Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7698–7702, October 1990
Immunology

The distinct surface of human blood dendritic cells, as observed after an improved isolation method

(mixed leukocyte reaction/antigen presentation/colony-stimulating factor 1 receptor/leukocyte integrins/CD45R molecule)

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Communicated by Maclyn McCarty, June 28, 1990 (received for review May 10, 1990)

ABSTRACT **Prior studies have identified a subset of dendritic cells in human blood, as well as their stimulatory function for T-cell-mediated immune responses. However research has been limited by difficulties in isolation, since dendritic cells make up only 0.1–1% of blood mononuclear cells. We present a protocol that reliably yields preparations that are >80–90% pure. The method relies on the sequential depletion of the major cell types in blood and simultaneously provides T cells, monocytes, and B plus natural killer cells for comparison with dendritic cells. The last step in the procedure is the removal of residual contaminants on the basis of expression of a CD45R epitope. The enrichment of dendritic cells is evident by three criteria, each of which is related to the surface of these antigen-presenting cells. (*i*) All dendritic cells are motile, constantly forming large lamellipodia or veils. (*ii*) When analyzed with a large panel of monoclonal antibodies and the FACS, the cells express high levels of all known polymorphic major histocompatibility complex gene products, as well as a distinct combination of receptors and adhesion molecules. Unlike monocytes, for example, dendritic cells lack Fc receptors and the colony-stimulating factor 1 receptor (c-fms) but express much higher levels of ICAM-1 and LFA-3 adhesins. (*iii*) In functional assays, dendritic cells are at least 100 times more potent than monocytes or lymphocytes in stimulating the primary mixed leukocyte reaction. These properties help make the trace subset of dendritic cells more amenable to further functional and clinical studies.**

Prior reports (1–9) have described antigen-presenting dendritic cells in human blood. These cells resemble more extensively studied counterparts in the lymphoid tissues of other species. In particular, blood dendritic cells express high levels of major histocompatibility complex (MHC) products and actively stimulate primary T-cell responses to transplantation antigens (3, 4, 10–12). When we began to study dendritic cells in individuals infected with human immunodeficiency virus type 1, we encountered difficulties in obtaining sufficiently high purity and in monitoring the purification. To date, only partial purification of dendritic cells (<1% of blood mononuclear cells) has been achieved, and there has been no detailed study with the fluorescence-activated cell sorter (FACS). Here we present an approach for preparing highly enriched populations, which we then study to describe their distinctive motility, phenotype, and function.

METHODS

Culture Medium. RPMI medium 1640 supplemented with 5–10% heat-inactivated normal human AB^+ serum, 1 mM glutamine, 50 μM 2-mercaptoethanol, penicillin at 100 units/ml, and streptomycin at 100 μg/ml was used.

Cell Separation. See Fig. 1. Granulocytes were isolated from heparinized whole blood (13) and used immediately. For mononuclear cells, the blood or leukocyte-enriched "buffy coat" was layered onto Ficoll-Paque (1.0777 g/ml; Pharmacia) and sedimented at 1000 × *g* for 20 min at 21°C. Mononuclear cells were harvested from the interface and sedimented three times in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} to remove platelets (first at 650 × *g* then twice at 235 × *g*). T lymphocytes were separated by rosetting at 4°C with neuraminidase-treated sheep erythrocytes followed by Ficoll-Paque sedimentation. T cells were recovered from the pellet by lysing the erythrocytes in NH_4Cl and washing twice in RPMI 1640 (6). The T-cell-depleted erythrocyte rosette-negative (ER^-) fraction was washed twice in RPMI 1640 and cultured at 3–5 × 10^6 cells per ml at 37°C for 36 hr in 100-mm tissue culture dishes. After culture, most of the cells could be collected from the plates. By reculturing the cells twice for 30–40 min at 37°C on fresh dishes, the monocytes were selectively attached. Nonadherent cells were "panned" once or twice on bacteriologic plastic dishes coated with human immunoglobulin to remove residual Fc fragment receptor (FcR)-bearing monocytes (6). To recover the adherent monocytes, the dishes were washed three times with cold PBS and cultured with fresh medium for 3–4 hr, at which point the monocytes were easily dislodged from the surface. The monocyte- and T-cell-depleted fraction (5–8 × 10^6 cells per ml; 3–4 ml) was layered onto 2.5-ml columns of hypertonic 14.5% metrizamide (dissolved in RPMI 1640 with 10% heat-inactivated fetal calf serum) in 15-ml conical tubes and sedimented at 650 × *g* for 10 min at room temperature (14). The dendritic-cell-enriched interface was separated from the B- and natural killer (NK)-cell-enriched pellet. Both fractions were washed in two successively less hypertonic washes to return the cells to isotonicity [RPMI 1640 supplemented with 10% fetal calf serum and 40 mM NaCl (wash 1) or 25 mM NaCl (wash 2)].

CD45R Panning. The dendritic cell fraction was treated with a monoclonal antibody (mAb) specific for restricted CD45 antigen (CD45R) (Leu18; Becton Dickinson) at 20 μl of mAb per 10^6 cells in 1–2 ml of culture medium and on ice for 45–60 min. The cells were washed four times and sedimented twice at 50 × *g* (5 min, 4°C) onto plastic dishes coated with goat antibodies to mouse immunoglobulin (10). The plates were swirled gently prior to harvesting the dendritic cell preparation that was depleted of $CD45R^+$ contaminants.

Cell Sorting. An alternative final step in the dendritic cell enrichment procedure was to use the FACS. Cells in the metrizamide low-density fraction were combined with 20 μl of fluorescein-conjugated Leu18 per 10^6 cells in 1–2 ml of medium on ice for 45–60 min. The cells were washed four times and

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Abbreviations: MHC, major histocompatibility complex; ER, erythrocyte rosette; NK, natural killer; mAb, monoclonal antibody; MLR, mixed leukocyte reaction; APC, antigen-presenting cell(s); FcR, Fc fragment receptor; C3R, complement component 3 receptor; IL-2R, interleukin 2 receptor; CSF, colony-stimulating factor.

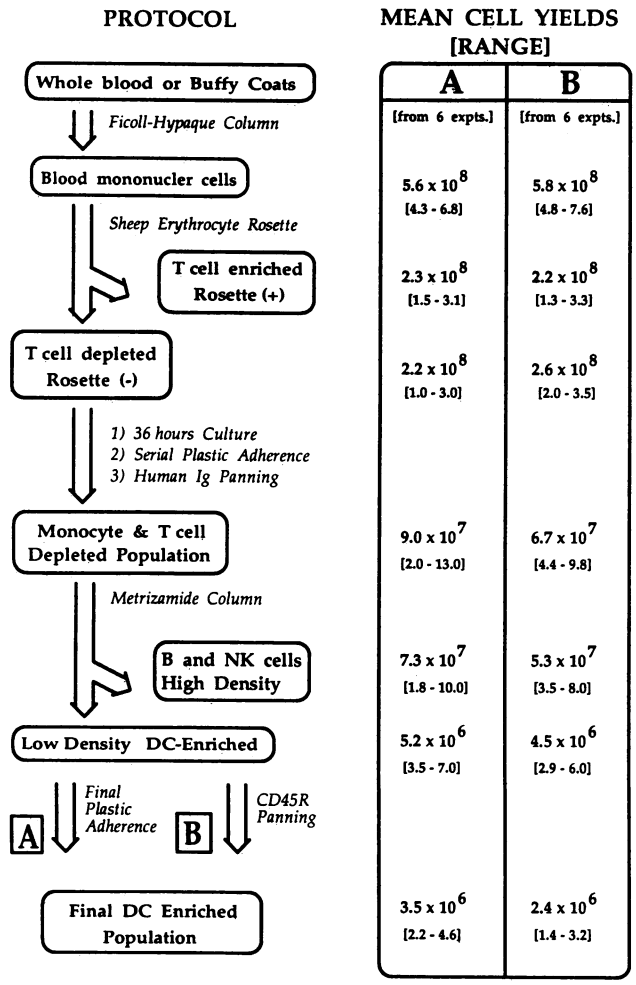

FIG. 1. Flow plan and yields of the dendritic cell (DC) isolation protocol. (*Left*) Outline of the steps in the isolation protocol. (*Right*) Mean and range of cell yields in two sets of six experiments. Each set (A, B) utilized a different final step.

sorted on a FACStar Plus (Becton Dickinson). Large (high forward scatter) Leu18$^-$ cells were selected as the dendritic cells. A "cocktail" of six phycoerythrin-conjugated mAb to other cell types (Leu4, Leu11c, Leu12, Leu16, Leu19, and LeuM3; Becton Dickinson), each added at 10 μl per 10^6 cells, could be used instead of Leu18. Dendritic cells were again collected as the large unlabeled cells.

FACS Analyses. Unless otherwise stated, populations were stained with mouse mAbs followed by fluorescein-conjugated goat antibodies to mouse immunoglobulin. The cells were analyzed on a FACScan instrument (Becton Dickinson).

Mixed Leukocyte Reaction (MLR). Stimulators (antigen-presenting cells, APC) for the MLR were derived from the separation protocol. The APC were irradiated [3000 rads (1 rad = 0.01 Gy) from ^{137}Cs] and added in graded doses to 1.5 $\times$ 10^5 allogeneic or syngeneic T cells in 96-well flat-bottom tissue culture plates in 0.2 ml of medium per well. Proliferation was measured by the uptake of [^{3}H]thymidine [1 μCi per well, 6 Ci/mmol (1 Ci = 37 GBq)] added for 8 hr on the 5th day. The responses are reported as the mean cpm of triplicates, omitting the standard deviations, which were <10%.

Motility and Morphology. Morphology and motility were monitored at 37°C on an incubated-stage inverted microscope (Nikon Diaphot) with a video camera, a video cassette recorder, and monitor attached.

RESULTS

Method and Monitoring Criteria for Enriching Human Blood Dendritic Cells. Fig. 1 outlines a sequence of steps that reliably enriched the trace population of human blood dendritic cells. Using protocol A, we obtained 3.5 $\times$ 10^6 cells that were 30–60% dendritic cells. Protocol B allows further enrichment (80–90%) by using the CD45R panning method discussed below. At the same time, three other dendritic cell-depleted populations were obtained: T cells by erythrocyte rosetting, monocytes by plastic adherence after 1–2 days in culture, and a mixture of B and NK cells by sedimentation in metrizamide (Fig. 1).

Two approaches were used to document the progressive enrichment of dendritic cells. First, observation of live preparations best revealed an irregular shape and motility, as outlined below. Second, cytofluorography displayed the enrichment of large cells (high forward scatter) that failed to stain with a cocktail of mAbs to other types of leukocyte (Fig. 2: CD14, monocytes; CD16 and CD56, NK cells; CD19 and CD20, B cells; and CD3, T cells). Such large "null" cells were not detected in the other fractions (Fig. 2 *A*, *B*, *E*).

To further purify the dendritic cells—i.e., to 80–90% purity or more—we took advantage of a finding that dendritic cells lack CD45R, but the majority of contaminants in the metrizamide low-density fraction express high levels. Contaminants could then be depleted by panning with the CD45R mAb, Leu18 (Fig. 2 *E–H*). Alternatively, one could sort the dendritic cells to an even higher purity on a FACS by marking the contaminants either with anti-CD45R or with the cocktail of mAbs (Fig. 3*A*). Sorting provided the highest purity, but panning provided higher yields of dendritic cells.

In summary, the above method enriched a trace subset of large, "null," irregularly shaped cells. This procedure can be used to isolate dendritic cells from small volumes of blood (60–120 ml) with yields proportional to those from buffy coat fractions (450–500 ml starting blood volume) (not shown).

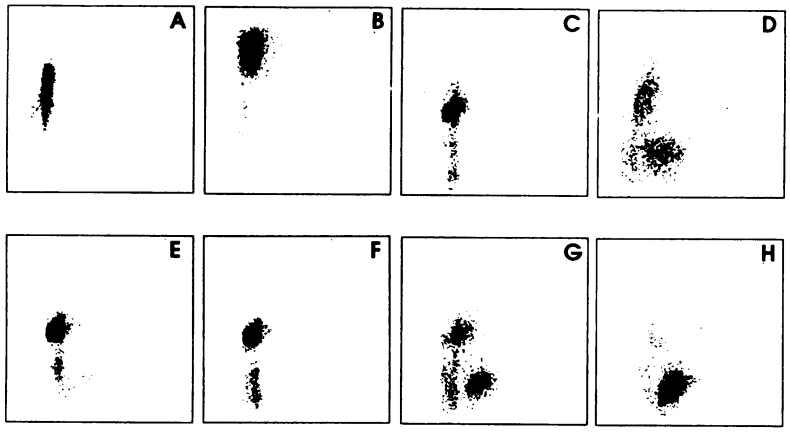

FIG. 2. (*A–D*) The four major cell populations generated from a typical separation protocol experiment. All populations were stained with the cocktail of mAbs to T-cell, B-cell, NK-cell, and monocyte markers. *x*-Axis, forward light scatter; *y*-axis, fluorescence intensity (logarithmic scale, 4 powers of 10). (*A*) ER$^+$/T-cell enriched. (*B*) Adherent/monocyte-enriched. (*C*) Metrizamide high density/B- and NK-cell-enriched. (*D*) Metrizamide low density/dendritic-cell-enriched. (*E–H*) Dendritic cell enrichment by using CD45R panning. All populations were stained with the mAb cocktail as above. (*E*) ER$^-$, monocyte-depleted population. (*F*) Metrizamide high-density population. (*G*) Metrizamide low-density population. (*H*) Panned (CD45R$^-$) enriched dendritic cell population.

Motility of Dendritic Cells. Each dendritic cell continually extended and reoriented processes, usually as lamellipodia or veils (Fig. 4). Individual veils extended 10–30 μm and could pass through an arc of 180°C in 30–60 sec. This pattern of veil formation and movement has been noted previously (15, 16). Our experiments indicated that this is a feature of all dendritic cells and is not seen in monocytes and lymphocytes studied in parallel.

Surface Composition of Dendritic Cells: FACS Analyses. With the populations that we obtained, it was feasible to analyze surface antigens with a large panel of mAbs (Table 1) and the FACS (Fig. 3*B*). Several conclusions were evident. (*i*) Dendritic cells had high levels of surface MHC products, including all three MHC class II loci (HLA-DR, -DQ, and -DP) and the class II-associated invariant chain. Monocytes, isolated from the identical cultures, expressed 1/10th to 1/100th as much HLA-DR and had low or undetectable levels of HLA-DQ, HLA-DP, and the invariant chain (not shown). (*ii*) Dendritic cells either lacked or expressed very low levels of most T-cell, B-cell, NK-cell, and phagocyte (monocyte and granulocyte)-restricted markers, but all cells expressed comparable levels of the leukocyte common antigen, CD45. (*iii*) Dendritic cells had a distinct repertoire of surface receptors and adhesion molecules. All known Fc and complement component 3 (C3) receptors (FcR and C3R) were weak or absent, as was the c-fms receptor for colony-stimulating factor (CSF)-1 or macrophage CSF, but the p55 interleukin 2 receptor (IL-2R) (CD25) was present. Adhesion molecules were abundant, including ICAM-1 (CD54), LFA-3 (CD58) and several integrins (β_1/CD29, β_2/LFA-1/CD11a, and p150/90/CD11c). In contrast, monocytes expressed CD32 FcR, CD11b C3R, and CSF-1R, and had much lower levels of ICAM-1, LFA-3, and IL-2R. (*iv*) The phenotype of dendritic cells was uniform in that most markers were either completely absent or expressed to a comparable degree.

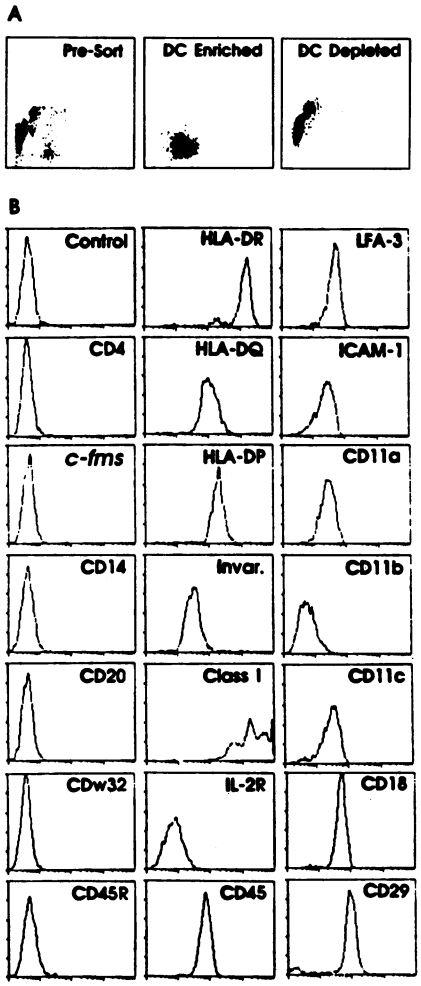

FIG. 3. (*A*) FACS separation of dendritic cells (DC) on the basis of CD45R expression. *x*-Axis, forward light scatter; *y*-axis, fluorescence intensity (logarithmic scale, 4 powers of 10). Dendritic cells are sorted as large CD45R$^-$ cells. (*B*) Highly enriched (sorted) dendritic cells stained for key surface markers. *x*-Axis, fluorescence intensity (logarithmic scale, 4 powers of 10); *y*-axis, frequency.

MLR Stimulating Activity of Enriched Populations of Blood Mononuclear Cells. The APC function of the populations prepared as in Fig. 1 were compared by testing stimulatory capacity for allogeneic T lymphocytes in the primary MLR. As one enriched for dendritic cells, stimulatory capacity was markedly enhanced (Fig. 5). Bulk blood mononuclear cells were 1/30th to 1/100th as active as enriched dendritic cells. Monocyte, B-, and T-cell populations were significantly less stimulatory in the MLR than bulk mononuclear cells. Only dendritic cells induced a syngeneic MLR (Fig. 5 *Right*).

DISCUSSION

Dendritic cells can be isolated by sequentially removing each of the other cell types in blood. Our method differs from prior methods in the degree of enrichment achieved and in two additional respects. First, all the blood mononuclear cells are retained in either dendritic cell-depleted or -enriched populations. Second, each fraction can be monitored with the FACS or by direct observation looking for large, "null," class II-MHC-rich cells or for motile dendritic cells.

The strategy (Fig. 1) utilizes more than one negative selection technique to deplete each cell type. This is necessary, because dendritic cells are outnumbered 30- to 300-fold by any other mononuclear cell type in blood. T-cell depletion occurs through erythrocyte rosetting, sedimentation in metrizamide, and panning or sorting with mAb; monocyte depletion requires a combination of plastic adherence and

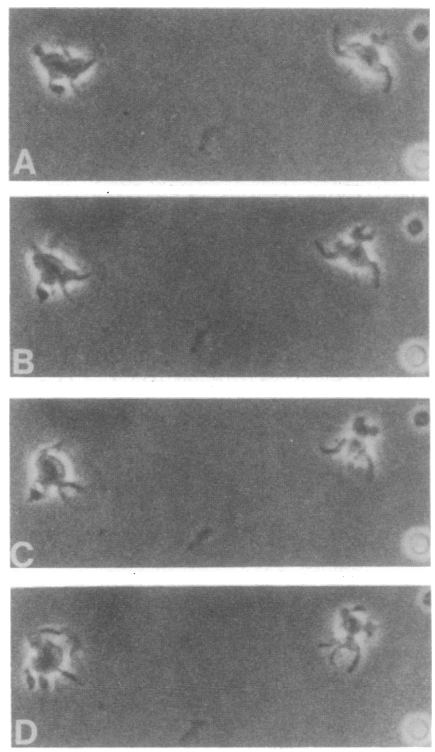

FIG. 4. Motility of dendritic cells. Four frames of two dendritic cells taken at 10-sec intervals. Note the rapid changes in morphology and veil position. (×380.)

Table 1. mAbs and their reactivities with various cell populations

Determinant	mAbs	Staining				
		DC	Monocytes	Granulocytes	B&NK	T cells
MHC						
HLA-A,B,C	W6/32	+++	++	++	++	++
HLA-DR	L243, L227, SG1.7	+++	+	−	++	−
HLA-DQ	HB103, 1A3	+++	−	−	+	−
HLA-DP	B7/21	+++	−	−	tr/+	−
Invariant chain	BU43, BU45	+/++	−	−	−	−
Leukocyte antigen						
CD45	4B2, VIT200	+++	++	+/++	++	++
CD45R	4G10, Leu18	−	−	−	++	−/++
Myeloid						
CD13	MoU28	−	+			−
CD14	3C10, LeuM3, VIM13, 26ic	−	+/++	tr	−	−
CD15	VIM10, VIM11, LeuM1	−	−	+/++	−	−
CD36	OKM5	tr	++	tr	−	−
CDw65	VIM2, VIM8	−	−	+++	−	−
Receptors						
CD16, FcRγIII	3G8	−	−	+/++	+/++	−
CDw32, FcRγII	IV.3, CIK-M5	−	+	+/++		−
CD64, FcRγI	10.1, 32.2	−	−	−		−
CD25, IL-2R$_{LOW}$	Am47.1, AM92.2, V-IL2Ra, V-IL2Rb	+/++	−	−	−	−/tr
c-fms, CSF-1R	2-4A5-4	−	+		−	−
Integrin/adhesin						
CD11a, LFA-1	TS1/22	++	+	+	+	+
CD11b, C3biR	OKM1, VIM12	tr	+	tr	tr	−
CD11c, p150/90	LeuM5, L29	++	+/++	tr	tr	−
CD18, β_2-integrin	TS1/18	++	+	+/++	+	+
CD29, β_1-integrin	A1A5	++	+			
CD54, ICAM-1	RR1/1, LB-2	++	+	tr	+	−
CD58, LFA-3	TS2/9	++	+	+	+	−
T-cell markers						
CD1a	OKT6	−	−	tr	tr	
CD1b	NU-T2	−	−	−	−	
CD1c	L161	tr	−	−	−	
CD2	TS2/18, OKT11, Leu5a	−/+	−	−	−	++
CD3	OKT3, Leu4	−	−	−	−	++
CD4	OKT4, Leu3	−	−	−	−	++
CD8	OKT8, Leu2	−	−	−	−	++
TcR α/β	WT/31	−	−	−	−	++
B-cell markers						
CD19	Leu12, HD-37	−	−	−	+/++	−
CD20	Leu16, L27	−	−	−	+/++	−
CD21	IF8	−	−	−	+	−
CD22	4KB128, Leu14	−	−	−	+	−
CD24	VIB-C5, VIB-E3	tr	−	−	+	−
CD40	G28-5	+/++	tr	−	+/++	−
NK-cell markers						
CD56	Leu19	−	−	−	+/++	−
CD57	HNK-1	−	−	−	+/++	−

DC, dendritic cells; B&NK, B and NK cells. The levels of mAb staining on the various cell populations obtained as in Fig. 1 are indicated as follows: −, no detectable staining; tr (trace), <5 × background; +, 5–15 × background; ++, 15–100 × background; +++, >100 × background. A range of staining is indicated with division of symbols by /. A blank means not done. mAbs are described in ref. 17.

FcR panning; and B- plus NK-cell depletion depends upon metrizamide sedimentation and a final panning or sorting step. At no point is there positive selection for cells with the unusual motility and phenotype of dendritic cells. The fact that such cells emerge upon negative selection supports the conclusion that they are a distinct element in human blood.

For a variety of reasons, it was advantageous to separate the monocytes from the dendritic cells after 36 hr in culture. (*i*) The monocyte is the main cell type that adheres firmly but reversibly to plastic after culture. This allows monocytes to be separated and subsequently recovered largely free from dendritic cells, many of which are adherent in fresh isolates. (*ii*) During culture, the phagocytic monocytes remove residual erythrocytes and other debris that are present after the T-cell depletion. Debris is a major contaminant of populations that are enriched by flotation on dense columns. (*iii*) The presence of monocytes or monocyte-derived factors seems to improve the viability of the final dendritic cell population. The criteria in this paper for identifying dendritic cells can now be used in fresh isolates of human blood.

The important functional role of dendritic cells is their initiation of primary responses such as the MLR. The data in Fig. 5 show the greatest differences that have been observed between dendritic cells and other cell types, presumably

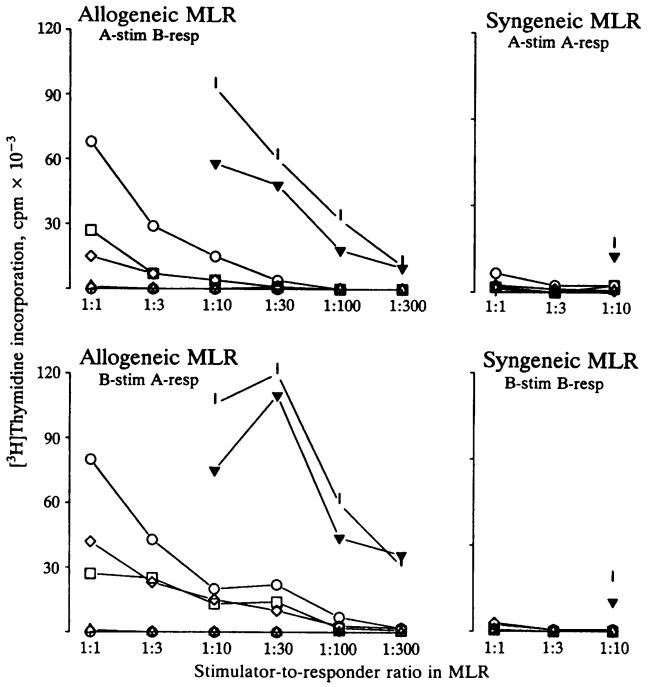

FIG. 5. MLR stimulatory function of the different cell populations isolated through the enrichment protocol. Typical reciprocal syngeneic and allogeneic MLRs from two donors (A and B) are shown. Responder cells (resp) are purified T lymphocytes. The stimulators (stim) were as follows: ○, bulk mononuclear cells; △, ER^+ T cells; □, monocyte-enriched cells; ◇, metrizamide high-density cells; ▼, metrizamide low-density cells; and ▮, $CD45R^-$, metrizamide low-density cells.

because of the greater purity of each population. In a recent report, granulocyte–macrophage CSF enhanced dendritic cell viability (18). This conclusion is puzzling, however, because the MLR-stimulating function of the dendritic cells in that report was only twice that of monocytes or other cells.

Because of the difficulty in enriching blood dendritic cells, it has not been possible previously to obtain a detailed semiquantitative analysis of their surface. The results of such an analysis are summarized in Table 1. The expression of high levels of all MHC class II (HLA-DR, -DQ, -DP) products is the most distinctive feature and helps to explain the observed potency of dendritic cells as APC. Given the high levels of HLA-DP, -DQ, -DR, and invariant chain, it is possible to generate mAbs that appear to be specific for dendritic cells, but are instead directed to these products. The repertoire of dendritic cell surface receptors is also unique. FcR (CD16, CD32, CD64) and C3R (CD11b, CD21, CD35), which are involved in particle binding and uptake, are all weak or absent, as is the lineage restricted c-fms receptor for macrophage CSF-1. p55 IL-2R (CD25) was detected by using any of the four mAbs we tested. The dendritic cells do not grow upon addition of interleukin 2 (1–100 units/ml), however. Dendritic cells have the highest levels among blood leukocytes of the adhesins CD29 (β_1-integrin), CD11a (LFA-1), CD54 (ICAM-1), and CD58 (LFA-3). Since adhesins are subject to up-regulation by cytokines, it is possible that blood dendritic cells are migrating from tissues where they have been exposed to such stimuli. The presence of adhesins helps to explain the capacity of dendritic cells to aggregate large numbers of T cells for long periods (10, 19).

Only granulocytes in blood move as rapidly and form such large lamellipodia as dendritic cells. However, granulocyte veils typically extend in a directed fashion in response to a chemotactic stimulus (20). Our enrichment protocol should facilitate the search for substances that influence dendritic cell movements. The potency of dendritic cells as APC might be augmented by rapid and vigorous movement, which would facilitate the sampling and formation of contacts with T lymphocytes.

We thank Mary T. Crowley for her expertise and assistance with cell sorting and William Swiggard for his helpful comments. Our thanks also go to the following colleagues for providing mAbs for this study: W. Knapp (University of Vienna), N. Romani (University of Innsbruck), S. M. Fu (University of Virginia, Charlottesville), and T. A. Springer (Dana–Farber Cancer Institute, Boston). This work was supported by National Institutes of Health Grant AI24540 (R.M.S.) and Training Grant GM07982-08 (P.S.F.) and by a grant from the David C. Scott Foundation (P.S.F.).

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