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## HEMATOLOGICAL OBSERVATIONS OF LEUKOCYTES COLLECTED BY WATER METHOD FROM BOVINE PERIPHERAL BLOOD

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An attempt was made to collect a large number of leukocytes without red cells from bovine peripheral blood by the use of the water method improved by the authors, then they were examined hematologically.

1) In the collection of leukocytes by the water method, it was found that using blood and water, in the ratio of 1 to 1.5, with the restoration of isotonicity after 20 seconds was the most favorable condition for bovine blood.

2) In the light microscopy of the collected leukocytes smears stained with Giemsa stain, a significant decrease of normal cells and an increase of destructed cells was observed in comparison with those of the untreated control blood, respectively, but in so-called ghost cells, no significant changes were observed.

3) A significant increase of so-called dead cells was observed in the collected cells in comparison with those of the untreated control blood.

4) By the treatment with water, the phagocytic activities of carbon particles were decreased significantly in monocytes and eosinophils, but in neutrophils no significant changes were observed in comparison with those of the untreated control blood.

5) In the electron microscopy of the collected leukocytes, the defects of the fine structure of the cells were not so marked that almost all the collected cells distinguished their kind of cells by the characteristics of their fine structures.

6) In the differential count of the collected leukocytes, a significant increase of neutrophils and a decrease of lymphocytes was observed, but no significant changes were observed in monocytes, eosinophils and basophils in comparison with the untreated control blood.

### INTRODUCTION

A collection of a large number of leukocytes without erythrocytes is very important for the detection of abnormal cells in the peripheral blood, for leukocyte culture and for making blocks in electron microscopy. However, it is especially difficult to obtain the buffy coat consisting of a large number of leukocytes from bovine peripheral blood<sup>2,7)</sup> because the erythrocyte sedimentation rate is extremely slow in cattle. As a method for simple collection of leukocytes

from bovine peripheral blood, BEHRENS & ESCH reported a hemolytic method by the use of water. WEINHOLD used the modified water method to collect bovine leukocytes for blood cell culture. However, in these reports, hematological observations of the leukocytes collected by the methods were not conducted in detail. In the present studies, first of all, methods for the collection of leukocytes from bovine peripheral blood by the use of water were re-examined and improved. Then the leukocytes collected by the improved method were examined hematologically.

## MATERIALS AND METHODS

### 1 Materials

**Cattle** Ten normal Holstein-Friesian oxen were provided for the experiments. They were all two years old.

**Blood** The blood taken by venipuncture from the jugular vein was anticoagulated by the addition of one part of 3.8% sodium citrate solution to 4 parts of blood.

### 2 Collection methods

Four beakers containing 5.0 ml of anticoagulated bovine blood were prepared. Distilled water was added to the anticoagulated bovine blood in the ratio of 1 to 1, 1.5, 2 and 2.5, respectively. They were mixed gently. After 30 seconds exactly, isotonicities were restored by the addition of 1.0 ml, 1.5 ml, 2.0 ml and 2.5 ml of 5.4% NaCl solution, respectively. Four conical tubes filled with the hemolysates were centrifuged at 2,000 rpm for 5 minutes. Immediately after that, thick smears of the sediments were made and stained with Giemsa solution. By the microscopy of the thick smear film, the most favorable ratio of anticoagulated blood to water was determined according to the absence of red cells. Then, only the times from the addition of water to the restoration of isotonicities were changed to 10 seconds, to 20 and 30 seconds and the most favorable time with the most favorable ratio of blood to water was determined on the basis of the absence of red cells and the morphological characters of the leukocytes. Consequent hematological observations were conducted on the leukocytes collected by the improved water method.

### 3 Morphology

Two hundred leukocytes were checked on thin smears made routinely from freshly drawn blood (control) and thick smears of leukocytes collected by the improved water method, respectively. They were divided into 3 cell groups, viz., normal cells without any morphological abnormalities, destructed cells with some nuclear or cytoplasmic abnormalities such as vacuoles in the cytoplasm, swelling and fusing in nuclei and abnormal staining in both cytoplasm and nuclei still with the original shapes of the cells, and so-called ghost cells which were undistinguishable their cell types. The appearance rates of the cells in each group were shown in percentages of the cells.

### 4 Viability

The eosin method<sup>13)</sup> was used for checking the viability of the leukocytes. In this

instance, the collected leukocytes were resuspended in their own plasma equivalent to the volume of the initial blood plasma, then the resuspended solutions and anticoagulated fresh blood (control) were mixed with the same volume of eosin solution, respectively. After preservation for 10 minutes, 200 cells in each of the mixtures were counted and classified into stained and unstained cells.

### 5 Phagocytic activity

Phagocytic activity was examined by TANABE's method modified by the authors. Namely, a kind of india ink was made by rubbing Kobaien sumi stick in 5 ml of physiological saline solution at the secondary grade pressure, at 100 revolutions per minute for 3 minutes. The india ink solution thus obtained was centrifuged at 3,000 rpm for 5 minutes and filtered with Tōyō filter paper No. 6 just before use. One part of the india ink solution was mixed with 9 parts of anticoagulated blood (control) or a suspension which contained collected leukocytes in their own plasma equivalent to the volume of the initial blood plasma. Each of the mixtures was poured into tubes with their insides coated with paraffin and incubated at 37°C for one hour, during which time they were mixed gently every 15 minutes. After one hour, anticoagulated blood with the india ink solution was hemolyzed by the water method and the leukocytes were collected. From them, thick smears were made, dried, fixed with methanol and stained with Giemsa solution (control). On the other hand, the suspensions containing collected leukocytes were centrifuged, and thick smears were made from them. In the examinations, 200 cells of neutrophils, monocytes and eosinophils respectively were counted, and in each of the cell types the percentage of the cells phagocytosing and non-phagocytosing carbon particles was calculated.

### 6 Electron microscopy

Collected leukocytes were fixed in 1% osmic acid solution (Millonig method) for 50 minutes and then were dehydrated by ascended acetone series and embedded in Epon. Ultra-thin sections were cut on a Porter-Blum MT 1 ultra-microtome and they were double stained with uranyl acetate and lead citrate solutions. The microphotographs of the cells were taken with a JEM 7 type electron microscope.

### 7 Differential count

A differential count was conducted on 200 leukocytes of both the usual thin smears made of fresh peripheral blood (control) and thick smears made of leukocytes collected by the improved water method.

### 8 Statistical analysis

The values obtained in the examinations of the morphology, viability, phagocytic activity and the differential count of the collected leukocytes and the control ones were analysed by a two-way layout statistically. In the calculations, each numerical value was transformed into arcsine.

## RESULTS

### 1 Collection method

In all the thick smears made from the sediments of the hemolysates in the 1 to 1 ratio of anticoagulated bovine blood to water, and restoration of isotonicity after 30 seconds, red cells were present among the leukocytes. After isotonicity was restored, the hemolysates were turbid and their color was dark red. In all the thick smears made from the sediments of anticoagulated bovine blood diluted with water in the ratio of 1 to 1.5, 2 and 2.5 and restoration of isotonicity after 30 seconds, no red cells were observed. The destruction of the leukocytes was smallest in the smears when the ratio of blood to water was 1 to 1.5. In these instances, after isotonicity was restored, the hemolysates were not turbid and their color was dark red.

From these findings, it was determined that the most favorable ratio of blood to water was 1 to 1.5. For the determination of the most favorable time for restoration of isotonicity when the ratio of blood to water was 1 to 1.5, further experiments were carried out. Namely, ten thick smears were made from sediments of the hemolysates restored after 10 seconds, 20 and 30 seconds respectively, and they were examined in microscopy. In ten thick smears made from the sediments of the hemolysates restored after 10 seconds, a considerable number of erythrocytes were seen among the leukocytes. In all the thick smears made from the sediments of the hemolysates restored after 20 and 30 seconds, no erythrocytes were observed.

From these findings, it was decided that the ratio of blood to water was 1 to 1.5 and the restoration after 20 seconds of mixing was the most favorable collection method for the bovine blood.

### 2 Morphology

The normal cells of thin and thick smears were 97.80 (95.50~99.50) and 95.40 (83.50~99.00) % on the average, respectively; the latter was 2.40 % lower than the former on the average. The destructed cells of thin and thick smears were 1.30 (0~4.50) and 3.40 (1.00~7.00) % on the average, respectively; the latter was 2.10 % higher than the former on the average. The so-called ghost cells of thin and thick smears were 0.90 (0~3.00) and 1.20 (1.00~11.00) % on the average, respectively; the latter was 0.30 % higher than the former on the average.

As the results of the statistical analysis of the appearance rates of these cell groups show, the differences in the normal and destructed cells were significant at the 5 and 1 % levels, respectively.

### 3 Viability

Eosin-stained cells of the control and treated leukocytes were 6.70 (2.00~10.50) and 11.50 (5.50~17.00) % on the average, respectively; the latter was 4.80 % higher than the former on the average.

By the statistical analysis of the appearance rates of the eosin-stained cells, it was revealed that the difference between the control and treated groups was significant at the 1 % level.

TABLE Results of hematological observations

ITEMS OF OBSERVATION	APPEARANCE RATES		
	Fresh blood	Collected leukocytes	
	(%)	(%)	
Morphology	normal cells*	97.80	95.40
	destroyed cells**	1.30	3.40
	ghost cells	0.90	1.20
Viability	Eosin stained cells**	6.70	11.50
Phagocytic activity	neutrophils	69.65	67.85
	monocytes**	70.50	61.95
	eosinophils**	33.00	20.95
Differential count	neutrophils*	22.55	31.35
	lymphocytes*	66.75	58.70
	monocytes	3.95	4.30
	eosinophils	6.75	7.35
	basophils	0	0

Note: \* Significant at the 5% level, \*\* Significant at the 1% level

#### 4 Phagocytic activity

The neutrophils phagocytosing carbon particles in the control and treated blood were 69.65 (32.00~100.00) and 67.85 (54.00~90.50)% on the average, respectively; the latter was 1.80% lower than the former on the average. In monocytes, the phagocytosing ones in both blood were 70.50 (58.00~77.00) and 61.95 (56.00~69.50)% on the average, respectively; the latter was 8.55% lower than the former on the average. In eosinophils, they were 33.00 (24.50~42.00) and 20.95 (10.00~33.00)% on the average, respectively; the latter was 12.05% lower than the former on the average.

In the statistical analysis of the appearance rates of the cells phagocytosing carbon particles between the control and treated blood, the differences in the monocytes and eosinophils were significant at the 1% level.

#### 5 Electron microscopy

In the electron microscopy of the collected leukocytes, generally speaking, cell membranes and micro-organelles of the cytoplasm were distinct and they were scarcely destroyed. In some cells, however, a vacant space between the nucleus and cytoplasm was sometimes observed and slightly destroyed nuclear membranes were seen.

Therefore, almost all the collected cells revealed a distinction between each kind of leukocytes by the characteristics of their fine structures.

#### 6 Differential count

The appearance rates of neutrophils of thin and thick smears were 22.55 (13.00~36.50) and 31.35 (16.00~42.50)% on the average, respectively; the latter was 8.70% higher than the former on the average. In lymphocytes, they were 66.75 (54.50~77.00) and 58.70 (47.50~

74.50) % on the average, respectively; the latter was 8.05 % lower than the former. In monocytes, they were 3.95 (2.00~7.50) and 4.30 (2.00~7.50) % on the average, respectively; the latter was 0.35 % higher than the former on the average. In eosinophils, they were 6.75 (3.00~21.50) and 7.35 (2.00~21.50) % on the average, respectively; the latter was 0.60 % higher than the former on the average.

In the statistical analysis of the appearance rates of the cells between the control and treated blood, the differences in both neutrophils and lymphocytes were significant at the 5 % level.

#### CONSIDERATIONS

For the collection of leukocytes from human peripheral blood, many methods have been devised. These are; the addition of substances with high molecular weights for the acceleration of rouleaux formation<sup>3,4,5,6,8,10,11</sup>), centrifuge methods based on the difference in the specific gravities of blood corpuscles<sup>1,15</sup>), selective destruction of erythrocytes by means of using some hemolytic solutions<sup>14,16,18</sup>). In the past several years, the authors have tried to collect bovine leukocytes without erythrocytes for the electron microscopy by the use of various methods reported already. However, favorable results have not been obtained. This may be due to the special characters of the bovine blood as emphasized by some investigators<sup>9,12</sup>). Therefore, the authors were very interested in the hemolytic method of BEHRENS & ESCH by the use of water in bovine blood. By the modified water method, WEINHOLD collected bovine leukocytes for cell culture. However, in these two reports, collection methods such as the ratio of blood to water and the time from the addition of water to the restoration of isotonicities, and the hematological observations of the leukocytes collected by the methods were not carried out in detail. Therefore, at first, the authors tried to re-examine the collection methods of the leukocytes from bovine peripheral blood, and an improved method was determined; viz., a ratio of 1 to 1.5, bovine blood to water, and a period of 20 seconds from the addition of water to the restoration of isotonicity was found to be the most desirable method. In this method, the ratio of water to blood is smaller and the restoration time is shorter than those of the methods of BEHRENS & ESCH and WEINHOLD. Therefore, it is thought that the effects of treatment with water on the collected leukocytes is slighter in our method than in the latter two methods. However, the numerical value of the appearance rate of the normal cells without any defects was as high as 95.40 % in light microscopy, and the numerical value of the appearance rate of the so-called dead cells stained with eosin solution was as low as 11.50 %. In the electron microscopy of the collected cells, it was shown that the defects of the fine structure of the leukocytes were not so marked, that almost all the collected leukocytes revealed a distinction between each kind of the cells by the charac-

teristics of their fine structures. WEINHOLD reported that the appearance rate of each cell type of the collected leukocytes did not show any difference in comparison with that observed before treatment and only 2% of the collected leukocytes were dead cells by trypan blue stain. And he concluded that the leukocytes collected by the water method were utilizable for the purpose of leukocyte culture. Consequently, on the basis of the results obtained in the present hematological observations, it may be said that the leukocytes collected by the water method improved by the authors will be useful for the check of abnormal or tumor cells in the peripheral blood, blood cell culture and making blocks for electron microscopy, in which all of the collected cells do not always need to be intact, though they are not utilizable for the differential count of leukocytes in which the accurate numerical values are regarded as important.

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#### EXPLANATION OF PLATE

- Fig. 1 A low magnification of a thick smear film of the leukocytes collected by the improved water method  
A lot of leukocytes are seen collectedly.  $\times 250$
- Fig. 2 A high magnification of an area of the same film  
Almost all the leukocytes in this figure are free from any destruction. An extra large mononuclear cell with dark bluish cytoplasm (arrow) is seen near the upper right corner.  $\times 1,000$
- Fig. 3 Two neutrophils phagocytosing a lot of carbon particles  $\times 2,000$
- Fig. 4 A monocyte and an eosinophil phagocytosing several carbon particles  $\times 2,000$
- Fig. 5 A micrograph of collected leukocytes  
In the fine structures of the cells in this figure, no effects of the water treatment are observable.  $\times 11,400$

