosis, bactericidal activity, lymphocyte proliferation and NK-activity.

Layers kept at room temperature before use (21°C) resulted in disturbances on the layer surface of Histopaque®-1077 when carefully layering whole blood on top of it and produced less cell purity ($< 80\%$) of the separated bands. When Histopaque®-1119 and Histopaque®-1077 were stored at 4°C until use and kept in an ice bath immediately before layering fresh blood carefully on top of the gradient, the gradient surface was not disturbed and cell purity was $> 95\%$ in the polymorphonuclear cells as well as in the blood mononuclear cells band. We suggest that the higher differences in density between the Histopaque layers and blood when overlayering, which are decreasing due to temperature equalization during centrifugation, were responsible for this effect. The low temperature of the Histopaque layers resulted in a good cell purity of $> 95\%$, whereas layers at room temperature produced less cell purity. Using whole blood instead of diluting blood samples 1:1 with PBS (Toth et al., 1992) when layering the sample onto Histopaque®-1077 also reduced problems with the layer surface and improved cell purity and furthermore increased the possible yield of blood cells.

After adaption of centrifugal speed, Histopaque temperature and blood dilution to the requirements of canine blood cell purity blood mononuclear cells vs. polymorphonuclear cells was $> 95\%$. However, as shown by Toth et al. (1992), Weiss et al. (1989) and others red blood cells (RBC) consistently contaminated the polymorphonuclear cells band. The contaminating RBC, which disturbed the subsequent functional tests, had to be removed by cell lysis. After various tests we adapted a cell lysis buffer described by Hunt (1989). Lysis of RBC in the polymorphonuclear cell pellet with this lysis buffer was performed for different lengths of time (5–10 min) and with various temperatures for the buffer (room temperature and 4°C). The technique of continuously and gently shaking for 10 min with an initial temperature of 4°C for the lysis buffer produced a complete lysis of the RBC together with good viability of the polymorphonuclear cells, whereas shorter or longer lysis periods as well as lysis with lysis buffer at room temperature (21°C) resulted in incomplete lysis and/or reduced viability ($< 80\%$).

Therefore, in this study we improved the technique with discontinuous Ficoll gradient described by Toth et al. (1992) for feline blood and used it for the separation of polymorphonuclear and peripheral blood mononuclear cell populations from whole blood of dogs with an acceptable yield and purity. The technique is simple and rapid and requires no preparative procedures such as unit gravity sedimentation. No equipment other than a standard benchtop centrifuge is required. In order to achieve maximal purity and yield, centrifugation speed, Ficoll temperature, dilution of whole blood and RBC lysis were varied and adapted to the requirements of canine blood. Using this technique an almost complete separation ($> 96\%$) of blood mononuclear cells from polymorphonuclear cells was achieved in the dog. RBC contamination was minimal and

disappeared after treatment with red blood cell lysis buffer. Generally the results of the present technique compare favorably with previously described techniques. Cell purity and viability equalled or exceeded that of other techniques, as did the yield of PBCM. The yield of polymorphonuclear cells was less than that described by Weiss et al. (1989), but it was not contaminated with RBC and the cells remained completely active, as was demonstrated by the subsequent bactericidal assay (unpublished data).

In conclusion, the technique described provides a simple, rapid method for the separation of blood mononuclear cells and polymorphonuclear cells from canine blood. Since separation is accomplished at low speeds using a tabletop centrifuge, this technique is readily adaptable for use in clinical and research laboratories.

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