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Immunological heterogeneity of human monocyte subsets prepared by counterflow centrifugation elutriation

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SUMMARY

Human peripheral blood mononuclear cells were separated into three fractions by means of counterflow centrifugation elutriation (CCE). The first fraction, eluted at a flow rate of 24 ml/min, was composed of lymphocytes with less than 2% contaminating esterase-positive cells. The cells in this fraction were incapable of responding to either soluble antigen (tetanus toxoid) or particulate antigen (cytomegalovirus-infected fibroblasts) unless recombined with accessory cells. The second fraction, eluted at a flow rate of 28 ml/min, was composed predominantly (72%) of small Ia, leu M3, and esterase-positive monocytes, which stained weakly with leu 10 antibody. Cells in this fraction efficiently presented soluble and particulate antigens to monocyte-depleted lymphocytes. Of the remaining cells, 87% were large esterase-positive monocytes that labelled strongly with Ia, leu M3, and leu 10. These cells were less efficient in antigen presentation than the small monocytes. However, lymphocytes activated with antigen-pulsed large monocytes exhibited more suppressor cell activity than those activated with antigen-pulsed small monocytes.

INTRODUCTION

Although the existence of distinct subsets among monocytes/macrophages is suggested by the diversity of functions performed by these cells (reviewed by Shevach, 1984), conclusive evidence supporting the concept of functional heterogeneity is lacking. A major problem that has plagued research into monocyte/macrophage heterogeneity has been the difficulty in isolating large numbers of unaltered cells with a high degree of purity. Routinely, monocytes are separated by surface adherence, and although the level of purity achieved with this technique is high, this procedure stimulates the differentiation of monocytes into macrophages (Van Ginkel *et al.*, 1977; Van Furth, Raeburn & Van Zevet, 1979). Moreover, subsequent steps that are required to detach these cells from surfaces introduce additional complications. Other procedures, primarily centrifugation through density gradients, have also been utilized. These procedures allow for the separation of monocyte subsets (Khansari, Chou, and Fudenberg, 1985), but the cell yield is limited. Recently, counterflow centrifugation elutriation [CCE) has been used successfully to isolate large numbers of monocytes from peripheral blood mononuclear cells (Sanderson *et al.*, 1977). Using CCE, several laboratories (Noga, Norman & Weiner, 1984; Mason & Weiner, 1985; Yasaka *et al.*, 1981) report the separation of at least two monocyte subsets that differ in superoxide release, myeloperoxidase activity and tumoricidal capacity. In view of these findings, this study was undertaken to examine whether these two subsets differ phenotypically and whether the important role of monocytes in antigen presentation could be mediated by one and/or both subpopulations.

Abbreviations: CCE, counterflow centrifugation elutriation; CMV_x, cytomagalovirus antigen; PBM, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; TT, tetanus toxoid.

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MATERIALS AND METHODS

Subfractionation of peripheral blood mononuclear cells

Mononuclear cells were isolated from defebrinated peripheral blood of CMV or TT seropositive healthy volunteers by Ficoll–Hypaque centrifugation and suspended in Hanks' balanced salt solution (HBSS) supplemented with 100 mg/l EDTA and 0.5% bovine serum albumin. Cells, $2–3 \times 10^8$ in 5 ml of medium, were introduced into a Beckman JE-6B elutriator rotor. The cells were loaded at a medium flow rate of 16 ml/min, at a rotor speed of 3000 r.p.m. and at a temperature of 4°. Rotor speed varied by no more than 10 r.p.m. during the procedure. After all the cells were loaded, the flow rate was increased to 24 ml/min and two 200-ml fractions were collected. The flow rate was then increased to 28 ml/min and a 200-ml fraction collected. The rotor was stopped and the remaining cells eluted and designated rotor-off (r/o) fraction. Each fraction was washed twice in RPMI-1640. Cytospin preparations were made of each fraction and these were stained with Wright's stain and with alpha-napthyl acetate for non-specific esterase activity by a modification of the procedure described by Yam, Li & Crosby (1971).

Formalin–acetone fixed cytospin-prepared slide were reacted with alpha-napthyl acetate for 30 min at 37°, washed in tap-water and counterstained in methyl green.

Antigen pulsing

Preservative-free tetanus toxoid (TT) antigen (Wyeth Laboratories, Marietta, PA) and cytomegalovirus antigen (CMV_x) prepared as described by Converse *et al.* (1983) were diluted in RPMI-1640 (Flow Laboratories Inc., McLean, VA) supplemented with 20% normal human serum, 100 μg/ml streptomycin and 100 U/ml penicillin. TT antigen was used at 2 μg/ml and CMV_x at 2×10^4 cells/ml. Small and large monocytes were irradiated at 1500 rads from a ^{137}Cs source and then incubated with TT or CMV_x for 1 hr. After incubation, the cells were washed three times and cocultured with lymphocytes as described below.

Assay for antigen presentation

Monocyte-depleted lymphocytes (2×10^6) were combined with antigen-pulsed small or large monocytes (1×10^6 cells), placed in 24-well macroculture plates, and incubated at 37° in 5% CO_2 in a final volume of 2 ml. After 5 days, 0·1-ml aliquots were removed and seeded in triplicate wells of flat-bottomed microtitre plates. These aliquots were pulsed with 0·5μCi of [^{3}H] thymidine and incubated for another 16–18 hr, then harvested with a Minimash II (Microbiological Associates, Walkersville, MD) onto fiberglass filters and placed in scintillation fluid. Radioactivity was measured with a Packard beta counter (Tricarb, Packard Instruments Co., Downers Grove, IL). In order to examine the kinetics of the proliferative response induced by antigen-pulsed monocytes, duplicate 0·1-ml aliquots were removed once very 24 hr starting 3 days after coculture. These aliquots were pulsed with [^{3}H]thymidine as described above.

PHA stimulation

PHA stimulation was performed essentially as described by Hess & Tutschka (1980).

Suppressor cell assay

Suppressor cell activity was assayed as previously described (Hess & Tutschka, 1980). Briefly, 2×10^6 lymphocytes were primed for 7 days with antigen-pulsed small or large monocytes. Graded quantities of these primed lymphocytes were harvested and added in 0·1-ml volumes to $2{\cdot}5 \times 10^5$ fresh autologous responder cells that were then stimulated with antigen in a final volume of 0·2 ml. Fresh autologous responder cells were obtained by Ficoll–Hypaque centrifugation of peripheral blood at the time of assay. The response of the fresh responder cells was measured after 6 days of incubation by [^{3}H] thymidine uptake as described above. Percentage suppression of the stimulation of fresh responder cells was then calculated as follows:

$$\% \text{ suppression} = 1 - \frac{\text{c.p.m. of test culture}}{\text{c.p.m of control culture}}.$$

Control cultures contained responder cells, antigen and irradiated primed cells to control for density.

Flow cytometry

Flow cytometry analysis was performed as described by Hess *et al.* (1983). Briefly, the cell fractions were washed three times in PBS supplemented with 0·1% azide. The cells (2×10^6/ml) were then pelleted and incubated with the following monoclonal antibodies: OKIa, T3 (Ortho Pharmaceutical Corp., Raritan, NJ), leu M3 and leu 10 (Becton Dickinson, Mountain View, CA), or with PBS (control). OKIa (IgG2, anti-human DR framework) reacts with 90% of human monocytes and B cells, while OKT3 (IgG2a) reacts with 95% of peripheral blood T cells. Leu 10 (IgG1) is specific for a D-region determinant and reacts weakly with peripheral blood lymphocytes and monocytes but strongly with some macrophages, and Leu M3 (IgG2b) reacts with 70–93% normal monocytes and does not react with lymphocytes. After 30 min incubation at 4°, the cells were washed three times in PBS–azide and incubated for 30 min at 4° with fluoresceinated (FITC) $F(ab')_2$ fragments of sheep anti-mouse IgG (Cappel Laboratories, Cochranville, PA). After three washes, the cells were analysed on the fluorescence-activated cell sorter (FACS II, Becton Dickinson, Rutherford, NJ). The percentage positive refers to the percentage of cells with higher fluorescence intensity then 99% of control cells.

Statistical analysis

Each experiment was performed at least three times. The results were analysed by the Student's *t*-test. Standard deviation between triplicate and duplicate measurements was always less than 10%.

RESULTS

Subfractionation of PBL

Sixty-five percent of the mononuclear cells eluted at a flow rate of 24 ml/min. Cells in this fraction were shown by Wright stain to have lymphocyte morphology and less than 2% were positive for non-specific esterase. Table 1 summarizes FACS analysis data and shows that 57% of this lymphocyte fraction labelled with anti-OKT3 antibody, while less than 2% was positive for leu M3, and 7·2% and 9·6% labelled with leu 10 and OKIa,

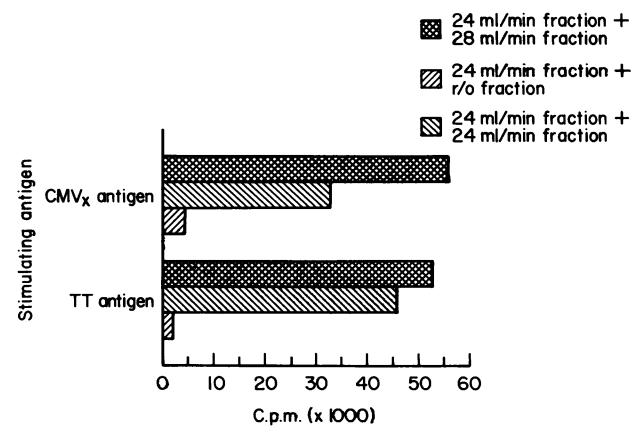

Figure 1. PBM cells were separated into three fractions that eluted at 24 ml/min, 28 ml/min, or when the rotor was turned off (r/o). 2×10^6 cells from the 24 ml/min fraction were then combined with 1×10^6 cells from the 28 ml/min, r/o, or 24 ml/min fraction and stimulated with either TT or CMV_x antigen. [^{3}H]Thymidine uptake was measured 6 days after coculture. Neither of the two monocyte fractions (28 ml/min and r/o) responded to the antigens (not shown). Mean c.p.m. of triplicate determinations are shown.

Table 1. Phenotype of unfractionated and CCE fractionated cells*

Fraction	% positive			
	OKT3	OKIa	leu 10	leu M3
Unfractionated	57·1	18·0	11·5	13·9
24 ml/min	61·5	9·6	7·2	1·8
28 ml/min	6·8	86·9	16·1	93·0
r/o	9·0	93·0	46·2	86·1

* Percentage positive cells were determined by labelling 2×10^6 cells from each fraction with the monoclonal antibodies or with PBS and then with FITC sheep anti-mouse IgG. Experimental and PBS (control) results were normalized for total cell number and compared. Percentage positive refers to the percentage of cells with higher fluorescence than 99% of the control.

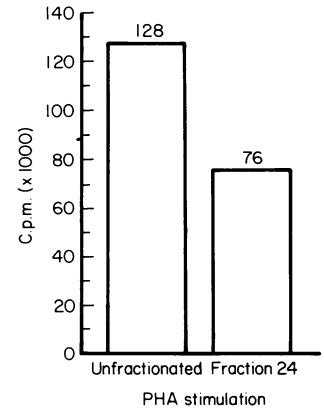

Figure 2. PHA stimulation of unfractionated PBM or CCE isolated lymphocytes (fraction eluted at 24 ml/min). $2{\cdot}0 \times 10^5$ PBM cells were placed in triplicate microtitre wells and then stimulated with 10 μg/ml PHA and incubated for 60 hr. These were pulsed with [^{3}H] thymidine and incubated for another 16–18 hr. Mean c.p.m. of triplicate determinations are shown.

respectively. Twenty-four percent of the cells eluted at 28 ml/min. The majority (72%) of cells in the 28 ml/min fraction appeared to be small monocytes by light microscopy and a corresponding proportion stained with alpha-naphthyl acetate. Eighty-six percent of these cells were Ia+, 93% were leu M3+, and only 16% were leu 10+. The rotor/off fraction constituted 5% of eluted cells and was composed mostly of large monocytes. Eighty-seven percent of the cells were demonstrated by light microscopy to be monocytes. These cells were Ia+ (93%), leu M3+ (86%), and leu 10+ (46%).

Induction of antigen-specific lymphocyte proliferation

Lymphocytes isolated through the above procedure contained less than 2% esterase-positive cells and could not respond to TT or CMV_x antigens (Fig. 1). These cells could still respond to PHA stimulation (Fig. 2). Proliferative responses to TT or CMV_x could be restored by the addition of cells from either monocyte fraction (Fig. 1). In order to compare antigen presentation efficiency of the small and large monocytes, both subsets were irradiated at 1500 rads, pulsed with TT or CMV_x for 1 hr at 37° in 5% CO_2, washed three times, and then combined with lymphocytes. As shown in Fig. 3, small monocytes were more efficient than large monocytes in presenting both types of antigens to lymphocytes. The monocyte fractions were not capable of proliferating in response to TT or CMV_x, and therefore, proliferation of contaminating lymphocytes in these monocyte preparations could not have accounted for the above differences. The data presented in Fig. 3 also show that cells in the lymphocyte fraction could not present antigens, further demonstrating that these cells were accessory-cell depleted. In order to determine whether changes in proliferative response kinetics accounted for the above differences, we measured [^{3}H] thymidine uptake for 7 days. The results (Fig. 4) show that there were no time-course differences; peak proliferative response triggered by antigen-pulsed small or large monocytes occurred on Day 6.

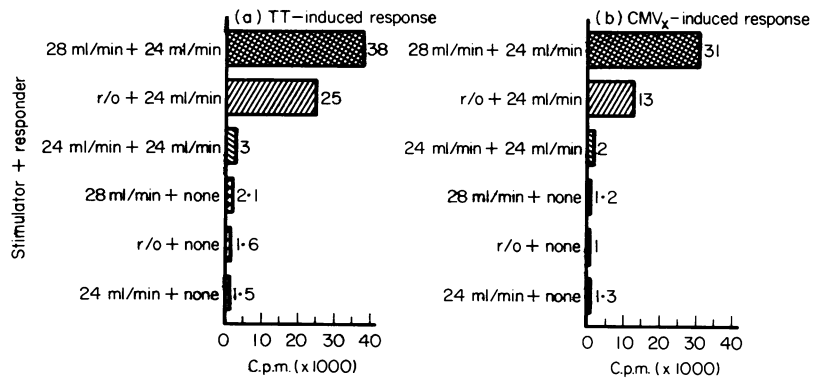

Figure 3. Comparison of the antigen-presentation capacity of the large and small monocyte subsets. After irradiation at 1500 rads, large (r/o fraction) or small (28 ml/min fraction) monocytes were incubated with either (a) TT or (b) CMV_x antigen for 1 hr at 37°. After incubation, they were washed three times and then combined with lymphocytes (24 ml/min fraction). Lymphocyte proliferative responses induced by the antigen-bearing monocytes were then determined 6 days after coculture by [^{3}H] thymidine uptake. The ability of cells in the lymphocytes fraction to present antigen was also similarily examined. $P > 0{\cdot}05$ for TT response and $P > 0{\cdot}01$ for CMV_x response. Values are mean c.p.m. of triplicate determinations.

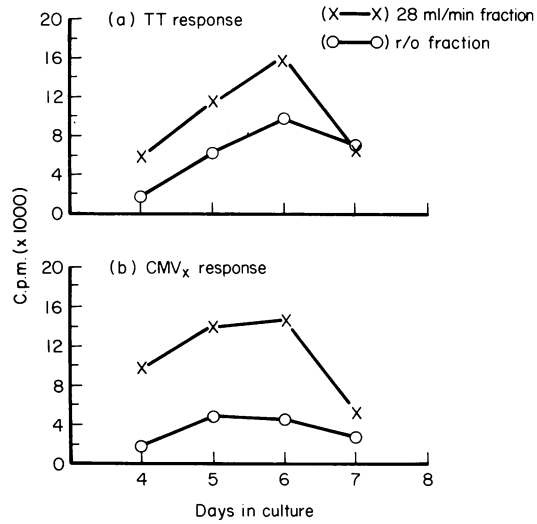

Figure 4. In order to determine the kinetics of the proliferative responses induced with large (r/o) and small (28 ml/min) monocytes, these cells were pulsed with (a) TT or (b) CMV_x and cocultured with lymphocytes (24 ml/min) in 24-well Costar macrotitre culture plates. Duplicate 0·1-ml aliquots were removed every 24 hr starting at Day 3 and these were pulsed with [^{3}H]thymidine. Mean c.p.m. for each day is shown.

Table 2. Generation of suppressor cell activity with antigen-pulsed large or small monocytes*

	% suppression	
	Ratio	
Priming environment	1:5	1:10
CMV-pulsed large (r/o) monocytes	92	85
CMV-pulsed small (28 ml/min) monocytes	72	63
TT-pulsed large (r/o) monocytes	83	67
TT-pulsed small (28 ml/min) monocytes	42	42

* Lymphocytes primed with CMV_x- or TT-pulsed small or large monocytes for 7 days were harvested, and their ability to suppress CMV_x or TT-induced proliferation of fresh antologous peripheral blood lymphocytes was assessed. Stimulation of the fresh responder lymphocytes was measured by [^{3}H] thymidine uptake 6 days after antigen addition, and suppression determined. The data shown are those obtained when the fresh responder cells were stimulated with the original antigens used to prime the putative suppressor cells (specific suppression). Non-specific suppression (i.e. proliferation induced with an antigen unrelated to that used in priming the putative suppressor cells) ranged between 33% and 40% in all cases.

Induction of suppressor cell activity

We were further interested in examining the suppressor activity of cells sensitized by antigen-pulsed large or small monocytes. Lymphocytes activated with antigen-pulsed monocytes were added at a ratio of 1:5 or 1:10 to $2{\cdot}5 \times 10^5$ fresh autologous mononuclear cells, which were then stimulated with TT or CMV_x antigens. Suppression of the proliferative response of these fresh responder cells was then determined. The results of these experiments (Table 2) show that higher suppression was achieved with lymphocytes activated with antigen-bearing large monocytes. The difference in suppressor cell activation was particularly significant when TT antigen was employed. In the case of TT, the level of suppressor cell activity induced with antigen-pulsed small monocytes was equal to background non-specific suppression.

DISCUSSION

The results of this study concur with those of Norris *et al.* (1979), and not only confirm that CCE is a rapid and reliable method of separating monocytes from lymphocytes, but also substantiate the existence of two subsets of monocytes. Lymphocytes isolated in this process were contaminated with less than 2% esterase-positive cells and, as is true of accessory-cell depleted cultures, could not respond to antigen stimulation. They were still capable of responding to mitogen stimulation. Mitogen stimulation requires the presence of relatively few (less than 1%) accessory cells (Habu & Raff, 1977). The ease with which this high level of lymphocyte purity was achieved makes CCE attractive.

The two monocyte subsets shared several properties. They were both Ia, leu M3, and esterase-positive. Approximately half of the large monocytes labelled with the leu 10 monoclonal antibody. Leu 10 antibody is specific for determinants that are distinct from HLA-DR (Chen *et al.*, 1984). It does not bind T cells, but strongly binds some marcrophage populations and dendritic cells. CCE-separated small and large monocytes have also been characterized by other investigators on the basis of phagocytosis and adherence (Noga, Norman & Weiner, 1984), superoxide and myeloperoxidase activity (Yasaka *et al.*, 1981), and FcR (Fc receptor for IgG) expression (Norris *et al.*, 1979; McCarley, Shah & Weiner, 1983). These studies indicate that large monocytes possess enhanced phagocytosis, superoxide and myeloperoxidase activity. The data regarding FcR expression are, however, inconclusive. Norris *et al.* (1979) reported that large monocytes were strongly FcR+ while small monocytes were largely FcR−. Using a modified CCE procedure, McCarley *et al.* (1983) could not demonstrate such differences in FcR expression between the two monocyte subsets.

Both monocyte subsets were capable of presenting soluble or particulate antigens to lymphocytes. Small monocytes were, however, significantly more efficient in inducing a proliferative response. Our finding that small monocytes are better antigen presenters than large monocytes is in agreement with previous results reported by Lee & Wong (1980). In that study, bone marrow-derived small macrophages were identified as being the antigen-presenting cell population. Data reported by Lee, Wilkinson & Wong (1979) and Lee, Kay & Wong (1979) also indicate that large differentiated macrophages are immunosuppressive when activated, while small monocytes are immunostimulatory. It is conceivable that, due to excessive catabolic activity, the large cells reduce the amount of immunogen available for T-cell activation. Alternatively, the small monocyte population may include cells that are innately suited to process antigens and interact with lymphocytes. The existence

of a distinct monocyte subset uniquely suited for antigen presentation has also been suggested by Gonwa *et al.* (1983) who found that only monocytes that label with the monoclonal antibody MAC-120 could present antigens to T cells. This population constituted between 40% and 60% of peripheral blood monocytes and expressed the HLA-DS determinant.

Surprisingly, antigen-pulsed large monocytes were relatively more active in the induction of suppressor cells in spite of the fact that these cells were poor stimulators in proliferative assays. Whether or not this large monocyte subset specifically interacts with T lymphocytes in the induction of suppression remains to be explored. In a recent study, Rosenson-Schloss & Reinisch (1985) reported data indicating that a unique subset of Ia^+ accessory cells could interact with suppressor cells. Taken together, these results may suggest that, in addition to the existence of specific suppressor T cells, immune suppression may be regulated at the level of antigen presentation. It is possible that the two monocyte subsets elaborate different immunomodulator substances. Khansari *et al.* (1985) have reported that interleukin-1-producing monocytes were distinct from prostaglandin-producing monocytes. It is not possible to compare our study to that of Khansari *et al.* (1985) since we used different separation procedures. However, such a division of IL-1 and PGE production between the small and large monocytes would explain our results and merits further investigation.

The observed functional differences reported here and elsewhere by others may only reflect maturational differences between small and large monocytes and do not necessarily suggest that these subsets follow independent pathways in ontogeny. Data from a very recent study by Norman & Noga (1986) do, however, suggest that guinea-pig monocyte subsets are derived from different precursor cells. Further studies are needed to confirm this observation and to elucidate the origin of these precursor cells in the bone marrow, as well as whether human monocytes subsets are also derived from different precursors.

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