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Isolation and characterization of bovine mammary endothelial cells

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Summary. The isolation and maintenance in culture of bovine mammary gland endothelial cells is described. The uptake of acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate was used to purify the endothelial cells by fluorescence-activated cell sorting. The morphology of the purified cells is similar to that of endothelial cells from other species. The isolation of bovine mammary endothelial cells is potentially significant to study the role of the endothelium during inflammatory responses.

Key words: Bovine, Cell culture, Endothelial, Mammary

1. Introduction

Vascular endothelial cells line the blood vessels and contribute to many biological processes such as hematosis, coagulation, trafficking of lymphocytes, and the inflammatory response. The endothelial cell is of primary importance in the extravasation of leukocytes from the circulation into sites of tissue inflammation. In the case of mastitis, the magnitude and duration of inflammation can be related to such parameters as endothelial adhesion molecule expression, cytokine secretion, as well as, changes in vascular permeability. As such, these cells play an important role in the study of mastitis and associated mammary gland immunopathology.

Endothelial cells have been cultured successfully by collagenase profusion from bovine aorta and human mammary adipose tissue by UEA-1 selection [1]. However, methods of bovine mammary endothelial cell (BMEC) isolation and culture have not been described. The objective of this study was to establish a simple and reliable method for the isolation of BMEC. Mammary tissue was digested in a collagenase-dispase solution and filtered through a nylon mesh. The cell isolates took up acetylated low-density lipoprotein, which identified them as those of endothelial origin and allowed their purification using a fluorescence activated cell sorter. The endothelial origin of these cells was further confirmed by the positive staining of von Willebrand factor and fluorescent microscopy. The technique we developed is uncomplicated and has consistently yielded a pure population of endothelial cells.

The isolation of endothelial cells from bovine mammary tissue and maintenance in culture will be useful in studies of mastitis pathogenesis. An in vitro culture system for these cells is imperative to study how the endothelial cells regulate the entrance of leukocytes into mammary tissue during mastitis. A reliable and reproducible model system for mammary gland inflammatory responses will enable researchers to focus on the mechanisms of leukocyte regression while eliminating the myriad of variants inherent to in vivo systems.

2. Materials

A. Equipment
1. Laminar flow hood, model SG-600[1]
2. CO_2 incubator, model 3326[2]
3. 37 °C water bath, model 25[3]
4. Benchtop refrigerated centrifuge, model GS-6R[4]
5. EPICS 753 flow cytometer[5]
6. Surgical blades, No. 10[6]
7. Universal scissors, No. 08-951-30, stainless steel[6]
8. Operating scissors, straight blade, No. 13-806-2, sterile, stainless steel[6]
9. Delicate scissors, No. 08-940, sterile, stainless steel[6]
10. Dressing forceps, No. 13-812-39, sterile, stainless steel[6]
11. 3-channel timer, No. 06-662-5[6]
12. 210 micrometer nylon mesh, No. K-CMN-210[7]
13. 30 micrometer nylon mesh, No. K-CMN-30[7]
14. Fluorescence microscope, model Ortholux[8]
15. Inverted microscope, model TMS[9]
16. Dissecting pins, No. 09-002-18[6]

B. Cultureware
1. Centrifuge tube, conical, 15-ml, No. 25319-15[10]

2. Tissue culture flask, 25-cm^2, No. 3055[11]
3. Tissue culture slides, 2-chambered, No. 9384-V52[12]
4. Cover glass, 22 × 30 mm, No. 12-548-5A[6]
5. Cryotubes, NUNC, No. 363401[12]
7. Two-chambered slides, No. 177380[12]
8. 100 mm petri dish, No. 3100[11]

C. Media
1. Minimum Essential Medium Eagle (MEM), liquid, No. M7647[13]
2. Fetal bovine serum, A-111-L[14]
3. Penicillin - Streptomycin solution, No. P0781[13]
4. L-glutamine, No. G7513[13]
5. Fungizone, No. 600-5295AE[15]
6. Collagenase, No. C9407[13]
7. Dispase, No. 40235[16]
8. Heparin, No. H3125[13]
9. Phosphate buffered saline (PBS) (modified), No. D4031[15]
10. Trypsin-EDTA, No. T5775[13]
11. Dil-Ac-LDL, No. BT-902[17]
12. Bovine serum albumin (BSA), No. 670-5260AG[15]
13. von Willebrand factor antiserum, No. A082[18]
14. Triton-X, No. X-100[13]
15. FITC goat anti-rabbit, No. 62-6111[19]
16. Normal rabbit serum, No. R4505[13]
17. Glycerol, No. G7757[13]
18. Formaldehyde, No. F165[13]
19. Paraplast, No. 6761-E75[12]

3. Procedures

A. Preparation of culture medium and solutions
1. Prepare culture medium as follows: to 439.5 ml MEM add the following to give a final concentration of: 50 ml FBS (10%), 5 ml penicillin (100 U/ml)/streptomycin (100 µg/ml), 5 ml of 200 mM L-glutamine (2 mM), and 0.5 ml fungizone (0.1%).
2. Prepare 1X Kreb's Ringer bicarbonate as follows: to 1000 ml of deionized water add 0.1 g magnesium chloride, 7.0 g sodium chloride, 0.1 g sodium phosphate dibasic (anhydrous), 0.18 g sodium phosphate monobasic (anhydrous), 1.26 g sodium bicarbonate, and 0.54 g D-glucose (3 mM). Filter to sterilize.
3. Prepare collagenase stock solution as follows: to 50 ml of 1X Kreb's Ringer bicarbonate add 100 mg of collagenase and 2 ml of 7.5% BSA.
4. Prepare collagenase-dispase solution by adding 4 ml of dispase solution to 4 ml of the collagenase stock solution.
5. Prepare a solution of 500 U of heparin/ml of media.
6. Prepare Dil-Ac-LDL labeling solution by diluting the stock solution (200 µg/ml) 1:20 with MEM.

B. Isolation of bovine mammary endothelial cells
1. Primary cultures of bovine mammary endothelia cells are prepared by using a modification of Kern's methods [2]. Obtain abattoir samples of the supramammary artery.
2. Place the artery in a petri dish containing pre-warmed (37 °C) PBS, then remove blood clots, fat and connective tissue aseptically.
3. Cut the artery lengthwise with scissors to expose the interior surface to a collagenase-dispase solution.
4. Pin the artery open in a paraffin-filled petri dish with dissecting pins, then add 8 ml of the collagenase-dispase solution.
5. Scrape the interior surface of the artery with a number 10 scalpel blade every 10 min for a total of 30 min.
6. Transfer the collagenase-dispase cell suspension to a 15 ml centrifuge tube containing 8 ml of MEM, 10% Hyclone FBS and 500 units/ml of heparin [3].
7. Centrifuge the tube at 1000 rpm for 10 min at room temperature, discard the supernatant and resuspend the cell pellet in 10 ml of culture medium.
8. Filter the resuspended pellet through 210 micrometer nylon mesh and reserve the filtrate.
   a) Refilter the filtrate through the 30 micrometer nylon mesh.
   b) Remove and collect cell aggregates by washing with culture medium into a sterile 50 ml beaker.
9. Plate the cell aggregates in a 25 cm^2 flask and incubate in a 37 °C, 5% CO_2 incubator.
10. Replace the culture medium every 3 days. Subculture the cells when the monolayer becomes confluent in about 10 to 11 days.
11. Evaluate the growth of endothelial cells from aggregates daily by phase contrast microscopy.
12. To subculture the cells, remove the medium and add 4 ml of trypsin-EDTA. Incubate the flask at 37 °C for 5 min. When the cells are detached from the flask, add 5 ml of culture medium and transfer the cell suspension to a 15 ml centrifuge tube. Pellet the cells by centrifugation at 1000 rpm for 5 min, discard the supernatant, and resuspend the cell pellet in 15 ml of culture medium.

C. Labeling cells with Dil-Ac-LDL for fluorescence microscopy
1. Culture cells on two-chambered slides until confluent.
2. Add 10 µg/ml Dil-Ac-LDL (acetylated low-density lipoprotein labeled with 1,1′-

dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) in MEM and incubate for 4 hours at 37 °C.
3. Remove media containing Dil-Ac-LDL and wash the cells with MEM.
4. Slides are mounted with FA mounting fluid (10 ml glycerol and 8 ml PBS). Slides of bovine aortic endothelial cells are included as positive control and chicken smooth muscle cells or fibroblasts should be included as negative controls.
5. Examine the cells for fluorescence using an epifluorescence microscope equipped with a rhodamine excitation-emission filter.
6. Bovine mammary endothelial cells demonstrate fluorescent deposits in their cytoplasm similar to that of bovine aortic endothelial cells. The avian smooth muscle cells or fibroblasts are not stained with the Dil-Ac-LDL labelling solution.

D. Labeling cells with Dil-Ac-LDL for fluorescence-activated cell sorting
1. Incubate the 25 cm^2 flask of bovine mammary endothelial cells with 3 ml of Dil-Ac-LDL for 4 hours at 37 °C.
2. Remove the Dil-AC-LDL and wash the cells with MEM.
3. Add 1 ml trypsin-EDTA to the cells and incubate the flasks at 37 °C for 5 min. When the cells detach from the flask, add 5 ml of culture medium to flask.
4. Transfer cells and medium to a 15 ml centrifuge tube and pellet the cells by centrifugation at 800 rpm for 5 min.
5. Discard the supernatant and resuspend cell pellet in 0.3 ml of culture medium.
6. Place cells on ice until analysis and sorting procedure are performed. Sorting should be performed immediately and not longer than 30 min after trypsin treatment.

E. Fluorescence-activated cell sorting
Measurements are made on an EPICS 753 flow cytometer[5] equipped with two argon ion lasers. Filters used for this procedure are: a 514 nm laser blocking filter, a 550 long pass filter. The laser is tuned to 514 nm at 100 mW of power and the high voltage is typically set at 900 volts.
1. Collect cells with fluorescent emission at 575 nm into a 15 ml centrifuge tube or directly into tissue culture plates containing culture media.
2. Collect cells into a 15 ml centrifuge tube, then pellet cells by centrifugation and plate into 25 cm^2 flasks.
(During the first analysis and sorting, cells collected from the right of the gate (Figure 3) will contain more endothelial cell aggregates than cells retrieved from the left gate).
3. At confluency, label cells with Dil-Ac-LDL and repeat the sorting procedure. The cells are sorted twice to obtain a pure culture of endothelial cells [4].

F. Staining with antisera to human von Willebrand factor
1. Prepare two-chambered slides containing bovine mammary endothelial or bovine aortic endothelial cells.
2. Aspirate medium from slide.
3. Fix cells by placing slides in a coplin jar containing 3.7% formaldehyde made in PBS. Incubate for 20 min at room temperature.
4. Remove excess formaldehyde by blotting.
5. Permeabilize cells by immersing slides in 0.5% Triton X-100 in PBS for 15 min at room temperature.
6. Remove excess Triton-X by blotting. Add von Willebrand factor antiserum at 1:40 dilution in PBS to one chamber of the slide. Use a 1:40 dilution of normal rabbit serum as a serum control and add to the other chamber.
7. Incubate slides in a humid chamber (box containing wet paper toweling) for 30 min, at 37 °C.
8. Wash slides in PBS (i.e. 3 min each change). Repeat three times.
9. Incubate slides with a 1:20 dilution of FITC goat anti-rabbit antibody in PBS for 30 min at 37 °C.
10. Wash slides in PBS for 10 min, repeat three times.
11. Blot excess PBS and mount with FA mounting fluid (1:1, glycerol:PBS).
12. Examine using fluorescent microscope equipped with a rhodamine filter.

4. Discussion

This paper has described a simple and reliable procedure for the isolation and characterization of BMEC from large blood vessels. In our initial attempts at isolating BMEC, tissue samples were collected from microvessels of the mammary gland. Briefly, the samples from freshly slaughtered animals were finely minced aseptically with scissors, digested in a collagenase-dispase solution, and filtered through a nylon mesh. This isolation procedure was time consuming, yielded a relatively low number of cells, and was often limited by overgrowth of contaminating cells such as fibroblasts, periocytes, and smooth muscle.

Previous researchers also encountered difficulty with contamination when tissue samples were extracted from the microvasculature [5]. Consequently, an additional attempt was made to isolate cells from larger vessels, namely the supramammary artery [5]. BMEC isolated from the supramammary artery as

described by the preceding method yielded an almost pure culture. The cells initially isolated grew to confluence within 10–14 days and demonstrated cobblestone morphology characteristic of endothelial cell density inhibition (Figure 1). The rate of proliferation in vitro was increased by raising the culture media serum level from 10% to 20%. These BMEC have been passed 5 times thus far without any signs of transformation; for, there have been no morphological changes or alteration in function as demonstrated by a panel of conventional endothelial markers. That the endothelial cell retains at least some of its characteristics in culture is imperative to its use to study many aspects of vascular biology and pathophysiology [1].

Isolated cells were determined to be of endothelial origin by the expression of von Willebrand factor and on the basis of Dil-Ac-LDL uptake, both classical endothelial markers [1]. We examined the use of rabbit antisera to human von Willebrand factor as a specific stain for endothelial cells through immunofluorescence microscopy from which we observed a positive reaction (Figure 2). Von Willebrand factor caused the cells to display intense granular perinuclear immunofluorescence [1]. The uptake of Dil-Ac-LDL by tissue cultured bovine mammary endothelial cells was displayed in a typical granular pattern of fluorescence in their cytoplasm when examined by an epifluorescence microscope equipped with a rhodamine excitation-emission filter (Figure 3). This staining pattern was due to the accumulation of acetylated lipoprotein in secondary lysosomes [1].

To reduce fibroblast contamination of these cultures, endothelial cells were further purified by FACS using Dil-Ac-LDL as a stain. A typical histogram of the fluorescence and size of these cells as analyzed by FACS is shown (Figure 4). Vascular smooth muscle cells and fibroblasts of bovine do not take up Dil-Ac-LDL and are used as negative controls [6]. The cells are sorted twice to obtain a pure culture. A histogram from the second subculture stained with Dil-Ac-LDL indicates the homogeneity of the cells (Figure 5). The use of Dil-Ac-LDL was further advantageous because the stain is maintained by the cells for a prolonged period and the staining procedure does not affect cell viability [7]. Staining with α-Naphthyl Acetate Esterase confirmed that there was no further contamination with monocytes, macrophages, or histocytes (data not shown).

Cell morphology was shown in the cobblestone appearance, the presence of von Willebrand factor, and the capacity to take up Dil-Ac-LDL establishes the identity of the isolated cells as endothelial. In general, there is considerable heterogeneity of the

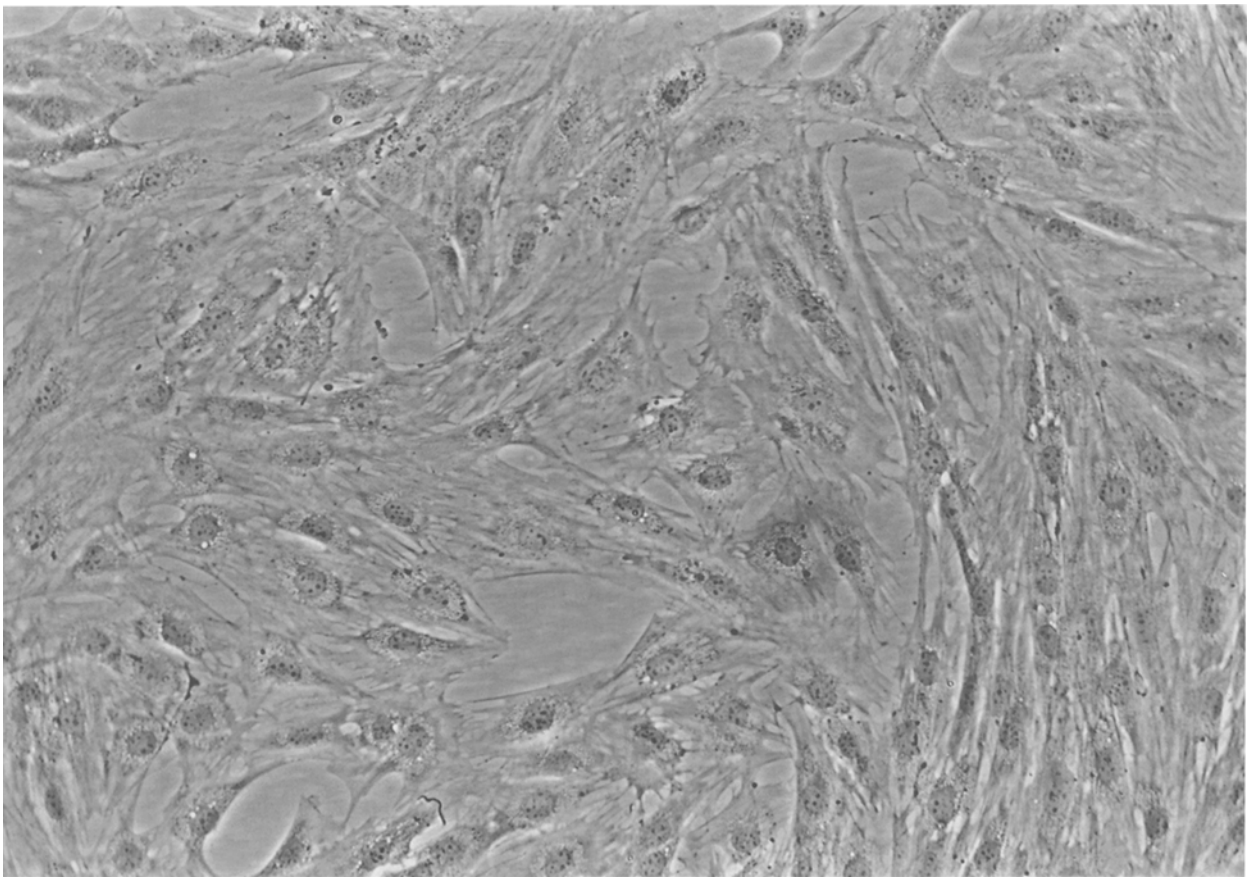

Figure 1. Bovine mammary endothelial cells 12 days after isolation showing the characteristic appearance of cells in a confluent monolayer. ×10

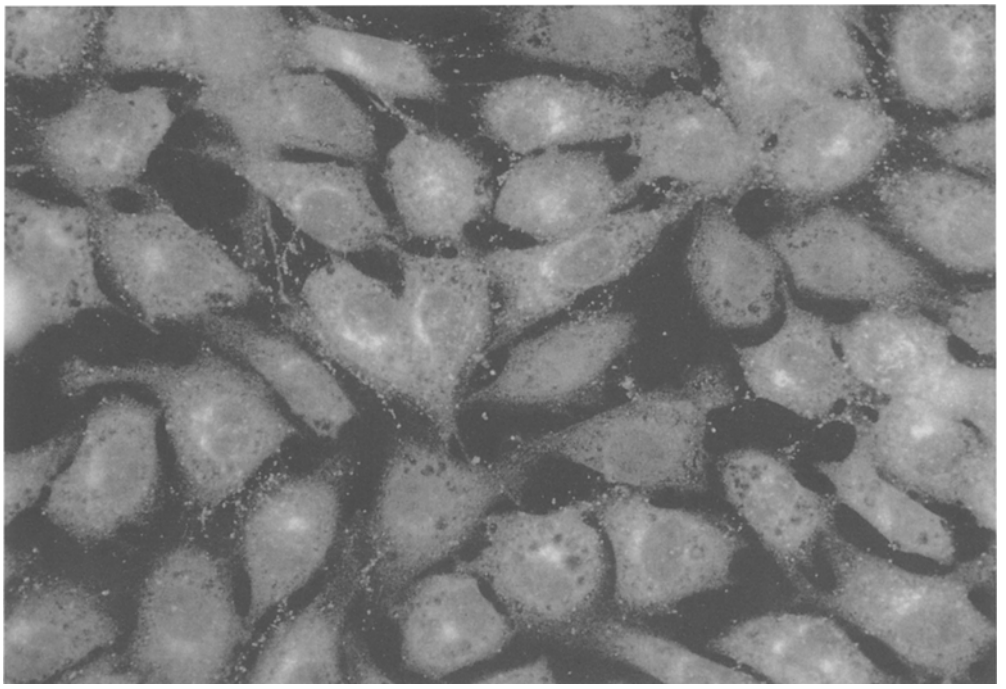

Figure 2. Photomicrograph of BMEC positively stained with von Willebrand factor showing granular immunofluorescence in the perinuclear. ×300

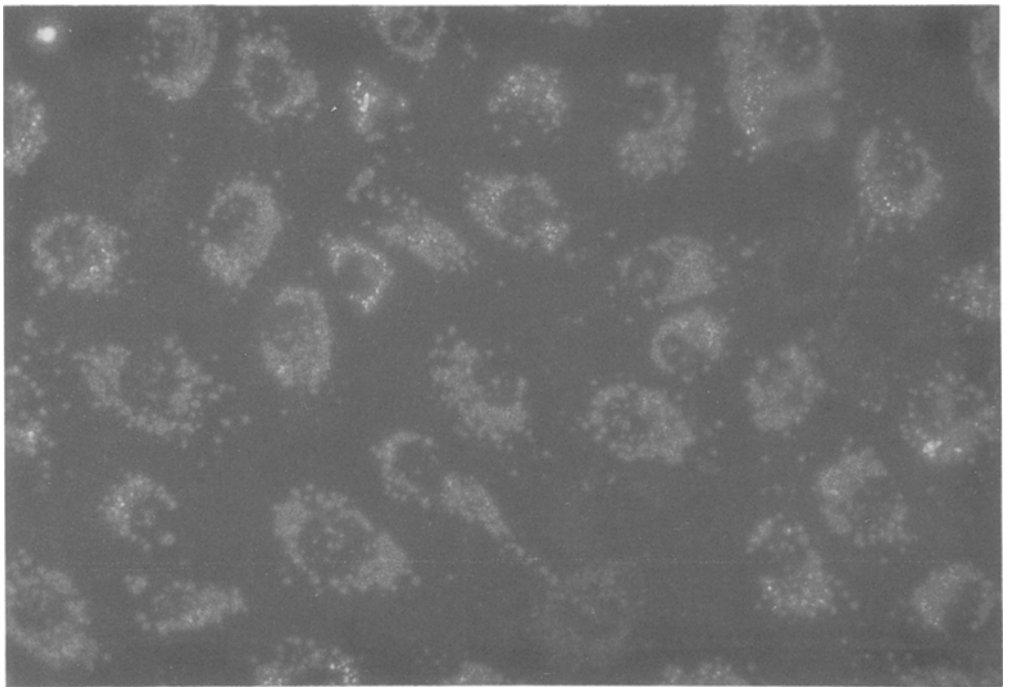

Figure 3. Photomicrograph of bovine mammary endothelial cells labeled with Dil-Ac-LDL showing the characteristic cytoplasmic fluorescence. ×300

vascular endothelium. Differences in structure, function, antigenic composition, metabolic properties, and response to growth factors can be seen in endothelial cells within the vasculature and between organs [5]. In order to effectively investigate the underlying mechanisms of host inflammatory responses, it is essential to use endothelial cells from the target organ of interest. The described in vitro culture system for BMEC will facilitate the study of leukocyte trafficking mechanisms into the bovine mammary gland during mastitis.

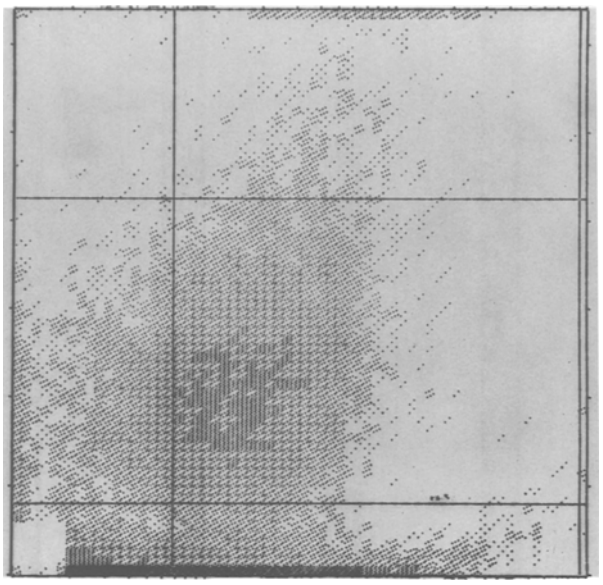

Figure 4. Histogram of bovine mammary endothelial cells stained with Dil-Ac-LDL and analyzed by FACS. Histogram indicates a population of cells with similar size and fluorescence.

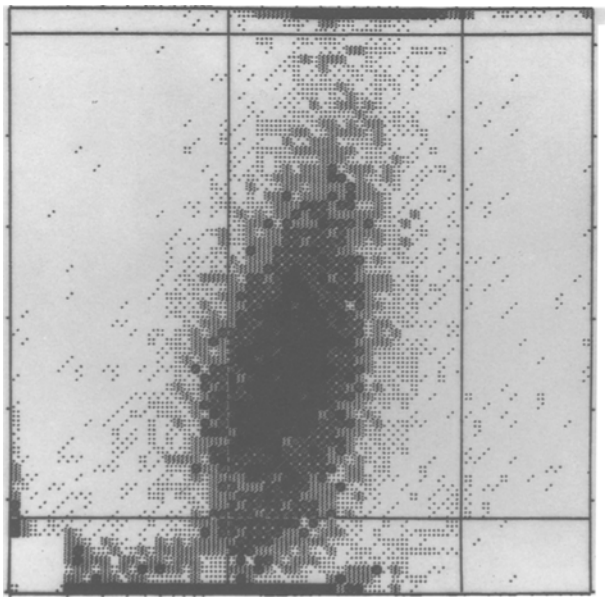

Figure 5. Histogram of bovine mammary endothelial cells from the second subculture stained with Dil-Ac-LDL and analyzed by FACS indicating the homogeneity of the cells.

Acknowledgements

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Notes on suppliers

1. Baker Company Inc., Sanford, ME, USA
2. Forma Scientific Inc., Marietta, OH, USA
3. Precision Scientific, Chicago, IL, USA
4. Beckman, Fullerton, CA, USA
5. Coulter Electronics, Hileah, FL, USA
6. Fisher Scientific, Pittsburgh, PA, USA
7. Small Parts Inc., Miami Lakes, FL, USA
8. Leitz, Overland Park, KS, USA
9. Nikon, Melville, NY, USA
10. Corning Inc., Corning, NY, USA
11. Costar, Cambridge, MA, USA
12. Falcon, Thomas Scientific, Swedsboro, NJ, USA
13. Sigma Chemical Company, St. Louis, MO, USA
14. Hyclone, Logan, UT, USA
15. Gibco Laboratories, Grand Island, NY, USA
16. Collaborative Research, Bedford, MA, USA
17. Biomedical Technologies Inc., Stoughton, MA, USA
18. Dako Corporation, Carpinteria, CA, USA
19. Zymed Laboratories Inc., South San Francisco, CA, USA

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