CHAPTER 30

Leucine Aminopeptidase Activity by Flow Cytometry

John J. Turek* and J. Paul Robinson†

*Department of Veterinary Anatomy
†Department of Physiology and Pharmacology
School of Veterinary Medicine
Purdue University
West Lafayette, Indiana 47907

I. Introduction

Macrophage activation or maturation is an important parameter in the study of cell-mediated immunity (Assreuy and Moncada, 1992; Buchmüller-Rouiller *et al.*, 1992; Fortier *et al.*, 1992; Hamilton *et al.*, 1991; Higginbotham *et al.*, 1992; Lim and Stewart, 1991; Michelacci and Petricevich, 1991; Murray, 1992; Nelson *et al.*, 1992; Oswald *et al.*, 1992; Singh and Sodhi, 1991; Valdez *et al.*, 1990). Some of the methods to assess macrophage activation include measurement of various enzyme levels (Morahan *et al.*, 1980), phagocytic ability (Abel *et al.*, 1989, 1991; Cannon and Swanson, 1992), chemotaxis (Litwin *et al.*, 1992), microbicidal activity (Fortier *et al.*, 1992; Kiderlen and Kaye, 1990; Murray, 1992), production of reactive oxygen intermediates (Decker, 1990; Johnson *et al.*, 1986), and nitric oxide production (Marotta *et al.*, 1992). One of

METHODS IN CELL BIOLOGY, VOL. 41

the enzymes associated with macrophage activation is leucine aminopeptidase (LAP). LAP is an ectoenzyme located in the outer cell membrane that is found in a variety of cells and tissues and appears to have higher levels of activity in phagocytic cells (Nagaoka and Yamashita, 1984). Measurement of LAP by methods other than flow cytometry has been used to assess macrophage or leukocyte activation/differentiation in a variety of studies (Dempsey *et al.*, 1988; Johnson *et al.*, 1986; Morahan *et al.*, 1980; Nagaoka and Yamashita, 1984; Volkman *et al.*, 1983).

A flow cytometric assay to stain for LAP activity was originally described by Dolbeare and Smith (1977) and the methods included here are essentially those of Haskill and Becker (1982). The flow cytometric assay has been used to study macrophage heterogeneity (Haskill and Becker, 1982; Becker *et al.*, 1983), the effects of interferon on monocyte and macrophage development (Becker, 1984), activation states of tumor-associated macrophages (Mahoney *et al.*, 1983), and the effects of dietary fatty acids on peritoneal macrophage activation (Turek *et al.*, 1991). The advantage of the assay is that it requires a minimum of preparation time and may be performed very rapidly. Further, the assay provides accurate information related to the activation status of macrophages, despite the heterogeneous nature of these cells, particularly when isolated from the lung or peritoneal cavity. An additional advantage is that the use of UV light excitation reduces the very real problem of autofluorescence levels associated with macrophages.

II. Application

This assay is primarily used to assess monocyte or macrophage activation, but it may also be used for other cell types. It is a simple analytical assay that is very reproducible if the conditions under Section V are followed.

III. Materials

Material	Supplier	Location
Leucine 4-methoxy-2-napthylamine	Enzyme systems products	Livermore, CA
5-Nitrosalicylaldehyde	Kodak Chemical	Rochester, NY
2-[N-morpholino]ethanesulfonic acid	ICN Biochemicals	Cleveland, OH
Ficoll (Histopaque-1077)	Sigma Chemical	St. Louis, MO

IV. Methods

1. Cell preparation: Alveolar and peritoneal macrophages and peripheral blood monocytes may all be assayed. Heparinized blood will require separation

of the monocytes using a Ficoll gradient. Lung and peritoneal macrophages should be collected by lavage with calcium and magnesium-free Hanks' balanced salt solution. The cells from peritoneal and lung lavage solutions often do not require separation on a Ficoll gradient, but gradient separation may be performed if the samples contain excessive debris. All cells should be washed twice with cold 0.15 *M* 2-[*N*-morpholino]ethane sulfonic acid (MES) buffer (pH 6.5) and adjusted to 2×10^6 cells/ml prior to the procedures listed below.

2. Prepare a 0.15 *M* solution of leucine 4-methoxy-2-napthylamine by dissolving 1 mg of this compound in 20 μl of *N,N*-dimethylformamide.

3. Prepare a 0.5 *M* solution of 5-nitrosalicylaldehyde by dissolving 1.67 mg of this compound in 20 μl of *N,N*-dimethylformamide.

4. Add 5 ml of 0.15 *M* MES buffer (pH 6.5) to 20 μl of each of the above (2 and 3).

5. Mix the two reagents 1 : 1 and add 1 ml of cells ($\sim 2 \times 10^6$) to 1 ml of the reagent mixture.

6. Incubate for 10–25 min. at 37°C.

7. Wash the cells one time in cold sodium acetate buffer (0.1 *M*, pH 5.2) containing 0.02% Triton X-100 and resuspend the cells in cold sodium acetate buffer without Triton X-100. The cells should be kept on ice and assayed within 2 hr. The cells may be assayed cold or warmed to room temperature just prior to analysis. Collect data on 20,000 to 50,000 cells.

V. Critical Aspects of the Procedure

For reproducibility of data, the instrument operating parameters, reagent concentrations, and cell incubation time need to be rigorously controlled. Since such small amounts of leucine 4-methoxy-2-napthylamine and 5-nitrosalicylaldehyde are required, it is best to weigh out more than is needed, add an amount of *N,N*-dimethylformamide that would be equivalent to the above concentrations, and then use 20 μl of that solution to prepare the staining reagents. Incubation time is also crucial. Longer incubation times (e.g., 25 min) will produce an increase in fluorescence and may decrease the sensitivity of the assay to detect small differences between cell populations. If freshly isolated cells are frozen and stored in liquid nitrogen using methods for preserving tissue culture cells, LAP activity may also be assayed after thawing of cells, but there is a slight decrease in overall LAP staining.

VI. Controls

Unstained cells that have been washed and incubated in the different buffers should be used to determine endogenous fluorescence levels.

VII. Instruments

A flow cytometer with a UV laser is required. The assay should be run with 352–363 nm excitation and an emission band pass filter at 525 nm. When the assay is first set up, the laser wattage and photomultiplier tube settings should be adjusted so that the endogenous fluorescence of control cells falls within the first decade of the log signal. Cells stained for LAP will then typically fall within the second and third decade of the log signal. These settings should remain the same for all samples to be compared.

VIII. Results

Figures 1 and 2 demonstrate LAP staining from 50,000 cells contained in lavage fluid from porcine lung. The ellipse in Fig. 1 denotes the gate chosen for analysis of a macrophage population. It is important that identical forward angle light scatter and 90° light scatter (90LS) gating be used to compare different samples as larger macrophages will be brighter due to a greater cell surface area. Figure 3 represents the log fluorescence signal due to LAP staining from

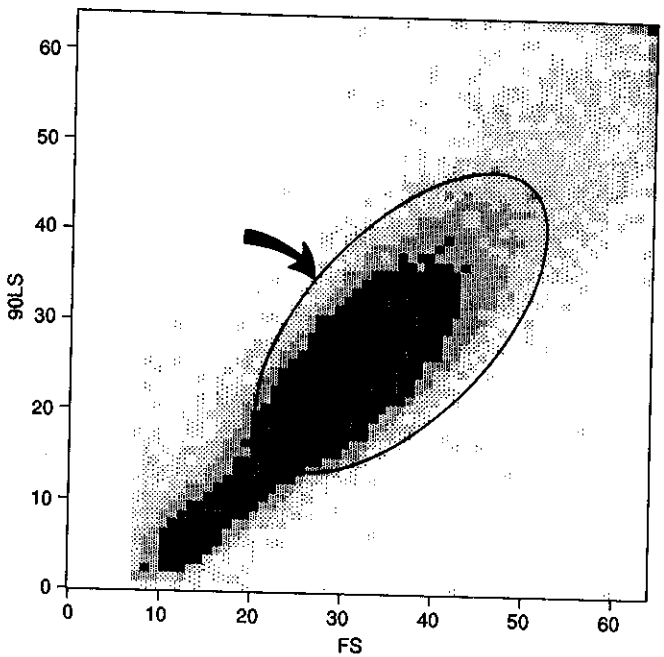

Fig. 1 Fluorescence resulting from staining for LAP in porcine alveolar cells. The ellipse (curved arrow) indicates the gating used for further analysis.

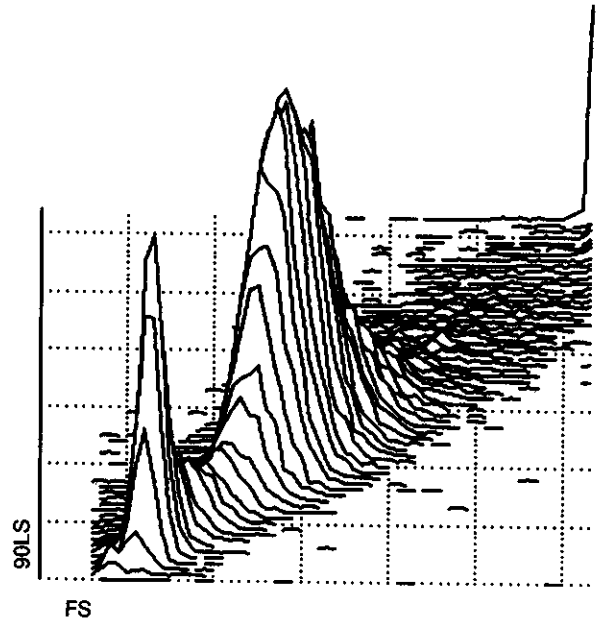

Fig. 2 Three-dimensional representation of the LAP staining of the entire cell population in Fig. 1.

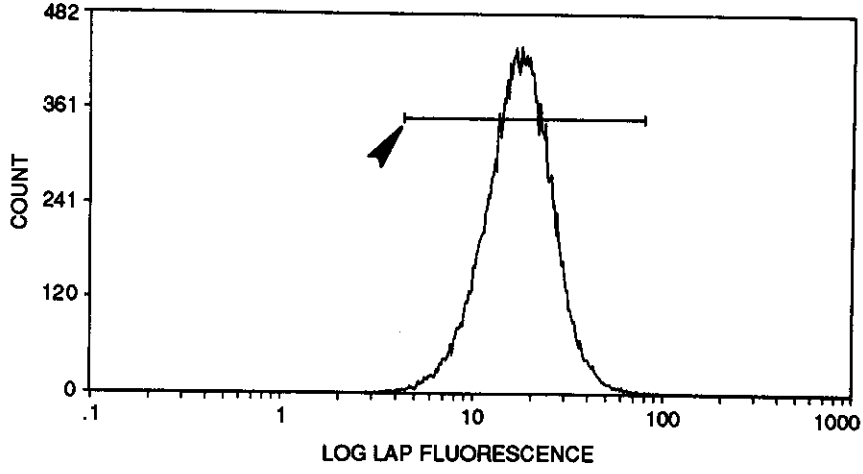

Fig. 3 Histogram of the log fluorescence due to LAP staining from the gated population in Fig. 1. The gate (arrowhead) is used to determine the linear equivalent of the mean log fluorescence channel.

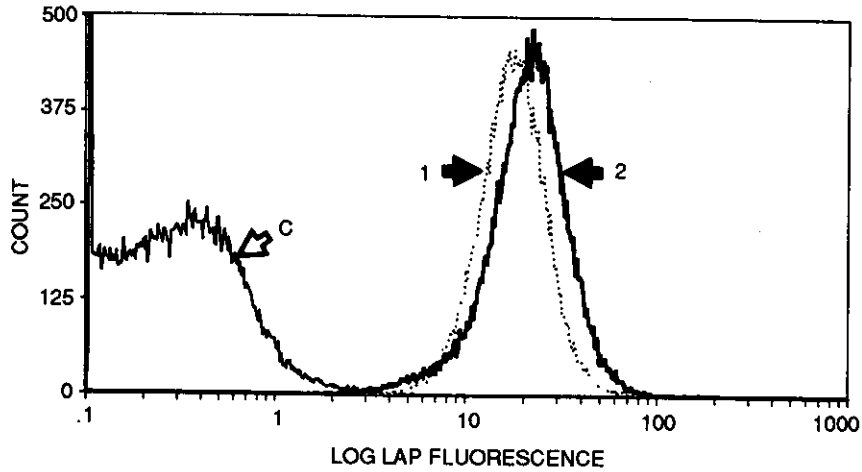

Fig. 4 Composite histogram showing endogenous fluorescence for a control sample (C) and from samples stained for LAP (1, 2). The linear equivalent of the mean log fluorescence channel is 17.8 for sample 1 and 22.9 for sample 2.

the gated population in Fig. 1. To determine the degree of fluorescence, a gate extending from base to base of the peak is used to determine the linear equivalent of the mean channel for the log fluorescence signal (Fig. 3). This gate will need to be centered over the peak for each sample. Figure 4 represents an overlay of the log fluorescence signals from a control sample (endogenous fluorescence) and samples from two different animals. The mean linear equivalent of the log channel difference of these two LAP-stained samples in Fig. 4 is typical of the results with this assay.

References

Abel, G., Szöllösi, J., Chihara, G., and Fachet, J. (1989). *Int. J. Immunopharmacol.* **11,** 615–621.
Abel, G., Szöllösi, J., and Fachet, J. (1991). *Eur. J. Immunogenet.* **18,** 239–245.
Assreuy, J., and Moncada, S. (1992). *Br. J. Pharmacol.* **107,** 317–321.
Becker, S. (1984). *Cell. Immunol.* **84,** 145–153.
Becker, S., Halme, J., and Haskill, S. (1983). *J. Reticuloendothel. Soc.* **33,** 127–138.
Buchmüller-Rouiller, Y., Corradin, S. B., and Mauël, J. (1992). *Biochem. J.* **284,** 387–392.
Cannon, G. J., and Swanson, J. A. (1992). *J. Cell Sci.* **101,** 907–913.
Decker, K. (1990). *Adv. Exp. Med. Biol.* **283,** 507–520.
Dempsey, W. L., Hwu, P., Russell, D. H., and Morahan, P. S. (1988). *Life Sci.* **42,** 2019–2027.
Dolbeare, F. A., and Smith, R. E. (1977). *Clin. Chem. (Winston-Salem, N.C.)* **23,** 1485–1491.
Fortier, A. H., Polsinelli, T., Green, S. J., and Nacy, C. A. (1992). *Infect. Immun.* **60,** 817–825.
Hamilton, J. A., Vairo, G., Knight, K. R., and Cocks, B. G. (1991). *Blood* **77,** 616–627.
Haskill, S., and Becker, S. (1982). *J. Reticuloendothel. Soc.* **32,** 273–285.
Higginbotham, J. N., Lin, T. L., and Pruett, S. B. (1992). *Clin. Exp. Immunol.* **88,** 492–498.
Johnson, W. J., DiMartino, M. J., and Hanna, N. (1986). *Cell. Immunol.* **103,** 54–64.

Kiderlen, A. F., and Kaye, P. M. (1990). *J. Immunol. Methods* **127,** 11–18.
Lim, W. H., and Stewart, A. G. (1991). *Int. Arch. Allergy Appl. Immunol.* **95,** 77–85.
Litwin, D. K., Wilson, A. K., and Said, S. I. (1992). *Regul. Pept.* **40,** 63–74.
Mahoney, K. H., Fulton, A. M., and Heppner, G. H. (1983). *J. Immunol.* **131,** 2079–2085.
Marotta, P., Sautebin, L., and Di Rosa, M. (1992). *Br. J. Pharmacol.* **107,** 640–641.
Michelacci, Y. M., and Petricevich, V. L. (1991). *Comp. Biochem. Physiol. B* **100B,** 617–625.
Morahan, P. S., Edelson, P. J., and Gass, K. (1980). *J. Immunol.* **125,** 1312–1317.
Murray, H. W. (1992). *J. Interferon Res.* **12,** 319–322.
Nagaoka, I., and Yamashita, T. (1984). *Comp. Biochem. Physiol. B* **79,** 147–151.
Nelson, B. J., Belosevic, M., Green, S. J., Turpin, and Nacy, C. A. (1992). *Adv. Exp. Med. Biol.* **319,** 77–88.
Oswald, I. P., Afroun, S., Bray, D., Petit, J.-F., and Lemaire, G. (1992). *J. Leukocyte Biol.* **52,** 315–322.
Singh, R. K., and Sodhi, A. (1991). *Immunol. Lett.* **28,** 127–134.
Turek, J. J., Schoenlein, I. A., and Bottoms, G. D. (1991). *Prostaglandins, Leukotrienes Essent. Fatty Acids* **43,** 141–149.
Valdez, J. C., De Alderete, N., Meson, O. E., Sirena, A., and Perdignon, G. (1990). *Immunobiology* **181,** 276–287.
Volkman, A., Chang, N. C., Strausbauch, P. H., and Morahan, P. S. (1983). *Lab. Invest.* **49,** 291–298.