ROSETTE FORMATION: A TOOL FOR T-LYMPHOCYTE ENUMERATION AND ASSESSMENT OF INNATE IMMUNITY


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Abstract: Rosettes formation technique (SRBC) was employed for the assessment of lymphocyte per cent. Development of accurate methods for enumeration of T and B lymphocytes in human and animal peripheral blood during the past decades has led to a plethora of papers on T and B lymphocytes in various pathologic states. Lymphocytes can be divided into T and B lymphocytes on the basis of selective binding capacity of T-lymphocytes with Sheep RBC (SRBC). On the basis of enumeration of T-lymphocyte innate immunity status of Non-discript (ND) and Sahiwal (S) cattle was compared. Rosette forming T-lymphocytes were enumerated in all the four groups of animals. The mean (±SE) values of T-lymphocytes (per cent) in ND-I, ND-II, S-I and S-II was 41.04±0.47, 56.83±0.99, 35.79±0.93 and 56.62±0.76, respectively. A comparative study on T-lymphocytes showed that there was significant increase (P<0.01) in T-lymphocytes count with respect to age in both breeds. The T-lymphocytes count of ND calves was significantly (P<0.01) high when compared with Sahiwal calves whereas breed difference was not observed in adult animals.

Key words: T-lymphocytes, Innate immunity

INTRODUCTION

Lymphocytes are relatively numerous in the blood of most species of domestic animals; they are most numerous in cattle, sheep, goat, swine and chicken. They are formed in lymphoid tissue (e.g. Lymph nodes, peyer’s patches, spleen, tonsil and thymus) and the main constituent of this tissue. Lymphocytes play a very important role in immunity [1]. Functionally they are of two types: T and B lymphocytes. B-lymphocytes are concerned with humoral immunity whereby antibodies are produced in response to stimulation by antigens. T-lymphocytes are concerned with cell-mediated immunity whereby T-lymphocytes are also activated in response to antigen stimulation. The origin of the B-lymphocytes is mainly in the lymphocytic follicles around the germinal centers of lymph nodes, spleen, bone marrow, and other places where they are formed. They account for about 15 percent of the circulating lymphocytes in the blood vessels. In contrast, the T-lymphocytes reside in the interfollicular areas of these organs. They account for about 85 percent of the circulating lymphocytes in the blood stream. Wilson [2] documented that the T-lymphocyte rosette forming cell have role in immunity. With the enumeration of T-lymphocytes innate immunity status can be evaluated.

The principle behind the rosette formation is that the suspected receptor bearing cells are mixed with the signal cells which carry the corresponding receptor building substance on their surface. That substance is either naturally occurring on it or artificially coupled to it. The receptor bearing cell will then bind the signal cells around their surface.
and form rosettes [3]. It is impossible to distinguish between T-lymphocytes and B-lymphocytes in a peripheral blood smear [4]. Normally flow cytometry testing is used for specific lymphocyte population counts. Whereas, the detection of bovine T-lymphocytes rosette formation between lymphocytes and sheep erythrocytes is a traditional method.

In the present study the innate immunity status of non-descript (ND) and Sahiwal (S) cattle of Chhattisgarh were compared on the basis of percent of T-lymphocyte in peripheral blood. This is the first ever attempt to assess the innate immunity status of non-descript cattle of Chhattisgarh. Sahiwal can be considered as useful candidate animal for comparison with ND cattle as the Sahiwal is one such breed which is an established breed in Chhattisgarh and probably in whole India. Rosette forming lymphocytes with SRBC were compared between the blood of Sahiwal and non-descript cattle of Chhattisgarh.

MATERIALS AND METHODS

Selection of animals: The study was planned to adjudge extent of innate immune response of village level local Non-descript (ND) cattle and findings were compared with Sahiwal reared in an organized farm. Non-descript cattle belonging to a Gaushala situated in Chhatagarh, Mohli, Durg (C. G.) and Sahiwal cows belonging to BMEF, Anjora, Durg (C. G.) were selected to study differential lymphocyte count of local ND cattle and Sahiwal cows. Sixteen apparently healthy local ND cows (Group A), and sixteen Sahiwal cows (Group B) were selected for study. The animals in each group were further divided into two subgroups viz. ND-I (6 months-2 years age), ND-II (above 2 years), S-I (6 months-2 years age) and S-II (above 2 years) comprising of 8 animals in each group.

Collection of blood samples: Five ml of blood was collected aseptically by jugular vein puncture from each cattle brought under the study. The sample was collected according to guidelines of Institute Animal Ethical Committee (IAEC). Freshly drawn blood sample was processed for identification and enumeration of lymphocytes within 2 hours of blood collection.

Separation of lymphocytes: Blood samples were processed for separation of lymphocytes as per the method described by Boyam [6]. For separation of lymphocytes, 1:2 dilution of the whole blood was made in isotonic phosphate buffered saline. 1.5 ml LSM (Lymphocyte Separating Medium- HiMedia) was taken in clean sterile centrifuge tube and overlayed with 4.5 ml diluted blood (Fig. 1A). A sharp interface was observed after centrifugation at 1800 rpm for 15 min at room temperature. Most of the plasma and platelet containing supernatant above the interface band was aspirated. Subsequently by using a clean glass Pasteur pipette the lymphocyte layer along with half of the LSM below was carefully aspirated. Three consecutive gentle washing were performed on the separated lymphocyte using PBS.

Preparation of sheep RBC (SRBC): For preparation of Sheep RBC suspension (0.5%) 5 ml of fresh blood was collected in Alsever’s solution in 1:2 ratio from the jugular vein of a healthy sheep along with taking all precautions. RBC were allowed to settle down and kept for at least 5 days at 4°C. Tube was centrifuged at 3000 rpm for 15 min. Supernatant was discarded and consecutive washing of RBC by using PBS was done thrice. Washed RBC suspension was kept in Alsever’s solution as stock RBCs suspension and 0.5% RBC suspension was prepared by taking 0.5 ml RBC and made the suspension of 100 ml in distilled water.

T-lymphocyte count by rosette technique: SRBC Rosettes formation technique was employed for the identification and enumeration of T-lymphocytes as per the method given by Talwar [7]. Equal volume (0.5 ml) of working SRBC and separated lymphocytes were mixed and incubated in water bath at 37°C for 10 min and spunned at 1500 rpm for 5 min. The suspension was kept at 4°C overnight. The pellet was gently re-suspended and equal volume of 0.2% glutaredehyde in PBS was added and this was left for 15 min at 4°C. The pellet was gently resuspended and a drop of suspension was placed under a coverslip on microscopic slide. Directly Rosette formation was counted and observed under light microscope at 1000X magnification. A minimum of 200 lymphocytes were counted and per cent of rosette forming lymphocytes (out of absolute lymphocyte count) was calculated and results were reported in per cent (%). Only cells with three or more tightly adherent SRBCs were scored as rosettes.

Statistical analysis: The data were analyzed statistically by Independent mean ‘t’ test to see the effect of age within a breed, if any, and to see the
Fig. 1: Separation of lymphocyte. A) Before centrifugation. B) After centrifugation, (i) Diluted blood (ii) LSM (iii) Plasma layer (iv) Settled RBC (v) Lymphocyte interface

Fig. 2: Rosette showing T-lymphocytes surrounded by Sheep erythrocytes

Table 1: T lymphocyte population in Non-descript and Sahiwal cattle. **Highly significant at 1% level (P<0.01).*Significant at 5% level (P<0.05). NS = Non-significant. Mean value bearing different superscripts within a row differ between different breed and within breed at different age level.

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-descript (ND)</th>
<th>Sahiwal (S)</th>
<th>Level of significance</th>
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<tbody>
<tr>
<td></td>
<td>ND-I</td>
<td>ND-II</td>
<td>S-I</td>
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<tr>
<td>T-cell (%)</td>
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<td>41.04±0.47b</td>
<td>56.83±0.099b</td>
<td>35.79±0.93c</td>
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RESULTS AND DISCUSSION

T-lymphocyte count by rosette technique: For the direct identification and enumeration of T-lymphocytes SRBC Rosettes formation technique was employed which is a simple and traditional technique. The specific adherence of sheep erythrocytes to lymphocytes to form rosette is a useful technique to study and differentiate the lymphocyte populations [9].

Separation of lymphocytes through LSM revealed a sharp interface of lymphocyte between plasma and LSM layer (Fig. 1B). Ficoll/Triosil mixture can also be used for separation of lymphocyte instead of LSM [10].

T-lymphocytes have the characteristic of forming E-rosettes when they bind selectively to sheep red blood cells (SRBC). So the Rosette formation is the surety for the presence of T-lymphocyte [11]. Further lymphocytes can be quantified by different labeling like acridine orange labeling and afterwards observed with a fluorescence or ordinary light microscope. If T and B lymphocytes are labeled with acridine orange, only T-lymphocytes form E-rosettes; B lymphocytes are actually stained but do not form rosettes. One percent methylene blue stain can also be used for the visibility of cell suspension [3].

In the present study rosette representing the T-lymphocytes (Table 1, Figs. 2,3) were enumerated...
directly in all the groups and expressed in per cent. Here directly the T-lymphocytes were demonstrated without any pretreatment of SRBC with any stabilizer. Whereas Paul et al. [12] demonstrated the significant usefulness of the chemicals namely Dextran and 2-aminoethylisothiouronium bromide (AET) and Kaupp, et al. [13] demonstrated the use of sulphydryl reagent which enhances rosette formation when pretreated with SRBC. The mean (±SE) values of T-lymphocyte (per cent) in ND-I, ND-II, S-I and S-II was 41.04±0.47, 56.83±0.99, 35.79±0.93 and 56.62±0.76, respectively. A comparative study on T-lymphocytes showed that there was significant increase (P<0.01) in T-lymphocyte count with respect to age in both breeds. The T-lymphocytes count of ND calves was significantly (P<0.01) high as compared to Sahiwal calves whereas breed difference was not observed in adult animals. This indicates that the local Non-descript cattle have the potential equally to the well established Sahiwal to withstand the environment adverse conditions and infections. Paul et al. [12] reported a similar finding of 63% T-lymphocytes in normal adult cattle. In another study Paul et al. [11] detected approximately 70% of peripheral blood leukocytes as rosette forming cells in bovine blood. They also presented that the rosette formation is an evidence of presence of T-lymphocytes. Binns et al. [14] reported a low value of T-lymphocyte in adult (37.3 ± 4.2%) animal along with rosette forming T-lymphocytes tended to increase with age.

Physiological conditions like parturition or any disease have significant effect on T cell population which could be evaluated with this technique. Levkut et al. [15] showed a significant decrease in absolute number of lymphocytes, and CD3+, CD4+, and CD8+ T-lymphocytes on days 7, 14, and 21 after parturition. Kimura et al. [16] experiment showed intact cows exhibited significant decline (P<0.05) in the percentage of all T-lymphocyte cell subsets from 27 day before parturition. The 51.9±1.9% was total T-lymphocytes population in normal intact cow is in conformity with those recorded in present study. However advancement in many scientific techniques Rosette formation is a simple technique and can be a useful as a first hand tool in further research on lymphocytes.

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