Identification and Quantification of Monocytes in Bovine Mononuclear Cells

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ABSTRACT. To identify and quantify bovine monocytes in peripheral blood mononuclear cells (MNCs), five procedures were used: neutral red-, latex- and yeast- ingestion methods, peroxidase staining and alpha naphthyl acetate esterase (ANAE) staining. The yeast ingestion method proved to be a simple and suitable procedure for discrimination between monocytes and lymphocytes. ANAE staining was also useful for identifying monocytes. Simultaneous application of phagocytosis of yeast and ANAE staining was the most reliable procedure for the identification of bovine monocytes.—KEY WORDS: bovine monocytes, mononuclear cell differentiation.

Monocytes/macrophages and lymphocytes play an essential role in host defenses against invading pathogens. The morphological distinctions between lymphocyte and monocyte, however, are not always clear owing to their close resemblance.

In bovine lymphocyte subpopulations, the identification of monocytes is a prerequisite for accurate evaluation of lymphocyte markers in mononuclear cell (MNC) preparations. Moreover, to investigate the effects of monocytes on lymphocyte functions, it is necessary to identify each cell type accurately and to separate both types. Furthermore, in the diagnosis of leukemic cattle, it is important to know whether leukemic cells are of lymphocytic or monocytic origin. However, procedures for the identification and quantification of bovine monocytes have not been satisfactorily developed. Therefore, this study was carried out for a comparison among five procedures to indentify phagocytic cells with the intention of developing a simple, accurate and widely applicable method for the identification of monocytes in MNC preparations isolated from bovine peripheral blood.

Blood was obtained from healthy, 2 to 7 years old, Holstein-Friesian cows. Blood samples were taken from the jugular vein and placed in tubes containing heparin (20 units/ml of blood). MNCs were isolated using Böyum’s method [2] modified for bovine lymphocytes as previously described [11, 12]. Cells were washed twice with phosphate-buffered saline (PBS) (pH 7.2) and once with Eagle’s minimum essential medium (MEM; Nissui Pharmaceutical Co., LTD., Tokyo) (pH 7.2). More than 98% of the cells were MNCs as assessed by the morphology of Giemsa-stained cells.

Monocytes were identified by the following procedures: 1) Neutral red staining [20]; neutral red (Kishida Chemical Co., LTD., Osaka) was dissolved in PBS (prior to use) at 1%, and then 10 µl of 1% neutral red solution was added to 2 ml of cell suspension (3–5×10⁶ cells/ml) in MEM containing 10% autologous serum. This mixture was kept at 37°C for 30 minutes, the cells were washed three times with PBS and then examined under a light microscope. MNCs which had ingested neutral red were referred to as monocytes. 2)
Fig. 1. Mononuclear cells were stained with neutral red (arrows). ×850.

Fig. 2. Monocyte (arrow) ingesting latex particles. ×850.

Fig. 3. Engulfment of yeast particles by monocytes (arrow). ×850.

Fig. 4. Peroxidase staining. ×850. Inset: Monocytes (M) show nonspecific and very weak peroxidase activity. Eosinophil (E) shows strong peroxidase activity. ×1140.

4 × 10⁶ cells) of the MNC preparations was washed twice with MEM and resuspended in 200 μl of prepared yeast suspension (2 × 10⁹/ ml) in MEM containing autologous serum, and then incubated for 1 hour at 37°C. After incubation, cells were washed once with PBS, centrifuged at 400 g for 10 minutes, and then 200 μl of 0.01% fuchsin solution were added to the cell pellet and resuspended by gentle shaking. Cells were examined by their ability to ingest yeast particles and were referred to as monocytes. 4) Peroxidase staining; peroxidase staining was performed according to Kaplow’s method [5]. 5) Alpha naphthyl acetate esterase (ANAE) staining; MNCs were examined for ANAE activity by Kulenkampff et al.’s method [7] with slight modifications. Cells in which the cytoplasm was of a diffuse reddish brown color were considered to be monocytes, according to the

Latex ingestion; monocytes were identified by their ability to phagocytize 0.81 μm latex particles (Difco Laboratories, Detroit, Michigan) as described by Paul et al. [15]. 3) Yeast ingestion; monocytes were identified by their ability to phagocytize yeast particles (Dried, Wako Pure Chemical, LTD., Osaka) according to the procedure described by Abo [1] with slight modifications. An aliquot (3-
criteria as described by Mueller et al. [10].

The results were summarized as follows: Neutral red ingestion; distinguishing monocytes from MNC preparations was difficult because lymphocytes were also stained by neutral red nonspecifically (Fig. 1). Latex ingestion; the number of latex particles ingested by monocytes was low (2–7) and there was no significant swelling of cells following latex ingestion (Fig. 2). Yeast ingestion; the yeast-ingesting phagocytic cells were twice or three times as large as their normal size (Fig. 3). Monocytes ingested 3 to 8 yeast particles, and were obvious because they became swollen following ingestion. It was easy to distinguish between ingested intracellular yeasts which appeared a light yellow color and the extracellular yeasts which were stained red by fuchsin. Peroxidase staining; bovine monocytes showed nonspecific and very weak peroxidase activity (Fig. 4), while lymphocytes showed no peroxidase activity at all. The occasional eosinophils and polymorphonuclear cells showed a strong peroxidase activity. ANAE staining; ANAE positive monocytes were diffusely and intensely stained reddish brown color, while lymphocytes showed a single or several granular spots of ANAE (Fig. 5). The number of monocytes distinguished by three selected procedures was estimated by counting 200 MNCs under a light microscope (Table 1).

Morphologically, monocytes and lymphocytes in bovine MNC preparations are generally considered to be indistinguishable under the light microscopy. However, certain characteristics such as the phagocytic ability and stain ability with cytochemical markers can be used to identify monocytes in suspension. But, neutral red ingestion is considered to be unsuitable for distinguishing these cells because lymphocytes are also stained by neutral red. In latex ingestion, it was difficult to distinguish whether latex particles adhered to the cell surface or not. The yeast ingestion procedure was found to be very useful since it was easy to perform and allowed for easy identification of monocytes. However, care should be taken to check the purity of the MNCs, because polymorphonuclear cells also ingest yeast particles. The results obtained from the phagocytosis of yeast and latex particles were almost the same for each cow (Table 1).

Monocytes in MNC preparations from 8 normal cows, identified by the yeast ingestion method contained 5.6±1.6% (mean± SD) (range: 3.5–8.2%). Peroxidase staining has been utilized extensively for the cytochemical identification of human monocytes [16]. However, peroxidase activity is not an absolute criterion for monocyte identification since not all monocytes are peroxidase positive [14, 16]. Bovine monocytes showed very weak and nonspecific peroxidase activity, therefore, this procedure was not appli-
cable for identification of bovine monocytes. ANAE activity is considered specific for human [4, 6, 7] and murine [10, 17] T lymphocytes which show granular spots of ANAE positive, however, it is not a specific marker for T lymphocytes in bovine [19], ovine [3] and swine [9]. It is, however, considered to be a specific marker for monocytes/macrophages [4, 6]. In general, the mean values of cells positive for ANAE activity are almost coincident with those of phagocytosis [8, 13]. The same cells were also identified by both methods [13]. In this study the mean values for each of the three procedures were almost similar (Table 1). However, differences between assays were observed in ANAE marker experiments but not in phagocytosis of yeasts and latex ingestion (Table 1). The main reason for this discrepancy may be related to the facts that cytochemical activity and surface marker expression are subtly dependent on cell differentiation and/or activation [4, 18].

In conclusion, in a comparison of five procedures, the yeast ingestion method was found to be the simplest and widely applicable procedure for the identification and quantification of monocytes in bovine MNC preparations, and besides, a cytochemical staining method using ANAE was also found to be useful. For the identification of bovine monocytes, a combination of phagocytosis assay and cytochemical staining for ANAE activity was more reliable than any one of the procedures.

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REFERENCES


要約

ウシ単核細胞中の単球の同定と測定に関する比較検討（短報）：永嶽 哲・寺嶋賢一・野田 寛（酪農学園大学家畜衛生学教室）——単球の同定を目的に，1）ニュートラルレッド，2）ラテックス，3）イーストの各食食法，4）ベルオキシダーゼ，および，5）アルファナフタールアセテートエステラーゼ（ANAE）染色法を実施した。イースト食食法が最も簡単で，リンパ球との鑑別も明瞭であったが，ANAE 染色法も応用可能であり，両法の併用によりウシ単球の正確な同定と測定が可能であった。