Identification and Phenotyping of Leukocytes in Bovine Bronchoalveolar Lavage Fluid

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A method is proposed to identify leukocyte subpopulations in bovine bronchoalveolar lavage fluid by dual-laser flow cytometry. The technique uses several parameters, i.e., exclusion of highly autofluorescent alveolar macrophages and inclusion of leukocytes on the basis of labeling by specific antibodies and light scatter characteristics.

Phenotyping of leukocyte subpopulations in bronchoalveolar lavage (BAL) fluid by flow cytometric analysis may be useful for characterization of pulmonary reactions; e.g., quantification of cellular adhesion molecules (CAM) on leukocytes reflects the migrating behavior and activation status of the cells (4, 14). Flow cytometric analysis of BAL fluid is, however, hampered by the high autofluorescence of alveolar macrophages (AM) and aspecific binding of fluorescent monoclonal antibodies (MAbs).

Several approaches have been described for the identification of leukocyte subpopulations in BAL fluid on the basis of light scatter characteristics, selection by one or more MAbs, or a combination of these. Procedures to identify neutrophils by flow cytometry have used similar techniques: light scatter profile characteristics (13) or differences in the expression of non-specific markers such as major histocompatibility complex class I or CD45 (7, 8, 13). Hitherto, techniques that have been used in the bovine system have been primarily aimed at detection and characterization of lymphocytes in BAL fluid.

The aim of this study was to establish a dual-laser flow cytometry technique that permits identification and subsequent phenotyping of leukocytes in bovine BAL fluid and that is applicable to healthy calves and calves with lower respiratory tract disease.

Animals. All animal procedures were carried out with the prior consent of the Faculty Ethical Review Committee of Utrecht University. BAL fluid samples were from Holstein Friesian calves, 6 to 15 weeks of age, kept at a farm in crates. Animals were identified as suffering from severe inflammation of the respiratory tract or as non-diseased on the basis of clinical examination results (i.e., respiratory rate and rectal temperature) as described earlier (15). BAL fluid samples were taken from 10 healthy calves and 10 with respiratory disease. The BAL procedure was performed on the standing, non-dated calves as described earlier (15). In pilot studies, radiological examination had ascertained reproducible positioning of the tube in seven healthy calves, confirming earlier findings (6).

Flow cytometer setup and data acquisition. BAL fluid cells were kept on melting ice, stained, and fixed in phosphate-buffered saline containing 1% formalin before analysis in a dual-laser flow cytometer (FACScalibur; Becton Dickinson, San Jose, Calif.). Viability (>95%) was determined by trypan blue exclusion. Staining was performed in buffer containing 25% bovine serum for blocking of non-specific binding. Unlike forward scatter (FSC)-side scatter (SSC) plots of peripheral blood leukocytes, BAL fluid cells did not show well-defined cell populations (Fig. 1) in samples of both pneumatic and non-pneumatic calves. However, in the lower left quadrant characteristically granulocytes and lymphocytes appeared, which was confirmed by application of the same settings to formalin-fixed peripheral blood leukocytes (data not shown). Broad neutrophil and lymphocyte gates in the light scatter plot were defined (Fig. 2 C1, C2, E1, and E2) and used in the procedure to identify neutrophils and lymphocytes in BAL fluid.

Since autofluorescence of AM in FL4 is low compared to that in FL1, FL2, and FL3 (7), FL3-FL4 plots were used to identify neutrophils with MAb IL-A110 (12), which was conjugated to biotin-X-NHS (Boehringer Mannheim, Mannheim, Germany) and visualized by streptavidin-allophycocyanin (PharMingen, San Diego, Calif.). Biotin-conjugated R73, specific for the rat T-cell receptor, functioned as an isotype control MAb (11). The FL3 signal was amplified such that high- and low-autofluorescence populations were discerned. The FL4 amplification was adjusted such that the low-autofluorescence population was present in the lower left corner (Fig. 2A1 and A2). The IL-A110 gate in the FL3-FL4 plot was set such that background staining with isotype control MAbs was minimal. IL-A110 (specific for neutrophils)-positive cells were identified on the basis of low autofluorescence in FL3, excluding the majority of AM, and positive staining for the identifying MAb in FL4 (Fig. 2B1 and B2). These IL-A110⁺ events were gated...
Identification of AM by selecting for autofluorescence was preferred to an AM-specific MAb because of aspecific binding of the MAb to other cell populations. In addition, as the FL4 channel was used for detection of a leukocyte-specific marker, the AM-specific marker was to be analyzed in FL1, FL2, or FL3, where autofluorescence hampered detection. Pilot studies had shown that fluorescence of AM, induced by a marker for CD14 such as VPM65 (3), was low compared to the background level (data not shown). Plotting autofluorescence against the isotype control-induced fluorescence in the FL3-FL4 plot showed an open area that was filled with events when MAbs were used that were specific for neutrophils or CD2⁺ lymphocytes. Correlation of these gated events to their respective FSC-SSC gates made the procedure highly selective and finally identified the neutrophil and lymphocyte populations.

The numbers of neutrophils and CD2⁺ lymphocytes were represented as percentages of the total number of events in the FL3-FL4 gate and the FSC-SSC gate for neutrophils and lymphocytes, respectively. The percentages of neutrophils in the FL3-FL4 gate were significantly lower than the percentages of lymphocytes in the FL3-FL4 gate (Table 1). Therefore, the final step in the isolation procedure, i.e., backgating of FL3-
FL4 positive events to the respective FSC-SSC gates, was more selective for neutrophils than it was for lymphocytes. Differences in binding affinity of the MAb specific for lymphocytes and neutrophils and differences in their cognate antigen expression may account for the specificity of the FL3-FL4 gate. In addition, it may be due to differences in autofluorescence, which is higher in granulocytes than in lymphocytes (7).

As leukocytes acquire an activated phenotype under inflammatory conditions, which may interfere with identification, the procedure was used both on BAL fluid from healthy calves and on BAL fluid from calves with respiratory disease. The percentages of finally identified neutrophils in the FSC-SSC of diseased calves increased compared to those in healthy calves, which were detected with a high but not significant probability ($P = 0.08$), while the percentages of lymphocytes in diseased and healthy calves were not different. Similar findings were reported for microscopic cell counts in either group (15), which suggests that microscopic and fluorescence-activated cell sorter analyses detect similar fluctuations in the leukocyte contents of BAL fluid.

**Phenotyping leukocytes.** To demonstrate the suitability of the method described for subsequent phenotyping, leukocytes in BAL fluid from a calf with respiratory disease were additionally labeled by MAbs, isolated from hybridoma culture supernatants, and conjugated to fluorescein isothiocyanate (FITC; Conjugation kit; Sigma, St. Louis, Mo.). To quantify CAM expression of neutrophils in BAL fluid, fluorescence was determined by FITC-labeled MAbs specific for CD18-dependent emigration in the lung. J. Immunol. 152:3095–3098.


9. Machugh, N. D., E. L. Taracha, and P. G. Toye. 1993. Reactivity of workshop FITC [1]) and CD8 (IL-A105-FITC [9]) (Fig. 3B). These additional labelings show that the procedure may be used to characterize leukocytes in BAL fluid.

In conclusion, the present report describes a method for the identification of leukocytes in bovine BAL fluid and subsequent phenotyping of the identified population by flow cytometry.

**REFERENCES**


