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Short communication

A simple method for the purification of human peripheral blood monocytes

A substitute for Sepracell-MN

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Sepracell-MN has provided a simple, rapid means of isolating peripheral blood monocytes. However this product is no longer available. Consequentially we have developed a Percoll gradient which matches Sepracell-MN in simplicity and yield of monocytes. Using this Percoll gradient, an average of 7×10^6 monocytes with a purity of 83% were obtained from 30–40 ml of blood. These monocytes were at least 97% viable and responded to chemotactic stimuli in comparable numbers to those prepared using Sepracell-MN

Key words: Monocyte; Purification; Sepracell

Introduction

The purification of monocytes from peripheral blood has been a long standing problem for which many methods have been developed to solve. Some of these methods such as counter-flow elutriation (Sanderson, 1977) require large amounts of blood and expensive specialized equipment and are very time consuming. Several methods utilizing Percoll (Gmelig-Meyling and Waldmann, 1980; Pertoft et al., 1980; Weiner and Mason, 1984) alone or in combination with Ficoll-Hypaque have been described which produce a highly purified monocyte population yet with yields too low to be useful for the performance of bioassays such as chemotaxis. While monocytes can be adequately purified by adherence to glass or plastic (Borregaard and Kragballe, 1980), adherence may activate these cells and thereby alter responses to biological stimuli. In addition it is often difficult to remove adherent cells without significant loss of viability.

The development of Sepracell-MN was a breakthrough in monocyte isolation methodology. It provided a means of obtaining several million monocytes of up to 90% purity from 20–50 ml of blood in less than 2 h (Vissers et al., 1988; Denholm et al., 1989). These monocytes have been

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Abbreviations: PBSA, phosphate buffered saline (PBS) + 1 mg/ml BSA; RBSA, RPMI + 2 mg/ml BSA; ZAS, zymosan activated serum.

used for assays of chemotaxis (Denholm et al., 1989), and for the study of superoxide, enzyme and cytokine release (Vissers et al., 1989a,b).

As of last year Sepracell-MN was no longer being manufactured and researchers using this product have been searching for a substitute. We report here, a Percoll gradient which matches Sepracell in ease and rapidity. This Percoll gradient is used almost exactly as Sepracell was and equals the purity and yield of monocytes.

Materials

Percoll and Percoll density marker beads were obtained from Pharmacia LKB Biotechnology, Piscataway, NJ. Sepracell-MN was purchased from Sepratech, Oklahoma City, OK. Hanks' (10 ×) salt solution and RPMI 1640 were from Grand Island Biological, Grand Island, NY. Deionized, sterile, endotoxin-free water was from Baxter Scientific, Deerfield, IL. BSA (Bovuminar low-endotoxin) was from Armour Pharmaceutical Co. of Kankakee, IL. Histopaque-1077, the α-naphthyl-acetate esterase kit (no. 90), trypan blue and Wright's stain were from Sigma Chemical Co., St. Louis, MO. Surfasil was purchased from Pierce Chemical Co., Rockford, IL. Round-bottom polypropylene tubes with caps were from Sarstedt Inc., Pennsauken, NJ.

All solutions, medium, and chemicals as listed above, including the water used to prepare the PBS were endotoxin-free or low in endotoxin, as per testing by the manufacturers. In addition endotoxin was undetectable (< 0.025 U/ml) when RPMI + BSA was tested in this laboratory using the E-Toxate kit from Sigma Chemical Co.

Methods

30–40 ml of blood from normal healthy human volunteers, giving informed consent, was collected in sodium citrate (3.8% w/v; 1 ml/10 ml blood) by venipuncture. Blood was mixed 1:1 with PBSA, layered over Histopaque-1077 (three parts diluted blood to two parts Histopaque) and centrifuged ($500 \times g$) at 25 °C for 30 min. The mononuclear cells collected from the plasma/Hypaque interface were washed once in PBSA, and finally resuspended in 4 ml of PBSA.

A Percoll solution was prepared by mixing 1.65 ml of 10 × Hanks' balanced salt solution with 10 ml of Percoll. The pH of the Percoll mixture was adjusted to 7.0 using approximately 30 μl of 0.1 N HCl, with gentle stirring. 8 ml of Percoll solution were added to 4 ml of mononuclear cells in PBSA in a 10 × 1.5 cm round-bottom polypropylene tube which had been previously silanized with Surfasil according to manufacturer's instructions. Mononuclear cells were mixed with the Percoll solution by inverting the tube 3–4 times then centrifuged at $370 \times g$ (1500 rpm) in a fixed angle SM-24 rotor in a Sorvall RC2-B centrifuge at room temperature for 25 min without braking; alternatively a Beckmann J2-21 centrifuge with a fixed angle JA14 rotor can be used at $345 \times g$ (1500 rpm) with the same results. Blank tubes containing density marker beads, PBSA and the Percoll mixture were also centrifuged.

Monocytes appeared as a cloudy layer in the top 5 mm of the gradient. The monocyte layer was collected, diluted to 50 ml with PBSA and centrifuged at $500 \times g$ for 5 min. The cell pellet was resuspended in RPMI + BSA (2 mg/ml) or PBSA and counted.

Cell viability was assessed as the ability to exclude trypan blue. The purity of the monocytes was determined by differential counts of Wright's stained cytocentrifuge preparations (Shandon, Pittsburgh, PA) and by staining for nonspecific esterase using the α-napthyl-acetate esterase kit according to manufacturer's instructions.

Assays of chemotactic response were carried out essentially as described previously (Denholm et al., 1989) using 48-well chambers (Neuroprobe, Cabin John, MD) and polycarbonate filters (PVP-free) with 5 μm pores. Monocytes purified as above or using Sepracell-MN as described by Vissers(1988) were resuspended in RPMI + BSA at a concentration of 1×10^6 cells/ml. Monocyte migration to medium alone (RPMI + BSA) was compared to that of 1% zymosan activated serum, prepared as described before (Denholm and Lewis, 1987). Cell migration was quantitated by counting the number of monocytes that had migrated through the filters in ten 1000 × fields,

with each substance assayed in triplicate for each experiment.

Results and discussion

A continuous self-generating gradient of Percoll with a density of 1.088 g/ml was created by mixing 1.65 ml of 10× Hanks' balanced salt solution with 10 ml of Percoll and adjusting the pH to 7.0 with approximately 30 μl of 0.1 N HCl. Adjustment of the pH was critical; the mixture of Percoll and Hanks' alone had a pH of 9 or greater, which caused cell lysis. In addition to affecting cell viability, the pH of Percoll solutions has been shown to have an effect on the distribution of cells and subcellular organelles in the gradient (Record et al., 1982). It was also important that the pH be adjusted using a very dilute solution of HCl (0.1 N) with gentle stirring to prevent clumping and precipitation. Percoll solutions in which precipitates formed were discarded.

Mononuclear cells from Hypaque gradients were washed and resuspended in 4 ml of PBSA then mixed with 8 ml of the Percoll gradient and centrifuged. Monocytes separated in a band in the top 5 mm of the gradient, which corresponded to a density of slightly greater than 1.063 g/ml as determined by the location of density marker beads. In this gradient, beads of 1.063 g/ml density remained at the surface of the gradient and those of 1.076 g/ml sedimented 5 mm into the gradient. Monocytes appeared as a band between these two densities; this is in agreement with estimates of monocyte densities made by others, including 1.062–1.064 g/ml (Umler and Flad, 1979) and 1.064 g/ml (Pertoff et al., 1980). Monocytes in gradients of Sepracell MN also appeared as a band just below a density of 1.063 g/ml. Also, as with Sepracell, the only band in the Percoll gradient was that containing the monocytes; lymphocytes were present throughout the gradient below the monocytes band and the few granulocytes and erythrocytes pelleted to the bottom of the tube.

We found that either of the two centrifuges and rotors listed in methods yielded monocytes of similar numbers and purity. Three experiments using a swinging bucket rather than a fixed angle rotor resulted in relatively low yields of less than 5×10^5 monocytes from 30 ml of blood.

TABLE I

PURIFICATION OF MONOCYTES FROM PERIPHERAL BLOOD

Data shown are from individual experiments. Mononuclear cell # was that recovered from the plasma/Hypaque interface. The number of monocytes was calculated by multiplying # cells in monocyte band by the % of monocytes present. Monocyte purity was assessed as described in the methods section. The (–) indicates counts not done

Blood (ml)	#mononuclear cells ($\times 10^7$)	#cells in monocyte band ($\times 10^6$)	%monocytes	#monocytes ($\times 10^6$)
(A)				
30	5.0	4.3	85	3.7
35	–	7.9	87	6.9
30	–	4.2	83	3.5
40	7.1	9.8	80	7.8
40	7.9	8.4	79	6.6
40	9.3	8.8	84	7.4
60	–	14.8	82	12.1
40	10.5	10.3	77	7.9
40	9.1	9.9	87	8.6
Mean	8.2	8.7	83	7.1
(B)				
15	2.6	5.0	79	4.0
15	4.7	3.7	44	1.6
15	–	6.4	81	5.2
10	1.3	2.2	70	1.5
12.5	1.9	3.1	42	1.3
15	4.5	1.7	53	0.9
15	–	6.3	75	4.7
12.5	–	2.7	69	1.9
Mean	3.0	3.9	64	2.6

Using the fixed angle rotors, the factor which most affected yield and to some extent purity was the number of mononuclear cells mixed with the Percoll solution (Table I). The best results were obtained with $5–11 \times 10^7$ mononuclear cells isolated from 30–40 ml of blood (Table I(A)); this yielded $4.3–10.3 \times 10^6$ cells, 83% of which were monocytes. Smaller numbers of monocytes, $1–4.7 \times 10^6$, were obtained when less than 5×10^7 mononuclear cells (from 10–15 ml blood) were used (Table I(B)). Monocyte viability in both instances was between 97 and 99%.

The average yield of monocytes per ml of blood was 1.73×10^5 which is within the range of the $1.5–2.25 \times 10^5$ that we had been able to obtain previously with Sepracell (Denholm et al., 1989). Vissers (1988) reported higher yields of monocytes (4.9×10^5/ml) from Sepracell when the starting volume of blood was 100 ml. The one experiment which used 60 ml of blood suggested that the yields of monocytes/ml of blood from the Percoll gradient may also be increased slightly with larger amounts of blood.

The percentage of monocytes present in the mononuclear cells recovered from the initial Ficoll-Hypaque gradient was found to be $20 \pm 1\%$ monocytes. This is in close agreement to the findings of Gmelig-Meyling and Waldmann (1980) who found an average of 21% monocytes in the mononuclear cells obtained from Ficoll gradients. Of the monocytes loaded onto the Percoll gradient, an average of $50 \pm 4\%$ ($n = 15$) were recovered.

Since this Percoll gradient was developed primarily to separate monocytes for use in chemotaxis assays, a comparison was made between the chemotactic response of cells obtained with Percoll to those obtained using Sepracell. Monocytes isolated by both methods responded in comparable numbers and proportions to the positive (1% ZAS) and negative (RPMI + BSA) controls (Table II). These results suggested that monocytes isolated by both methods were representative of the same monocyte population. It is therefore likely that monocytes separated on this Percoll gradient can also be substituted for Sepracell-isolated monocytes in studies of enzyme, superoxide and cytokine release (Vissers et al., 1989a,b).

TABLE II

COMPARISON OF CHEMOTACTIC RESPONSES OF MONOCYTES ISOLATED ON SEPRACELL OR PERCOLL

Monocytes isolated from gradients of Sepracell-MN or Percoll were resuspended in RBSA then assayed for chemotactic activity as described in the methods section. RBSA (RPMI + 2 mg/ml BSA) was the negative control and 1% ZAS (zymosan activated serum) was the positive control. ZAS:RBSA is the ratio of monocytes migrating to ZAS divided by the number migrating to RBSA. Data is the number of monocytes counted in ten 1000× fields and is shown as the mean ± SEM of five (Percoll) or six (Sepracell) experiments

	Sepracell	Percoll
RBSA	41 ± 7	51 ± 3
1% ZAS	209 ±31	277 ±42
ZAS:RBSA	5.3± 0.03	5.4± 0.5

Percoll has been widely used as a separation medium for monocytes and other cells. Since Percoll is particulate in nature, it is possible that monocytes may ingest some of the gradient material during separation; however evidence of internalized Percoll is lacking. The polyvinylpyrrolidine(PVP) which coats the silica particles in Percoll would more likely make ingestion difficult since monocytes and other leukocytes do not adhere to PVP (Harvath et al., 1980). Regardless of the possible uptake of Percoll by monocytes, there is ample evidence that the function and viability of these cells is unaffected by this gradient medium. Monocytes separated by several different Percoll gradient systems have been found to be completely viable, adhere to glass and plastic, have cytolytic capabilities, phagocytize latex beads and erythrocytes, and (as shown here) to respond to chemotactic stimuli (Gmelig-Meyling and Waldmann, 1980; Pertoff et al., 1980; Peri et al., 1990; Weiner and Mason, 1984).

Initially the Percoll gradients described here were prepared fresh daily. We have since found that larger batches can be prepared and stored at 4 °C in polypropylene tubes for up to two weeks, which further enhances the convenience of this method. This gradient can also be used without silanizing the tubes, however better yields (up to 4×10^6 more monocytes) were obtained with silanized tubes.

In summary, we have developed a simple and rapid method of purifying monocytes from peripheral blood. This method was developed to closely mimic the separation of monocytes using Sepracell-MN, a product which is no longer available. Trials of this method in two independent laboratories have produced the same yields and purity of monocytes. It is hoped that the method described here will prove similarly useful to other investigators searching for a Sepracell replacement.

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