Modification of the Recalde method for the isolation of human monocytes[1]

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Summary A modification of the method for monocyte isolation reported by Recalde (1984. *J. Immunol. Methods.* **69:** 71–77) is described. Application of the modified method to 36 consecutive healthy adult donors gave a monocyte purity of 73 ± 8% monocytes with less than 1% polymorphonuclear leukocytes and a yield of 3.44 ± 0.93 × 10^5 monocytes/ml blood. While the monocyte purity of the modified Recalde method was lower than that obtained by elutriation (method BB in Fogelman et al. 1981. *J. Lipid Res.* **22:** 1131–1141) in 15 donors (71 ± 10% vs. 83 ± 6%) the monocyte yield and the viability of the cells before and after culture were similar in both methods. The modified Recalde method does not require the expensive or complicated equipment required for elutriation and permits the isolation of human monocytes for culture in autologous serum from multiple donors in a single day.—**Fogelman, A. M., F. Elahi, K. Sykes, B. J. Van Lenten, M. C. Territo, and J. A. Berliner.** Modification of the Recalde method for the isolation of human monocytes. *J. Lipid Res.* 1988. **29:** 1243–1247.

Supplementary key words elutriation • human monocyte-macrophages

Lipid metabolism in monocyte-macrophages has been an area of intensive study in recent years (1). However, the isolation of large numbers of human monocytes has remained difficult. In 1984 Recalde (2) reported a simple ingenious method for isolating monocytes from buffy coats. The technique is based on the ability of monocytes to resist a change in density in the presence of high salt concentrations as compared to other leukocytes that rapidly become more dense under such conditions. Unfortunately, in our hands the technique yielded fewer monocytes than reported and upon being placed in culture the protein content of the resulting macrophages was substantially less than that seen with monocytes prepared by elutriation (3, 4). We report here a modification of the technique that produces human monocytes in yields comparable to elutriation (3, 4) and with comparable cell viability before and after culture.

MATERIALS AND METHODS

Collection of blood

After obtaining written permission, 500 ml of blood was drawn through a 19-guage E-Z Set infusion set (Cat. No. 38-9026-01, Deseret Pharmaceutical Co., Sandy, UT) from 36 consecutive healthy adult donors. The blood was col-

Abbreviations: CPD, citrate phosphate dextrose solution; Na_2 EDTA, ethylenedinitrilotetraacetic acid disodium salt; LDL, low density lipoprotein; MDA-LDL, malondialdehyde-altered low density lipoprotein; PBS, Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}.

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lected into plastic syringes that were prepared the night before and kept sterile in the refrigerator. Initially five 60-ml syringes, each containing 50 units of heparin sodium injection USP, were collected and 30-ml aliquots were transferred to 40-ml conical, graduated, siliconized glass centrifuge tubes each containing 150 units of heparin. After collecting the heparinized blood, an additional blood sample was obtained in a syringe containing 8.5 ml of citrate phosphate dextrose solution (CPD) and filled to the 60 ml mark. The ratio of blood to CPD was 6:1 (v/v). The CPD solution contained trisodium citrate (89.4 mM), citric acid (17 mM), dextrose (129 mM), and monobasic sodium phosphate (16.1 mM) at pH 7.4. A further 148.5 ml of blood without anticoagulant was collected and dispensed to glass centrifuge tubes for the preparation of autologous serum.

All manipulations of blood and cells were sterile, and precautions to protect laboratory workers included appropriate gloves and the use of a biohazard hood.

Preparation of leukocytes

Each 30 ml of the heparinized blood was taken up into 60-ml plastic syringes each containing 10 ml Plasmagel (Laboratoire Roger Bellon, Neuilly, France). After mixing, the syringes rested on their plungers for 30 to 45 min at ambient temperature in order to sediment the red cells (5). The supernatants were collected into 50-ml plastic centrifuge tubes in ice by depressing the syringe barrels and expressing the supernatants through 19-gauge E-Z Set infusion sets (Cat. No. 38-5316 Deseret Pharmaceutical Co.) as previously described (5). The tubes were centrifuged at 200 *g* for 10 min at 5°C and the platelet-enriched supernatant was discarded. Using Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS) containing 1.1 mM ethylenedinitrilotetraacetic acid disodium salt (Na_2 EDTA) at pH 7.4, the cell pellets were combined into a single tube and half was dispensed into each of two 50-ml plastic centrifuge tubes which were filled to 40 ml, and centrifuged at 600 *g* for 10 min at 5°C. The supernatant was carefully removed and discarded; the cell pellets were resuspended in 40 ml of PBS containing 1.1 mM Na_2 EDTA at pH 7.4 and centrifuged at 600 *g* for 10 min at 5°C. The supernatant was carefully and completely removed and discarded; the cell pellets were resuspended in CPD plasma to give a total volume of 10 ml in each of the two tubes.

Preparation of CPD plasma

The CPD plasma was prepared while the leukocytes were sedimenting in Plasmagel by centrifuging 30 ml of the CPD blood in each of two 50-ml plastic centrifuge tubes at 600 *g* for 10 min at 5°C. The resulting supernatant was centrifuged at 9,500 *g* for 15 min at 5°C, passed through a 0.45-μm filter into a sterile tube, and the CPD plasma was held on ice.

Hypertonic treatment of cells, density gradient centrifugation, and preparation of monocytes for culture

Fifty μl of 1.54 M NaCl at pH 7.4 was added to each of the tubes containing 10 ml of leukocytes in CPD plasma. After a gentle swirl the tubes were incubated at 37°C in a small water bath. After 10 min at 37°C, an additional 100 μl of 1.54 M NaCl was added and the tubes were mixed with a gentle swirl. Ten minutes later a third addition of 1.54 M NaCl (100 μl) was made and the cells were mixed with a gentle swirl. At the end of this third 10-min incubation period (total time at 37°C was 30 min) the tubes were removed from the water bath and the cells were diluted by the addition of 15 ml of hypertonic PBS previously equilibrated to 25°C. Hypertonic PBS was made by adding 1.54 M NaCl to PBS in a ratio of 1:36 (v/v). The cells were mixed by pipeting up and down four times with a 10-ml plastic pipet and 10-ml aliquots were dispensed into five 40-ml conical, graduated, siliconized glass centrifuge tubes. An additional 15 ml of hypertonic PBS was added to each of the tubes and the cells were mixed by pipeting up and down 10 times with a plastic pipet. Each tube containing 25 ml was then underlayered with 15 ml of hypertonic Ficoll-Hypaque previously equilibrated to 25°C. The hypertonic Ficoll-Hypaque was prepared by adding 2.8 mg of crystalline NaCl to each ml of Ficoll-Hypaque (Cat. No. 17-0840-02 Pharmacia, Piscataway, NJ), the pH was adjusted to pH 7.4, and the solution was filtered through a 0.45 μm filter. The tubes were centrifuged at 600 *g* for 15 min at 25°C. The monocytes were found at and below the interface in a broad band. Therefore, the interface region from each tube was removed with a wet siliconized Pasteur Pipet with repeated circular motions of the pipet tip applied to the interface until a volume of approximately 15 ml was removed and placed into a 40-ml conical, graduated, siliconized glass centrifuge tube. No more than 15 ml of the Ficoll-Hypaque band was placed in any one tube. The contents of each tube were diluted to 40 ml with ice-cold PBS and centrifuged at 600 *g* for 15 min at 5°C. (We found that these steps were a potential source for monocyte loss. If too little of the broad Ficoll-Hypaque band was taken, cells were lost. If too much of the Ficoll-Hypaque band was placed in any given tube, the density of the diluted solution was too high and the cells did not sediment.) The supernatants were removed and discarded; the cell pellets were resuspended in ice-cold PBS and combined into one 40-ml conical, graduated, siliconized glass centrifuge tube in a total volume of 20 ml. Aliquots were removed for cell and differential counts. The number of monocytes was determined by multiplying the number of cells by the percent monocytes as determined from a Wrights-stained smear prepared with a Shandon-Southern cytospin centrifuge (Model SCA-0031, Shadon Southern Products, Ltd., Runcorn, Cheshire, England). The cells were centrifuged

at 600 *g* for 10 min at 5°C and resuspended in 30% autologous serum at a density of 10^6 monocytes/ml of incubation medium and plated as previously described (4).

The major differences between the original Recalde method (2) and the modification reported here are the Plasmagel separation of the heparinized blood, the removal of the platelets by a low speed centrifugation, the volumes of hypertonic PBS and Ficoll-Hypaque, and the collection of a broad Ficoll-Hypaque band.

Other techniques and assays

Cell classification, viability, and culture, chemotaxis, lipoprotein preparation, iodination and degradation by cells, and protein assays were performed as previously described (3–6). Monocyte-macrophages were also identified with the monoclonal antibody HAM 56 kindly provided by Dr. Allen M. Gown, University of Washington, Seattle, WA and used as described by Tsukada et al. (7).

RESULTS

Purity

The composition of the cells obtained from 36 consecutive donors with the modified Recalde procedure was 73 ± 8% monocytes (range 45 to 86%); 27 ± 8% lymphocytes (range 15 to 55%), and polymorphonuclear leukocytes 0.4 ± 0.5% (range 0 to 1%). Fifteen of these 36 donors had recently provided blood for monocyte isolation by elutriation (method BB in reference 4). The composition of the cells obtained from these 15 donors by elutriation was compared to the composition of the cells obtained from the same donors by the modified Recalde procedure (**Table 1**).

The distribution of cells obtained from the same donor by the modified Recalde procedure as compared to the original Recalde procedure (2) is shown in **Table 2** for five donors.

Yield

The number of monocytes recovered from each ml of blood for the three methods is shown in **Table 3.** Approximately 10^8 monocytes were obtained from each donor by the modified Recalde Method and by elutriation.

TABLE 2. Comparison of cells obtained by the modified Recalde method and the original method in five donors

	Modified Recalde Method	Original Recalde Method
% Monocytes	74 ± 7[a] (65–83)[b]	63 ± 9 (60–83)
% Lymphocytes	26 ± 7 (17–35)	32 ± 9 (17–40)
% PMNs[c]	0.3 ± 0.3 (0.0–0.7)	0

[a]Mean ± 1 SD.
[b](Range).
[c]Polymorphonuclear leukocytes.

Viability

Monocyte chemotaxis (6) was equivalent for cells prepared by the modified Recalde procedure and elutriation (data not shown). As was the case for elutriated cells (4) after culture for 1 week with the attendant media changes, and after the usual washing that precedes experiments (4), more than 95% of the cells obtained by the modified Recalde procedure were viable monocyte-macrophages as determined by nonspecific esterase staining, by staining with the monoclonal antibody HAM 56 and by the percent cells ingesting heat-killed yeast.

Equal numbers of monocytes obtained by the modified Recalde procedure and the original Recalde procedure (2) were plated in the experiments described in **Table 4**. After 6 days of culture, virtually all of the cells remaining in all of the wells were monocyte-macrophages (data not shown). However, the wells containing the monocytes from five out five donors obtained by the modified Recalde procedure had substantially more protein per well (Table 4). LDL receptor activity as measured by the specific uptake and degradation of ^{125}I-labeled LDL and scavenger receptor activity as measured by the specific uptake and degradation of ^{125}I-labeled MDA-LDL appeared to be somewhat less

TABLE 1. Comparison of cells obtained by the modified Recalde method and elutriation in 15 donors

	Modified Recalde Method	Elutriation
% Monocytes	71 ± 10[a] (45–83)[b]	83 ± 6 (69–91)
% Lymphocytes	29 ± 10 (17–55)	11 ± 5 (3–24)
% PMNs[c]	0.4 ± 0.5 (0–1)	6 ± 3 (3–16)

[a]Mean ± 1 SD.
[b](Range).
[c]Polymorphonuclear leukocytes.

TABLE 3. Comparison of the number of monocytes recovered by the modified Recalde method, original Recalde method, and elutriation

Modified Recalde Method	Original Recalde Method	Elutriation
	10^5 monocytes/ml blood	
3.44 ± 0.93[a] (n = 36)[b] (1.7–5.9)[c]		3.27 ± 0.97 (n = 15) (2.3–5.5)
4.0 ± 1 (n = 5) (2.7–5.3)	0.67 ± 0.4 (n = 5) (0.19–1.32)	

[a]Mean ± 1 SD.
[b]Number of donors.
[c](Range).

TABLE 4. Comparison of protein content and receptor activities of cells obtained by the modified Recalde method and by original Recalde method

	Modified Recalde Method	Original Recalde Method
Protein (μg/well)	47.5 ± 13.6[a] (n = 5)[b] (31-59)[c]	22.0 ± 11 (n = 5) (8-38.5)
LDL receptor activity (μg ^{125}I-labeled LDL specifically degraded · 4 hr · mg protein^{-1})	0.53 ± 0.46 (n = 3) (0.21-1.1)	0.25 ± 0.21 (n = 3) (0.02-0.44)
Scavenger receptor activity (μg ^{125}I-labeled MDA-LDL specifically degraded · 4 hr · mg protein^{-1})	1.64 ± 0.96 (n = 3) (0.81-2.7)	1.18 ± 0.52 (n = 3) (0.60-1.6)

Blood was drawn from five healthy donors and cells were prepared from 120 ml of blood anticoagulated with CPD by the original Recalde method (2) or from 120 ml of blood anticoagulated with heparin by the modified Recalde method as described in Materials and Methods. Equal numbers of monocytes obtained by each method (1×10^6 monocytes/ml) were resuspended in 30% autologous serum and 5×10^5 monocytes were plated in each well and cultured as previously described (4). After 6 days, the medium was removed, the cells were washed, and 20 μg/ml ^{125}I-labeled LDL (276 cpm/ng) or 10 μg/ml ^{125}I-labeled MDA-LDL (276 cpm/ng) was added in the presence or absence of a 25-fold excess of nonradioactive lipoprotein as previously described (4). After 4 hr at 37°C, the content of ^{125}I-labeled acid-soluble material was determined. The values obtained in the presence of a 25-fold excess of nonradioactive lipoprotein were subtracted from the values obtained in the absence of nonradioactive lipoprotein.

[a]Mean ± 1 SD. (The average of quadruplicate wells from each of the five donors was used to compute the mean ± 1 SD for the values of all five donors).

[b]Number of donors studied.

[c](Range of values).

in cells obtained by the original Recalde procedure (2) as compared to the modified Recalde procedure (Table 4). However, the small number of subjects studied (n = 3) precludes statistical evaluation.

DISCUSSION

The modified Recalde procedure described here produced a cell population of purity comparable to that obtained by the original Recalde procedure (Table 2) but the number of monocytes obtained (Table 3) and their viability as determined by the protein content after culture were greater for the modified Recalde procedure (Table 4). The reason(s) for the lower protein content after plating equal numbers of monocytes obtained by the original method as compared to the modified method is not clear. The lower protein content may have been due to the detachment of more of the cells obtained by the original method.

The modified Recalde procedure gave a cell population of somewhat lower purity than that obtained by elutriation (4) (Table 1). However, the number of monocytes obtained and their characteristics after culture were similar. The advantages of the modified Recalde procedure over elutriation (4) include a lower contamination by polymorphonuclear leukocytes, the elimination of expensive and complicated equipment, and a shorter time for cell preparation. After drawing 500 ml of blood from each of two donors, one person is able to process the cells by the modified Recalde procedure (keeping syringes and tubes separate by color coding) and within 7 hr approximately 2×10^8 monocytes have been plated in autologous serum. A single donor (yielding approximately 1×10^8 monocytes) can be processed within 5 hr. We believe that this modification of the Recalde procedure (2) will be useful to many laboratories for the study of human monocyte-macrophages.

We thank Mr. Greg Hough for the esterase and HAM 56 monoclonal antibody staining, Ms. Laura Almada for the chemotaxis assays, Dr. Allen M. Gown for generously providing HAM 56, Drs. Peter A. Edwards and Margaret E. Haberland for critical review of this manuscript, and Susan C. Murphy for the manuscript preparation. This work was supported in part by United States Public Health Service Grants HL-30568, IT-32-HL-07412, and RR-865, the Laubisch Fund, and the M. K. Grey Fund.

Manuscript received 11 December 1987 and in revised form 18 March 1988.

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