HUMAN DENDRITIC CELLS

Enrichment and Characterization from Peripheral Blood*

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Specialized dendritic cells (DC)[1] have been identified in lymphoid organs of mouse and rat (1–3). In all tissues, the frequency of DC is <1% of nucleated cells, as confirmed using a monoclonal antibody (Ab) specific for mouse DC (4). Methods for enriching DC are available (2, 3, 5). As a result, several distinctive functions of DC have been established. DC are potent stimulators of mixed leukocyte reactions (MLR) (3, 6–8) and accessory cells for several immune responses (2, 9–11). Recently, it has been shown that selective elimination of DC with monoclonal cytotoxic Ab and complement ablates MLR-stimulating capacity and accessory function in mouse spleen and spleen-adherent cells (12). The study of immune responses in man requires that the equivalent of the rodent DC be identified.

We have chosen peripheral blood mononuclear cells (PBMC) to begin the characterization of human DC for several reasons. Large numbers of blood leukocytes are readily obtained from blood-banking facilities, making it possible to enrich for a trace cell type like the DC. If the functional data on rodents are applicable to man, then DC should be present in PBMC, which are active MLR stimulators and proliferate when modified by oxidizing agents. One would also predict that some stage of the DC lineage would be present in the circulation, because DC are bone marrow-derived and undergo rapid turnover in mouse spleen (13).

We show here that 0.1–0.5% of human PBMC have the same cytologic features as rodent DC. Enriched (20–60%) populations of DC can be obtained with an approach modeled after the techniques used to purify mouse DC. Human DC are Ia positive and lack surface Ig, F_c receptors, the receptor for sheep erythrocytes, and several new lymphocyte and monocyte antigens defined by monoclonal Ab. Functional studies indicate that human DC are 10–30 times more active than other leukocytes as MLR stimulators and as accessory cells for oxidative mitogenesis.

Materials and Methods

Culture Media and Serum. Cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 1 mM glutamine (Gibco Laboratories),

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[1] *Abbreviations used in this paper*: Ab, antibody; BPA, bovine plasma albumin; DC, dendritic cells, E, erythrocyte; EA, sheep E coated with anti-sheep E Ab; E_{AET}, sheep E modified with 2-aminoethylisothiouronium bromide; EIgM, sheep E coated with anti-sheep E IgM Ab; EIgMC, sheep E coated with anti-sheep E IgM Ab and complement; EM, electron microscopy; E_n, sheep E modified with neuraminidase; FCS, fetal calf serum; HRP, horseradish peroxidase; MLR, mixed leukocyte reaction; NBCS, newborn calf serum; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PLL, poly-L-lysine.

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5×10^{-5} M 2-mercaptoethanol (Eastman Kodak, Rochester, NY), 100 U/ml penicillin, 20 μg/ml gentamycin (Schering Corp., Kenilworth, NJ), and 5–10% serum. Fetal calf serum (FCS; Flow Laboratories, Inc., Rockville, MD) and newborn calf serum (NBCS; North American Biologicals, Miami, FL) were heat inactivated at 56°C for 30 min. Human serum was prepared from 1 U of whole A^+ blood with no additives (Greater New York Blood Center, New York) after 4 h of clotting at 4°C, clarification by centrifugation, and storage at −70°C.

Microscopy, Staining, and Cytochemistry. To examine cells in suspension by light microscopy, we centrifuged (400 *g*, 10 min, room temperature) 2×10^5 cells onto 12-mm circular cover slips (Propper Manufacturing Co., Long Island City, NY, CGS, distributed by SGA Scientific, Bloomfield, NJ) coated with 75 μg/ml poly-L-lysine (PLL, type VII, Sigma Chemical Co., St. Louis, MO), and incubated the cover slips for 20 min in RPMI 1640 to allow spreading. For phase contrast, cells were fixed with 1.25% glutaraldehyde in phosphate-buffered saline (PBS) for 15–30 min. To make lysosomes visible, cells were exposed to acridine orange (Allied Chemical Corp., Morristown, NJ), 2.5 μg/ml culture medium at 37°C for 20 min, rinsed, and mounted in medium for immediate viewing with a fluorescent microscope as described (14). Giemsa (10% in PBS, Fisher Scientific Co., Pittsburgh, PA) staining was for 10–20 min on cytocentrifuged specimens fixed 10 min in absolute methanol. Endogenous myeloperoxidase and exogenous horseradish peroxidase (type II, Sigma Chemical Co.) were visualized in glutaraldehyde-fixed cells exposed to 0.4 mg/ml 3,3′diaminobenzidine tetrahydrochloride (grade II, Sigma Chemical Co.) and 0.1% hydrogen peroxide (Mallinckrodt Inc., St. Louis, MO) in 0.1 M tris, pH 7.5, for 10 min at 20°C. Nonspecific esterase staining with alpha-naphthyl-butyrate (Sigma Chemical Co.) was exactly according to Li et al. (15).

For transmission electron microscopy (EM), cells were fixed in a mixture of 1.25% glutaraldehyde and 1% osmium tetroxide, stained en bloc with 0.25% uranyl acetate, and embedded in Epon as described (1). For scanning EM, cells on cover slips were fixed in 1.25% glutaraldehyde/PBS, washed, and stored in 0.1 M sucrose in 0.1 osmium cacodylate pH 7.4. Just before examination, the cover slips were dehydrated in ethanol, transferred to amyl acetate, and critical-point dried (Hitachi CTI, Tokyo, Japan) in CO_2. Specimens were coated with gold (Polaron EM coating unit, E5000, Polaron Instruments Inc., Doylestown, PA) and examined with a high resolution Hitachi scanning electron microscope (HHS/2R) at 20 kv, at a tilt angle of 30° (16).

Endocytic Tests. Soluble horseradish peroxidase was used as a marker for pinocytosis as described (17). Zymosan (Sigma Chemical Co.) and latex (1.1 μm, The Dow Chemical Co., Indianapolis, IN) were used to monitor phagocytosis at particle/cell ratios of 20:1 and 200:1, respectively, for 1 h at 37°C.

Indicator Erythrocytes (E): Sheep erythrocytes (Scott Labs, Fiskeville, RI) and bovine erythrocytes (Rockland Inc., Gilbertsville, PA) were obtained in alsevers and used within 2 wk of arrival. For E rosetting, washed sheep E at 5% vol/vol in RPMI 1640 were treated with 0.02 U of *Vibrio cholerae* neuraminidase (E_n) (Calbiochem-Behring Corp., LaJolla, CA) per ml at 37°C for 45 min and washed three times (18); alternatively, washed sheep E were treated with 2 aminoethylisothiouronium bromide (E_{AET}) (Calbiochem-Behring Corp.) exactly by the method of Kaplan et al. (19). Both E_n and E_{AET} rosetted >80% of nonadherent PBMC, but <5% of B cell-enriched fractions. Sheep E coated with antibody (EA), IgM (EIgM), and IgM plus mouse complement (EIgMC) were prepared as described (5). E-anti-human Ig were prepared using saline-washed bovine E and chromic chloride coupling (20). Rabbit anti-human $F(ab)'_2$ IgG fragment serum was affinity purified on human IgG linked to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. Pharmacia Inc., Piscataway, NJ). E anti-human Ig bound >80% of B cell and <5% of T cell-enriched fractions. Rosettes were formed in suspension (50 indicator cells per leucocyte were spun into a pellet) or on monolayers (1×10^7 to 2×10^7 indicator cells were spun onto 12-mm cover slips in 13-mm glass cylinders).

Immunofluorescence. All reagents for immunofluorescence were diluted in 5% NBCS-RPMI 1640–0.02% sodium azide. Two heteroantisera were directly labeled with fluorochrome: rhodamine-coupled $F(ab)'_2$ goat anti-human IgG, IgM, and IgD (N. L. Cappel Laboratories, Cochranville, PA), and rhodamine-coupled $F(ab)'_2$ rabbit anti-human glycoprotein 29/33 (anti-HLA-DR [21], a generous gift of Dr. Shu Man Fu of The Rockefeller University). A large group of mouse monoclonal Ab were visualized by indirect immunofluoresence: anti-HLA

(61D2 [22], 1 μg/ml, Bethesda Research Laboratories, Rockville, MD), anti-HLA-DR (59.5, 1 μg/ml; and OKIal [23], 1/40 of ascites, Ortho Pharmaceutical, Raritan, NJ); antimonocyte (63D3, 10 μg/ml; 61D3 [22], 1 μg/ml BRL; OKMl [24], 50 μg/ml, Ortho Pharmaceutical; and Ml/70, rat anti-mouse "Mac-1" [25], culture supernatant kindly supplied by Dr. T. Springer, Harvard University, Boston, MA); anti-F_c receptor (clone 3G8 [26], 30 μg/ml, kindly provided by Dr. J. Unkeless and Dr. H. Fleit of The Rockefeller University); anti-leukocyte common antigen (T29/33 [27], 1:300 dilution of ascites kindly supplied by Dr. I. S. Trowbridge, Salk Institute, La Jolla, CA); anti-human T cells (OKT 3 [28, 29], pan T cell Ag, 0.6 μg/ml; OKT6 [29, 30], common thymocyte and Langerhans cell Ag, 1.2 μg/ml; OKT8, [28, 29], suppressor cytotoxic Ag, 50 μg/ml, Ortho Pharmaceutical). Cells on PLL-coated cover slips were exposed to the primary Ab for 60 min at 4°C, rinsed in PBS, exposed to biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) at 50 μg/ml for 30 min at 4°C, rinsed in PBS, and overlaid with fluoresceinated avidin (Vector Laboratories) at 100 μg/ml for 30 min at 4°C, rinsed, fixed in 10% formalin/PBS for 20 min, mounted in glycerol, and viewed by epifluorescence (Zeiss photomicroscope II, Carl Zeiss, Inc., New York). To detect intracellular Ig, cells on PLL-coated cover slips were formalin fixed, rinsed in PBS, exposed to absolute methanol for 30 s, rinsed in PBS, and treated with rhodamine anti-human Ig, as above. This technique detects intracellular Ig in plasma cells from tonsil.

Enriched Populations of T Cells, Monocytes, B Cells, and DC (Table I). Our starting population was a concentrated leukocyte fraction (buffycoat) derived from 400 ml of blood by the Greater New York Blood Center. PBMC were recovered on Ficoll 400/sodium diatrizoate (Histopaque 1.077, Sigma Chemical Co.) with a yield after three washes of 4×10^8 to 10×10^8 mononuclear cells per buffycoat. Leukocyte subpopulations were identified by standard cytologic and surface markers as described in results.

5×10^7 PBMC were plated in 5% FCS medium on 100-mm plastic tissue culture plates (Falcon 3003, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) for 1 h at 37°C. Nonadherent cells were removed with five rinses of RPMI 1640 and used as a source of T cells. The T cells were purified on nylon wool according to Julius et al. (31). 10×10^7 to 20×10^7 nonadherent PBMC in 10% NBCS medium were loaded onto 3 g of nylon wool (Leukopak, Fenwal Lab, Division of Travenol, Deerfield, IL) in a 30-cc disposable plastic syringe, incubated at 37°C for 45 min, eluted dropwise over 15 min, and washed three times in RPMI 1640 before use. The population was >97% T cells, <3% monocytes, and <1% DC. Alternatively, nonadherent PBMC were separated into E rosette positive and negative fractions after rosetting with a 50:1 ratio of E_n to PBMC and centrifugation on Ficoll/diatrizoate (17). E_n were lysed in ammonium chloride and the fractions washed three times before use. The E^+ fraction was >90% T cells, <10% monocytes, and <1% DC. The E^- or non-T cell fraction was <20% T cells, 30–50% Ig-positive cells, 8–30% monocytes, and 2–10% DC.

Adherent PBMC were incubated for 16 h at 37°C in 5% FCS medium. About one-half the cells had released from the plates (released once) and were reattached in fresh medium to another 100-mm plastic plate for 1 h. The populations that adhered after overnight culture, or after the readherence step, were highly enriched in monocytes (80–90%) and had some small lymphocytes (5–15%) and DC (1–6%). Monocytes were eluted from the plates after treatment with phosphate saline supplemented with 10 mM ethylenediaminetetraacetic acid (Fisher Scientific Co., Pittsburgh, PA), pH 7.4, for 30 min at 4°C, or were dislodged by vigorous pipetting.

Cells that did not readhere (released twice) after overnight culture were used as a source of enriched B cells and DC. The population was either rosetted with E anti-human Ig (50:1 ratio of E anti-Ig/PBMC; 4°C, 20 min) or floated directly in bovine plasma albumin (BPA; Armour Pharmaceutical, Chicago, IL). 2×10^7 to 4×10^7 cells (+/− rosettes) were resuspended in 4 ml BPA, $d = 1.082$, and overlaid with BPA at 1.047 (2 ml). The columns were centrifuged at 10,000 *g* for 15 min at 4°C, to provide low density (1.047:1.080) interface and high density (pellet) fractions that were harvested and washed 3 times in RPMI 1640. The low density population could be additionally depleted of monocytes by a final adherence on plastic. The low density fraction (2×10^6 to 5×10^6 cells/buffycoat) contained 20–60% DC, 10–25% monocytes, and the balance small cells. If the fractionation included E anti-Ig rosetting, <1% of the low density cells had surface Ig, even after 4 d of additional culture. Without rosetting,

TABLE I

Enrichment Procedure for Dendritic Cells in Human Blood

	Concentrated leukocyte fraction (buffy coat)	Cell yield (percent of starting)	Percent DC
Granulocytes ← erythrocytes	Ficoll-Diatrizoate		
	PBMC	100	<1
Nonadherent PBMC ←	Adherence		
E_n rosetting or nylon wool → T cell enriched			
	Adherent PBMC	20	1–2
Firmly adherent, monocyte enriched ←	Culture 16 h		
	Released 1 ×	11	2–4
Readherent, monocyte enriched ←	Readhere		
	Released 2 ×	6	3–6
High density, released 2 × B cell enriched ←	Flotation in dense BPA; +/− rosetting E α-human Ig		
	Low density, released 2 ×	0.8	10–30
Readherent, released 2 × ←			
	Low density, released 3 × DC enriched	0.5	20–60

20–40% of the low density population was Ig positive cells. The high density fraction was always >80% Ig positive cells with small numbers of monocytes (2–5%) and DC (1–4%). DC in the high density fraction would not float if centrifuged in an additional BPA column.

Functional Assays. Functional assays were done in triplicate microtest wells with 1.5×10^5 responder T-enriched lymphocytes per flat-bottomed well (Costar, Data Packaging, Cambridge, MA) in 200 μl culture medium, or in round-bottomed wells (Linbro Chemical Co., Hamden, CT) in 150 μl medium. A+ leukocytes and 10% A+ human serum were used. T cells were prepared from nonadherent PBMC by E_n rosetting, or passage over nylon wool, as described above. For MLR assays, stimulator cells were irradiated with 3,000 rad (Cs^{137}, Gamma cell 1000, Atomic Energy of Canada, Ottawa, Canada). For oxidative mitogenesis, the accessory cells were irradiated, and the T cells were modified with 2 mM sodium periodate (Sigma Chemical Co.) in PBS for 30 min at 0°C, followed by washing. Proliferation by unmodified (not periodate-treated) controls was <10% of periodate-treated samples. Proliferation was measured with 1 μCi [^{3}H]thymidine in 50 μl/well (Schwarz/Mann Div., Becton, Dickinson &

Co., Orangeburg, NY, 6 Ci/mM) at 104–120 h (MLR) and 56–72 h (periodate). Cells were harvested in a multisample harvester, and the data displayed as mean counts per minute ± standard deviation of the mean.

Results

A small percentage (1–2%) of adherent human PBMC had similar morphologic features to the DC isolated from mouse lymphoid organs (1). These features included: irregular shape, abundant phase-dense granules (mitochondria), irregular nucleus with small nucleoli, and a prominent rim of heterochromatin. There were few surface ruffles, pinocytic vesicles, or lysosomes, organelles that were abundant in adjacent monocytes (Fig. 1 a). We first devised a multistep procedure for enriching these "candidate" DC. This involved successive depletion of T cells, monocytes, and B cells. We could then show that the cytologic, surface, and functional properties of human DC were similar to their counterparts in rodents.

Enriched Preparations of Human DC. The purification of human DC was monitored by phase-contrast microscopy of specimens fixed in glutaraldehyde. At each stage of the protocol (Table I), samples were attached to PLL-coated cover slips and incubated at 37°C to allow cells to spread. DC exhibited many processes, mitochondria, and irregular nuclei (Fig. 1 b, c). Monocytes spread circumferentially and had clear ruffles, pinocytic vesicles, lysosomes, and oval- or kidney-shaped nuclei (Fig. 1 d). Small lymphocytes showed little spreading (Fig. 1 c), except in the case of B cells, which

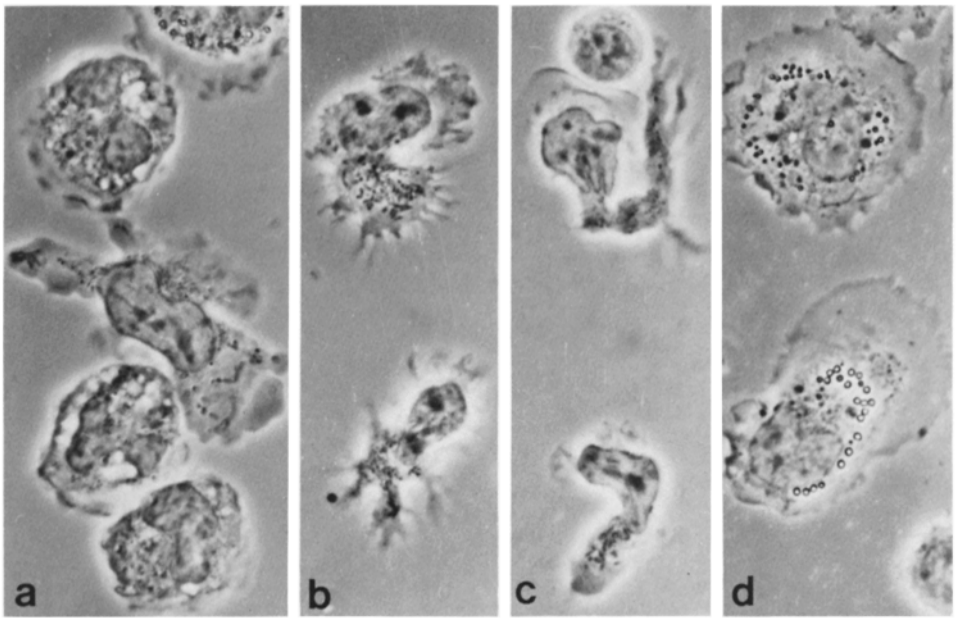

FIG. 1. Phase-contrast microscopy of glutaraldehyde-fixed, adherent mononuclear cells (× 1,080). (a) Fresh mononuclear cells adherent to glass. A DC is surrounded by four monocytes, which exhibit typical ruffles, lysosomes, and pinocytic vesicles. (b, c) DC from DC-enriched fractions, after attachment and spreading on PLL-coated glass cover slips. Dendrites extend in several directions, and the cytoplasm is full of granules that are mitochondria. At the top of Fig 1 c is a typical small lymphocyte, which does not have processes or ruffles when attached to PLL. (d) Monocytes adherent to PLL-coated glass. The cells spread more actively than on non-PLL-coated glass or plastic, and the characteristic ruffles and organelles are more readily visualized.

could form cell processes when attached to anti-Ig-coated erythrocytes. Subsequent studies with cell surface markers confirmed that these cytologic criteria were appropriate for differentiating DC, monocytes, and lymphocytes (see below).

DC enrichment required several steps and stimultaneously provided populations that were predominately monocytes, B cells, or T cells (Table I). Mononuclear cells (<1% DC) were obtained from Ficoll-Diatrizoate columns and attached to plastic. The adherent population contained 70–90% monocytes, 5–15% lymphocytes, and <5% DC. The nonadherent fraction contained most of the T cells and small percentages of monocytes (<5%), B cells (5–10%), and DC (<1–2%). The adherent population was cultured overnight and the once-released cells reattached to plastic. This provided an adherent fraction rich in monocytes and a nonadherent (twice-released) fraction containing B cells, most of the DC, and variable percentages (10–40%) of monocytes. The twice-released cells were then floated on dense BPA columns to obtain a high density B lymphocyte fraction and a low density population enriched in DC. Further enrichment of DC could be obtained by depleting B cells with E anti-Ig and by additional adherence to monocytes to plastic.

This procedure reproducibly provided populations that were 20–60% DC, so that a >100-fold enrichment over PBMC had been obtained. The principle contaminating cells were monocytes (10–20%) and small cells of uncertain type that lacked B and T lymphocyte markers. Attempts to further purify DC led to unacceptable losses. We also analyzed specimens obtained from fresh venipuncture, rather than buffycoat. In 10 samples from 8 donors, we obtained a yield of low density, once-released DC corresponding to 0.3 ± 0.2% of total PBMC.

Staining Techniques and Electron Microscopy. DC-enriched fractions were compared with other cell types using several cytologic techniques. On Giemsa stain, the DC cytoplasm was a pale blue-gray, like monocytes, and lacked azurophilic granules. Both DC and lymphocytes had small numbers of lysosomes, detected by acridine orange staining, whereas all monocytes had abundant granular staining. DC and lymphocytes were peroxidase negative, whereas >80–90% of monocytes had typical peroxidase-positive granules. Finally, DC and lymphocytes were esterase negative, or had weak staining in the perinuclear region, while >80% of monocytes were strongly positive.

By transmission EM (Fig. 2 a), the DC-enriched fraction contained small cells (~50%) with the features of lymphocytes, a minor component (<20%) of typical monocytes with many lysosomes and surface ruffles, and cells (25–40%) resembling mouse DC (Fig. 2 a). The nuclei of presumptive DC were irregular in shape, with a thick rim of heterochromatin and small nucleoli. The surface was irregular in contour but lacked the discrete microvilli and ruffles seen on lymphocytes and monocytes. Mitochondria were numerous, but rough endoplasmic reticulum, free polysomes and ribosomes, and lysosomes were scanty. Multivesicular bodies and chains of small, smooth vesicles were noted. Birbeck granules, which are found in epidermal Langerhans cells, were not detected in either DC- or monocyte-enriched fractions.

By scanning EM (Fig. 2 b, c) 30% of the cells in the DC-enriched population had similar features to adherent mouse DC. When attached to PLL, the smooth DC surface formed many dendrites and large lamellipodia. Monocytes were entirely different in appearance because they spread circumferentially and were covered with surface folds (Fig. 2 d). We conclude that the DC in human PBMC have similar

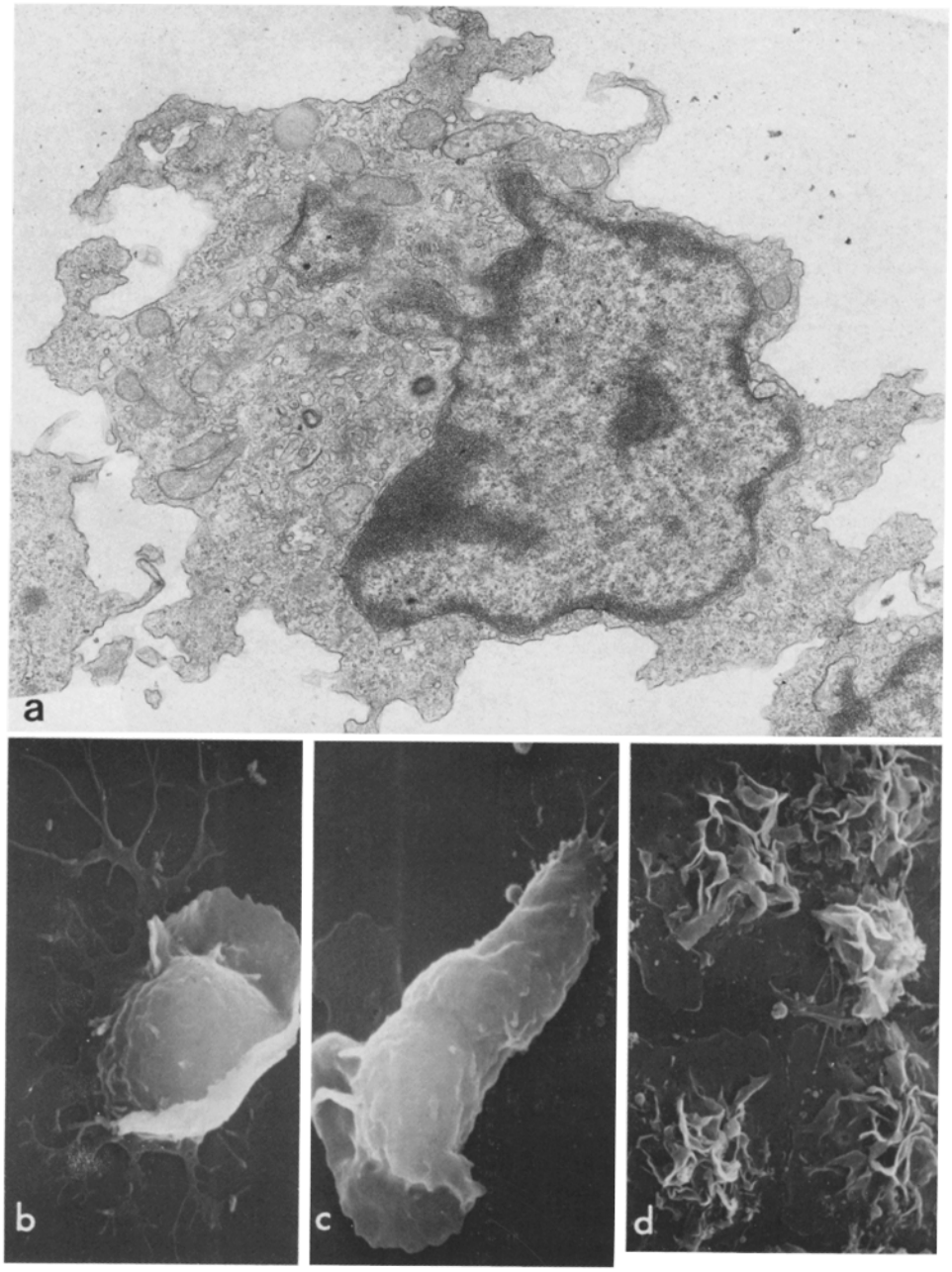

FIG. 2. EM of glutaraldehyde- and osmium- ixed mononuclear cells. (a) Transmission EM of presumptive DC fixed in suspension. Cells with similar cytologic features (see text) account for about one-third of DC-enriched fractions. × 11,300. (b, c) Scanning EM of DC fixed 20 min after attachment to PLL-coated cover slips. The smooth surface forms many dendrites and/or large lamellipodia. × 5,900 and 6,300. (d) Scanning EM of monocytes fixed 20 min after attachment to PLL. The surface is covered with folds, and the cells spread circumferentially. × 1,900.

cytologic features to mouse DC, and can be distinguished from monocytes and lymphocytes.

Endocytic Capacity and Rosette Assays. DC behaved more like lymphocytes than monocytes in standard endocytic tests. DC did not phagocytose zymosan particles or latex, although some sticking of latex to DC (one to three beads per cell) was evident. In contrast, monocytes actively phagocytosed both particles. DC pinocytosed little or no horseradish peroxidase (HRP), as assessed by diaminobenzidine cytochemistry, after exposure to 1 mg/ml of enzyme for 1–2 h. Although monocytes had endogenous peroxidase activity, addition of exogenous HRP for 1 h at 37°C resulted in dramatically increased granular peroxidase staining, due to active pinocytosis.

DC and monocytes were also compared in rosette assays with appropriately modified erythrocytes (see Materials and Methods). DC failed to bind E_n, E_{AET}, E anti-human Ig, EIgM, and EA. However, 2–10 EIgMC bound to DC identified by cytologic criteria. Mouse spleen DC failed to bind EIgMC, as previously reported (5). Monocytes did not bind E_n, E_{EAT}, and EIgM. 3–5 E anti-human Ig bound to 10–20% of monocytes. Large numbers of EA and EIgMC rosetted to monocytes. The EA were also phagocytosed. Thus, human DC have weak endocytic capacities, are Fc receptor, surface Ig, and sheep erythrocyte receptor negative, but express complement receptors.

Cell Surface Markers—Indirect Immunofluorescence. A large group of Ab, primarily monoclonals, were used to visualize surface antigens by immunofluorescence, usually with sensitive biotin-avidin reagents (Table II). All cells expressed the leukocyte-common antigen (clone T29/33) and HLA (clone 61D2). Most of the cells in the DC-enriched fraction were strongly Ia positive using OKIa 1, anti-HLA-DR (clone 59.5), and an $F(ab)'_2$ rabbit anti-GP 29/33 (20). Ia was found on most B cells, 30–80% of

TABLE II

Fluorescent Staining of Subpopulations of PBMC

Antibody (antigen)	T cell enriched*	Monocyte enriched‡	B cell enriched§	DC enriched ‖
	%	%	%	%
OKT3 (PAN T cell antigen)	96	10	8	11
OKM1 (monocyte/null cell antigen)	5	91	5	10
OKIa1 (HLA-DR antigen)	3	92	92	89
T29/33 (Human leukocyte-common antigen)	100	100	100	100
αIg (surface Ig)	<1	2	85	<1
63D3 (monocyte antigen)	1	87	ND¶	9
OKT6 (Thymocyte-common antigen and Langerhans cell antigen)	<1	<1	<1	<1
61D3 (monocyte antigen)	ND	96	ND	9
Ml/70 (mac-1; granulocyte, monocyte antigen)	ND	96	ND	10

Data are representative of three or more experiments with each cell type and antibody, and are expressed as percent positive (fluorescent) of 200 or more total cells counted. Cell populations were prepared as in Table I and included:

* Nylon wool-purified T cells. Phase-contrast morphology: 98% lymphocytes, 2% monocytes, 1% DC.

‡ Firmly adherent cells. Phase-contrast morphology: 87% monocytes, 11% lymphocytes, 1% DC.

§ Released twice, high density cells. Phase-contrast morphology: 95% lymphocytes, 4% monocytes, 1% DC.

‖ Low density released three times, E anti-human Ig-negative cells. Phase-contrast morphology: 37% DC, 10% monocytes, 53% small cells.

¶ Not done.

monocytes, and few T cells. Several monocyte antigens (clones 63D3, 61D3, OKM 1, and Mac-1, or M1/70) were detected on all monocytes and few of the cells in the B- and T-enriched fractions. 10–20% of the DC-enriched population stained with the monocyte Ab. This corresponded exactly to the percentage of monocytes enumerated by cytologic features on well-spread, glutaraldehyde-fixed specimens; also, many of the positive cells in the fluorescent specimens showed cytologic features of monocytes. A monoclonal anti-F_c receptor reagent, clone 3G8, did not stain cells in the DC-enriched fraction, but it is known that this reagent stains neutrophils primarily, and relatively few monocytes and B cells (25). All B cells stained for surface Ig, but staining was rare in purified monocytes and DC fractions. Less than 2% of the latter stained for intracellular Ig as well. T cell-enriched populations contained >95% OKT 3- (pan T) reactive cells, whereas DC, monocytes, and B cells had ~10% OKT 3 positive cells. DC were also OKT 8 negative (not shown). OKT 6, a monoclonal that reacts with thymocytes and Langerhans cells, did not stain any PBMC. We conclude that DC are HLA- and HLA-DR-bearing leukocytes that lack many of the known differentiation antigens of B cells, T cells, and monocytes.

DC in Culture. In two experiments, enriched preparations of DC were maintained in culture for 4 d. The distinctive cytologic features of DC persisted, and the cells did not acquire surface Ig. B cells maintained surface Ig in culture, and monocytes retained their cytologic features. Therefore, DC do not convert into lymphocytes or monocytes in vitro.

Stimulation of the Primary MLR. DC-enriched fractions were strong stimulators of both syngeneic and allogeneic primary MLR, and were at least 10 times more efficient than other cell populations on a per cell basis (Table III; each of the populations has been tested in three or more additional experiments with similar results to experiment 2). The potency of the DC-enriched fraction was not reduced after B cell depletion with E anti-human Ig rosetting (Table III, experiment 2). The use of BPA columns to enrich DC was not itself responsible for the activity of DC as MLR stimulators. That is, for both allogeneic and syngeneic MLR, the total stimulating activity recovered from the BPA columns was similar to the activity in the applied twice-released cells (not shown).

Other cell fractions were evaluated for MLR-stimulating activity. Contaminating DC in each fraction were enumerated by cytologic criteria. Monocyte fractions were at least 10 times less active than DC. Typically, the monocyte fractions contained 1–6% DC and 80–90% monocytes, and the DC populations had 25–40% DC and 10–25% monocytes. Therefore, stimulatory capacity correlated with Ia^+ DC content, rather than content of Ia^+ monocytes (as most monocytes are Ia^+, e.g., Table II). Nonadherent, E rosette-negative or non-T cells were 10–30 times less active than DC-enriched fractions in MLR stimulation, and again, small numbers of DC (2–10%) could be identified in this population. High density B cells, obtained from the twice-released population (Table I) were consistently 30–50 times less active than enriched DC (Table III). However, increased stimulating capacity was noted when B cells were sedimented after E anti-Ig rosetting of twice-released cells (Table III, experiment 2). This was not attributable to rosetting itself, because B cells that were first sedimented and then rosetted were not stimulatory (not shown). More likely, the stimulating activity was due to DC trapped during the rosetting procedure, and in fact, 1–3% nonrosetted DC were observed in the B cell fractions. Lastly, enriched T cells were

TABLE III

Stimulating Capacity of Subpopulations of Mononuclear Cells for the 1° Syngeneic (Syn) and Allogeneic (Allo) MLR

	Source of responder T cells	Dose of irradiated stimulator cells				
		10^5	3×10^4	10^4	3×10^3	10^3
Experiment 1						
DC enriched (without EαIg rosetting)	Syn	11,983 ± 605	7,119 ± 2,555	3,172 ± 664		
	Allo	119,506 ± 4,663	105,350 ± 15,849	62,922 ±6,106	32,670 ± 1,243	13,073 ± 2,292
Monocyte enriched (firmly adherent)	Syn	1,131 ± 630	2,034 ± 1,976	670 ± 800		
	Allo	31,148 ± 226	44,232 ± 4,712	18,509 ± 3,431		
Monocyte enriched (readherent)	Syn	1,706 ± 425	762 ± 243	660 ± 359		
	Allo	38,361 ± 1,038	29,937 ± 3,655	16,461 ± 706		
B cell enriched (high density released without EαIg rosetting)	Syn	219 ± 5	526 ± 584			
	Allo	2,766 ± 1,143	2,765 ± 1,301	735 ± 353		
T cell enriched (E_n^+)	Syn	ND*	869 ± 522			
	Allo	ND	11,682 ± 1,669			
Non-T cells (E_n^-)	Syn	6,012 ± 5,413	2,755 ± 1,517			
	Allo	36,331 ± 2,105	16,130 ± 2,149	4,378 ± 2,875	2,732 ± 782	
Experiment 2						
DC enriched (with EαIg rosetting)	Syn	11,621 ± 2,397	8,489 ± 983	5,858 ± 2,225	2,548 ± 1,688	1,795 ± 994
	Allo	ND	35,511 ± 4,882	26,797 ± 3,999	18,322 ± 6,110	11,642 ± 1,722
DC enriched (without EαIg rosetting)	Syn	14,943 ± 363	7,403 ± 856	7,222 ± 2,668	1,620 ± 285	882 ± 620
	Allo	112,435 ± 5,571	45,464 ± 2,787	32,161 ± 7,375	23,330 ± 6,648	9,992 ± 1,553
Monocyte enriched (firmly adherent)	Syn	5,474 ± 412	1,562 ± 899	677 ± 325	458 ± 326	
	Allo	42,893 ± 4,891	8,433 ± 461	3,324 ± 1,258	2,188 ± 740	
Monocyte enriched (readherent)	Syn	999 ± 185	641 ± 402	422 ± 285	855 ± 627	
	Allo	15,032 ± 1,496	2,943 ± 1,042	1,546 ± 348	1,514 ± 739	
B cell enriched (high density after EαIg rosetting)	Syn	3,291 ± 1,088	1,981 ± 721	1,268 ± 566	595 ± 331	
	Allo	31,280 ± 4,371	19,684 ± 552	9,317 ± 2,058	5,008 ± 1,989	
B cell enriched (high density without EαIg rosetting)	Syn	1,130 ± 443	611 ± 444	1,917 ± 1,159	1,166 ± 1,130	
	Allo	17,138 ± 4,393	7,199 ± 3,143	445 ± 125	492 ± 195	
T cell enriched (nylon wool filtered)	Syn	1,334 ± 760	2,124 ± 1,333	1,226 ± 1,106	ND	
	Allo	6,091 ± 483	763 ± 103	275 ± 218	ND	

Experiment 1: E_n-rosetted T cells and irradiated stimulator cells were cultured in round-bottomed wells. [^{3}H]Thymidine uptake without stimulators was 600 ± 287 for Syn responders and 3228 ± 1746 for Allo responders. The DC-enriched fraction was 35% DC, 10% monocytes, and 55% small cells.

Experiment 2: Nylon T cells and irradiated stimulator cells were cultured in flat-bottomed wells. [^{3}H]Thymidine uptake without stimulators was 638 ± 464 for Syn responders, and 245 ± 132 for Allo responders. The DC-enriched fraction was 23% DC, 24% monocytes, and 53% small cells.

* Not done.

weak or totally inactive as stimulators. We conclude that human DC are the most potent stimulating cells for both allogeneic and syngeneic MLR. It is possible that all other cell populations would be nonstimulatory if depleted of DC.

Accessory Cell Function in Oxidative Mitogenesis. Human T cells, modified with periodate, will not proliferate unless irradiated accessory cells (not periodate modified) are added (32). The accessory cell activity of our different cell populations paralleled MLR-stimulating capacity in most experiments. Again, DC-enriched fractions were the most active accessory cells, and this was not reduced by B cell depletion (Table IV, experiment 2). Monocyte-enriched fractions varied in activity (compare experiments 1, 2, and 3, Table IV). In four out of five experiments, monocyte populations were 10–30-fold less active than enriched DC. Monocytes did not inhibit DC when co-cultivated, unless added at high doses (20%, see Table IV, experiment 3). B and T cell fractions were again weak or inactive. We conclude that human DC, like rodent DC (2, 9), are potent accessory cells for the proliferation of periodate-modified T cells.

TABLE IV

Ability of Subpopulations of Mononuclear Cells to Act as Accessory Cells for Oxidative Mitogenesis

Accessory cell population	Doses of irradiated accessory cells			
Experiment	10^5	3×10^4	10^4	3×10^3
DC enriched (without EαIg rosetting)	108,024 ± 2,625	68,297 ± 6,811	34,301 ± 1,890	13,796 ± 936
Monocyte enriched (firmly adherent)	42,170 ± 27,057	46,162 ± 1,653	24,048 ± 1,958	
Monocyte enriched (readherent)	51,470 ± 2,353	39,427 ± 4,290	18,285 ± 1,183	
B cell enriched (without EαIg rosetting)	2,180 ± 571	2,228 ± 886		
T cell enriched (E_n^+)	ND*	5,245 ± 1,219		
Non-T cells (E_n^-)	15,384 ± 2,395	5,146 ± 718	3,145 ± 718	
Experiment 2	5×10^4	1.5×10^4	5×10^3	1.5×10^3
DC enriched (with EαIa rosetting)	84,518 ± 9,624	69,385 ± 1,714	44,854 ± 1,522	25,335 ± 3,840
DC enriched (without EαIg rosetting)	98,293 ± 8,348	66,560 ± 8,023	42,463 ± 1,091	27,402 ± 2,278
Monocyte enriched (firmly adherent)	17,379 ± 2,385	8,485 ± 1,064	7,274 ± 840	4,961 ± 982
B cell enriched (with EαIg rosetting)	38,527 ± 6,498	18,831 ± 1,345	8,463 ± 1,603	5,809 ± 2,346
B cell enriched (without EαIg rosetting)	5,743 ± 1,493	3,757 ± 236	2,536 ± 865	3,691 ± 1,162
T cell enriched (nylon wool T)	7,801 ± 1,468	5,525 ± 1,215		
Experiment 3	3×10^4	10^4	3×10^3	10^3
DC enriched (with EαIg rosetting)	63,691 ± 3,224	39,013 ± 4,010	19,977 ± 4,457	10,985 ± 1,724
Monocyte enriched (firmly adherent)	5,966 ± 754	7,317 ± 904	4,610 ± 1,150	3,237 ± 214
DC-monocyte mixture‡	36,899 ± 754	36,320 ± 904	22,616 ± 1,150	9,484 ± 214
B cell enriched (with EαIg rosetting)	19,438 ± 1,914	8,815 ± 1,866	5,218 ± 957	3,735 ± 744
T cell enriched (nylon wool T)	4,779 ± 683	4,215 ± 683		

Experiment 1: E_n-rosetted T cells (+IO_4) and irradiated accessory cells were cultured in round-bottomed wells, and the same populations used as MLR stimulators in Table III experiment 1 were used as irradiated accessory cells. Proliferative response of T cells only (no IO_4) was 177 ± 59; T cells only (+IO_4) was 5,772 ± 3,935; and T cells (no IO_4) + 10^5 DC enriched was 2,501 ± 380.

Experiment 2: Nylon T cells (+IO_4) and irradiated accessory cells were cultured in flat-bottomed wells. The twice-released fraction (see Table I) was divided in half and treated with bovine E or bovine E anti-human Ig, and floated in BPA. This produced two DC-enriched fractions (low density) and two B cell fractions (high density). The DC-enriched fraction with E anti-human Ig was 31% DC, 45% monocytes, and 24% small cells. The DC-enriched fraction without E anti-human Ig was 28% DC, 34% monocytes, and 38% small cells. Proliferative responses of T cells only (no IO_4) was 358 ± 157; T cells only (+ IO_4) 3,418 ± 754; and for T cells (no IO_4) + 5×10^4 DC was 3,856 ± 810.

Experiment 3: Nylon T cells (+IO_4) and irradiated accessory cells were cultured in flat-bottomed wells. The DC-enriched fraction was 34% DC, 16% monocytes, and 50% small cells. Proliferative responses of T cells only (no IO_4) was 2,584 ± 485; T cells only (+IO_4) was 2,648 ± 737; and for T cells (no IO_4) + 3×10^4 DC was 5, 231 ± 389.

* Not done.

‡ The DC-monocyte mixture (1:1) had 6×10^4 total cells at the highest dose, 2×10^4 cells at the next dose, etc.

Discussion

Human blood mononuclear cells have a small subpopulation (0.1–0.5%) of DC that are virtually identical to DC in mouse and rats. Human DC were enriched to 20–60% purity and compared with enriched populations of monocytes, B cells, and T cells by several criteria. First, the morphology of DC is distinctive. DC extend smooth cytoplasmic processes in several directions, have irregularly shaped nuclei and many mitochondria, but exhibit few lysosomes or endocytic vacuoles. Second, DC express histocompatibility (HLA, HLA-DR) and leukocyte-common antigens, but lack the principle differentiation markers of monocytes (phagocytic capacity, peroxidase, esterase, F_c receptors, four surface antigens detected with monoclonal Ab), B cells (surface and intracellular Ig), and T cells (E-rosette receptor; OKT 3, 6, and 8 antigens). Third, DC are at least 10 times more active than other cells as MLR stimulators and as accessory cells for periodate-induced mitogenesis.

A clear difference between DC of man and mouse is that human DC express complement receptors, i.e., they rosette erythrocytes coated with IgM and fresh mouse serum. Complement receptors have not been detected on mouse DC with the same reagent. Conceivably this difference is related to the species or tissue studied, the possibility that the receptor in mouse may be masked or occupied by endogenous complement, or the possibility that expression of complement receptors may be associated with an activation or differentiation step.

This paper provides the first description of DC in the circulation. Circulating cells are likely to be part of a widely spread DC system. For example, we have found DC in human spleen and tonsil that are indistinguishable from blood DC in morphology, low bouyant density, and surface markers. In mice and rats, DC have been enriched from spleen and node (2–5) and can be detected in thymus, liver, and peritoneal cavity (33 and R. Steinman, unpublished observations). Hart and Fabre have visualized Ia-bearing DC in sections of all rat nonlymphoid tissues except brain (34). Mason et al. (3) have demonstrated a substantial flux ($>10^6$ cells/d) of typical DC into the afferent lymph draining rat intestine. These DC probably are identical to Langerhans cells in the afferent lymph of guinea pigs (35) and "veiled" cells in afferent lymph of rabbits (36). DC in all sites (lymph, lymphoid, and nonlymphoid organs) are bone marrow derived, bear Ia and leukocyte-common antigen, and lack the differentiation markers of macrophages and B cells (3, 5, 34, 37, 38).

Relatively little is known about the kinetics and sites of DC production and their movement between different organs. Are all DC in tissues end-stage elements derived from proliferating bone marrow precursors? Can DC recirculate from one compartment to another, or at least move continuously from tissues to lymphoid organs via afferent lymph? Can DC proliferate locally in tissues, or be elicited from the marrow in increased numbers during inflammatory responses? The behavior of DC in inflammation is an important unknown, since entry of such potent accessory cells into a lesion would intensify immune responses at the local level. For example, cells resembling DC have been described in synovial tissue from patients with rheumatoid arthritis (39, 40). In preliminary experiments on rheumatoid synovial exudates, we have found relatively large numbers of cells that have the same cytologic features and surface markers as blood DC.

There are difficulties with the study of DC from human blood compared with mouse spleen. First, it is difficult to study DC in fresh, adherent blood mononuclear

cells because they are far outnumbered by B cells and monocytes. In contrast, mouse spleen adherent cells contain 10–50% DC. Second, human DC cannot be significantly enriched by density gradient separation of fresh blood mononuclear cells, because almost all monocytes are of low density as well. Mouse splenic DC are enriched by this technique because most splenic macrophages are of high density. Third, human DC cannot be enriched to the same degree of purity as mouse (>90%). The majority of the contaminating cells are small, Ia-bearing cells, which lack differentiation markers of B cells, T cells, and monocytes and do not differentate into these in culture. We have assumed, because of analogy to the rat and mouse, that the cells with the cytologic characteristics of DC account for the functional activity of DC-enriched fractions, but small, Ia-bearing cells may also contribute. Fourth, it is more difficult to deplete DC from human monocyte and lymphocyte fractions. Monocyte-enriched fractions contained 1–6% DC, B cells enriched with anti-Ig rosetting contained 1–4% DC, and non-T cells contained 2–10% DC. These levels of DC contamination could account for the variable functional activity of these populations.

We propose that DC are the principal MLR stimulators and accessory cells in human blood mononuclear cells, because DC fractions are much more potent than other fractions and because the small numbers of DC in the other fractions could explain their activity. Reagents that would selectively deplete human DC are needed to pursue this hypothesis. In mouse, a DC-specific cytotoxic monoclonal Ab ablates MLR-stimulating activity from mouse spleen (12). It is hoped that the DC-enriched populations described in this paper will be useful, as in the mouse, to immunize and screen for human DC-specific monoclonal Ab.

Summary

Previous studies demonstrated that lymphoid tissues of mice and rats contain small numbers (<1% of nucleated cells) of dendritic cells (DC) with special cytologic, surface, and functional properties. We show here that similar DC represent 0.1–0.5% of human peripheral blood mononuclear cells. DC can be enriched to 20–60% purity by a multistep procedure analogous to that used in mice. Adherent peripheral blood mononuclear cells are cultured overnight, and the released cells are depleted of monocytes and B cells by readherence to plastic, rosetting with erythrocytes coated with anti-human IgG, and centrifugation in dense albumin columns. Enriched DC have similar cytologic features to rodent DC by light and electron microscopy.

DC express HLA, and HLA-DR and the leukocyte-common antigens. They lack phagocytic capacity, receptors for antibody-coated and neuraminidase-treated erythrocytes, surface and intracellular Ig, esterase, peroxidase, and azurophilic granules. DC do not react with several monoclonal antibodies directed to phagocytes (OKM 1, "mac-1," 63D3, and 61D3) and T cells (OKT 3, 6, 8). Unlike the mouse, human DC express complement receptors. When maintained in culture for 4 d, human DC did not give rise to either B cells or monocytes. Therefore, DC identified by cytologic criteria are distinct from other leukocytes.

Enriched populations of DC have been compared to fractions enriched in monocytes, B cells, and T cells in three functional assays: stimulation of the primary allogeneic mixed leukocyte reaction, stimulation of the primary syngeneic MLR, and accessory function for the proliferation of periodate-modified T cells. In each case, the DC fraction was 10-fold or more active than other cell fractions. We conclude that

DC circulate in man, and represent the principal cell type required for the initiation of several immune responses.

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