Original Article

Differential Analysis of Rat Bone Marrow by Flow Cytometry

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Abstract. A procedure for rat bone marrow differential analysis using flow cytometry and commercially available monoclonal antibodies is described. The method uses a combination of the differential expression of leucocyte common antigen (CD45) on different cell lineages and the expression of transferrin receptor (CD71). This is coupled with the side scatter measurement (SSC) for morphological complexity and nucleic acid staining with LDS-751 for the separation of all nucleated cells from mature red blood cells. The relative number of erythroid, myeloid and lymphoid cells as well as the M:E ratio was generated by the flow cytometric method with acceptable reproducibility and a good agreement with microscopic analysis. There were no significant differences in mean values of the three cell populations between males and females or between bone marrow samples collected from left and right femurs. The flow cytometric analysis of bone marrow provides a semi-automated and rapid method that is suitable for the evaluation of rat bone marrow in the preclinical toxicity investigations.

Keywords: Bone marrow; CD45; CD71; Flow cytometry; Rat

Introduction

Bone marrow analysis provides diagnostic and prognostic information regarding haematopoietic function. Compounds and certain compound classes, which are found to cause haematological changes in preclinical safety evaluation studies, require a careful assessment and evaluation of bone marrow alterations. Haematological parameters such as peripheral blood cell count and bone marrow cytology and histology are monitored routinely in preclinical animal toxicity studies. The microscopic examination of cell populations in bone marrow smears can involve the classification of up to 1000 cells per specimen, which is labour intensive and depends greatly on the quality of bone marrow preparations, and requires appropriate analyst experience.

Flow cytometric immunophenotyping of leukaemias has become a standardised and valuable diagnostic tool in human medicine (Drexler 1987). A panel of monoclonal antibodies against selected cell surface antigens can be used to classify the various haematopoietic cell types at different stages of differentiation and maturation. In veterinary medicine the use of flow cytometry for bone marrow analysis is limited by the availability of cell surface markers for the various species of animals used in preclinical toxicology evaluations. Differential analysis of bone marrow by flow cytometry have previously been described for rats using staining reagents alone (Martin et al. 1992) or in combination with monoclonal antibodies (Criswell et al. 1998a) and for mice using double labelling with antiprecursor monoclonal antibodies (de Bruijn et al. 1998).

The combination of leucocyte common antigen (CD45) expression and intrinsic light scatter properties has been utilised to distinguish eight discrete clusters of normal human bone marrow cells (Stelzer et al. 1993; Rainer et al. 1995). These clusters correspond to lymphocytes, lymphoblasts, monocytes, segmented and band neutrophils, metamyelocytes and myelocytes, promyelocytes, myeloblasts and nucleated erythroid cells. A modification of this approach is described in

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the present study for rat bone marrow differentials by flow cytometry which provides a semi-automated and rapid method that is suitable for the evaluation of a large number of rat bone marrow samples in preclinical toxicity investigations. The procedure is based on a combination of the differential expression of CD45 and transferrin receptor antigen (CD71) on different cell lineages coupled with the side scatter (SSC) signal for morphological complexity.

**Materials and Methods**

Wistar rats (10 males and 10 females) of approximately 9 weeks of age (Møllegaard Breeding & Research Centre A/S, Denmark) were used in the present study. The rats were kept two to a cage at 17–23 °C and 40%–70% relative humidity. All animal care procedures were in accordance with the ‘European convention for the protection of vertebrate animals used for experimental and other scientific purposes (1988)’.

The rats were killed by exsanguination from a common carotid artery under enflurane (Efrane®; Abbott Laboratories, USA) and nitrous oxide anaesthesia. Both femurs were immediately removed and trimmed of excessive muscular tissue. The femoral head and the distal epiphysis of each femur were cut off using an electrical saw. The bone marrow tissue was gently flushed out (approx. five times), with 2 ml phosphate buffered saline (PBS) containing 50% fetal calf serum by using a 19-gauge needle and a 2 ml syringe. A single cell suspension was prepared by a gentle back and forth drawing of the bone marrow suspension through the 19-gauge needle.

The bone marrow cell suspension was filtered through a 100 μm disposable filter device (Falcon 2054, Becton Dickinson Inc., USA) in a small tube and then underlayered with 1 ml of fetal calf serum (Sigma Chemical Co., St Louis, MO). After centrifugation at 300 g for 5 minutes at 4 °C, the supernatant containing most of the fat cells was discarded and the cell pellet was resuspended in 4 ml of ice-cold PBS containing 0.5% bovine serum albumin (BSA).

The concentration of nucleated cells in the bone marrow cell suspension was determined by using a haematology analyser (Sysmex F-800, TOA Medical Electronics Co., Ltd, Japan). The total nucleated cell count (TNC)/femur was calculated and the cell concentration was adjusted to 10⁷ nucleated cell/ml using PBS containing 0.5% BSA.

Two cytospin preparations were made from the adjusted bone marrow cell suspension. Briefly, 100 μl of adjusted bone marrow sample was transferred to a cytofurnel sample chamber (Shandon, England). After centrifugation at 900 rpm for 3 min (Cytospin 3, Shandon, England), the slides were air dried and then stained with May–Grunwald–Giemsa, and microscopical classification of 200 cells into erythroid, myeloid and lymphoid cells was performed by an experienced haematopathologist.

A 100 μl sample of the adjusted bone marrow cell suspension was transferred into each of 10 small tubes (Falcon 2054, Becton Dickinson Inc., USA). To each tube, 5 μl of fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45 and 10 μl of phycoerythrin (PE)-conjugated mouse anti-rat CD71 monoclonal antibodies (Serotec Ltd, England) were added. After a thorough mixing, the tubes were incubated on an ice bath in the dark for 20 min. Cells were washed once with ice-cold PBS containing 0.5% BSA. The supernatant was discarded and the cell pellet was resuspended in 0.4 ml ice-cold PBS containing 0.5% BSA. This was followed by the addition of 20 μl LDS-751 staining solution (Molecular Probes Inc., USA) and the tubes kept in the dark at room temperature for at least 20 min prior to flow cytometric analysis. LDS-751 is a cell-permeant nucleic acid stain that can be excited by the argon-ion laser at 488-nm and is particularly useful in multicolour analyses in combination with FITC and PE fluorochromes due to its long-wavelength emission, maximal at 670 nm (McCarthy and Macey 1993). The LDS-751 staining solution was prepared by diluting 20 μl of stock solution (1 mg LDS-751/ml methanol) with 3 ml PBS.

The flow cytometric analysis was performed in a FACSscan flow cytometer (Becton Dickinson Inc., USA) using an excitation wavelength of 488-nm from a 15-mW argon-ion laser. The cytometer was interfaced to the data analysis system CellQuest (Becton Dickinson Inc., USA). Data were collected from 10’000 cells in a list mode file to save values of forward scatter (FSC) in a linear scale and side scatter (SSC), FL1, FL2 and FL3 in a logarithmic scale. Defined instrument settings were used throughout the study. The FACSscan was calibrated using CalibRITE beads and FACSComp software (Becton Dickinson Inc., USA) to adjust the photomultiplier voltages for fluorescence, side-scatter channels, and to check the instrument signal-to-noise discrimination.

Cell sorting was conducted with a FACStarPlus flow cytometer (Becton Dickinson Inc., USA) supplemented with an argon-ion laser tuned to 488 nm wavelength. Cells were sorted from various regions (10000–20000 cells) into collection tubes containing PBS with 50% fetal calf serum, cytopsin slides were prepared as previously described and the identification of sorted cells was microscopically verified.

All results are presented as mean ± SD of the mean. Statistical methods used in the present study were Pearson correlation and paired t-test.

**Results**

Figure 1 shows dot plots and gating strategy of rat bone marrow cells labelled with CD45:FITC, CD71:PE and LDS-751. Nucleated cells (LDS positive cells) occupying region 1 (R1) in Fig. 1(a) were readily separated from mature non-nucleated erythrocytes (LDS negative