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IDENTIFICATION OF A NOVEL CELL TYPE IN PERIPHERAL LYMPHOID ORGANS OF MICE*

II. Functional Properties In Vitro

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We have previously demonstrated (20) that a novel cell type can be identified in the glass-adherent cell population of mouse peripheral lymphoid organs. Its most abundant source is spleen, where it comprises about 1% of the total nucleated cells. The distinctiveness of the dendritic cell was established by morphological criteria. On the basis of phase and electron microscopy, cytochemistry, and cinematography the dendritic cell was clearly differentiated from other previously described components of lymph nodes and spleen.

In this report, we describe several functional tests performed on dendritic cells in vitro which corroborate their novel nature. Dendritic cells lack the surface properties of lymphocytes and mononuclear phagocytes, as well as many of the functions which have been postulated for "reticular cells" in situ.

Materials and Methods

Animals.—Outbred NCS mice were obtained from The Rockefeller University colony and inbred DBA/2 and C3H mice from The Jackson Laboratories, Bar Harbor, Maine. The C3H mice were only used to assess the presence of plasma cell antigen (PC-1) on lymphoid cells (22).

Dendritic Cells.—Single-cell suspensions of mouse spleen and lymph node were prepared in two ways, as previously outlined in detail (20). One method involved manual teasing of the lymphoid organ with fine forceps, and the other utilized treatment with Clostridial collagenase (Fx I, Sigma Chemical Co., St. Louis, Mo.), 0.05% in serum-free medium for 15 min at 37°C. The latter method greatly enhances the yield of cells with the typical morphological features of macrophages. Adherent cell preparations were made by cultivation of the cell suspensions on glass or plastic surfaces at a concentration of $1–2 \times 10^7$ nucleated cells/ml of 10% fetal calf serum (FCS)[1] (Grand Island Biological Co., Grand Island, N.Y.) in medium 199 (supplemented with 1,000 U/ml penicillin). Cells were incubated for 1 h at 37°C after which the nonadherent cells were removed with several washes of medium 199. Subsequent cultivation of the adherent cells was done in 10% FCS-medium 199. The viability of dendritic cells was assessed in several types of tissue culture medium and supplements, obtained from

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin, FCS, fetal calf serum; HRP, horseradish peroxidase; *H-2*, histocompatibility locus; MEM, minimal essential medium; NBCS, newborn calf serum; PC-1, plasma cell antigen; SRBC, sheep red blood cells.

either Grand Island Biological or Microbiological Associates, Inc. (Bethesda, Md). In some instances, dendritic cells were embedded in mouse plasma clots. 50 μl of citrated plasma was added to 13-mm coverslips containing the adherent cells and then clotted by 5 μl of 1.0% calcium chloride. The coverslips were then placed in culture dishes containing 10% FCS-medium 199.

Morphological Methods.—Phase microscopic observations were made on specimens fixed in 2.5% glutaraldehyde, in 0.1 M sodium cacodylate buffer, pH 7.4, for 5 min at room temperature, or 30 min for cells embedded in plasma clots. Dendritic cells were identified in the adherent populations by previously described morphological criteria (20). The number of dendritic cells in a given preparation was quantitated by counting a minimum of 100, × 800-fields on three or more coverslips. For electron microscopy, cells were fixed for 15 min at room temperature in 1.25 or 2.5% glutaraldehyde followed by postfixation in 1.5% osmium tetroxide, in 0.1 M cacodylate buffer, 30 min at room temperature. Specimens were stained en bloc with 0.5% magnesium uranyl acetate in saline, pH 5.0, and generally were embedded and sectioned in the plane of the cell monolayers, as outlined by Ross (16). For localization of horseradish peroxidase (HRP), glutaraldehyde-fixed cells were incubated in the diaminobenzidine-hydrogen peroxide mixture described by Graham and Karnovsky (7).

Enrichment of Dendritic Cell Populations.—Populations of spleen and lymph node cells were enriched in dendritic cells by equilibrium density centrifugation in dense bovine serum albumin (BSA) solutions. Preliminary observations with discontinuous gradients indicated that most dendritic cells had a density of 1.082 or less under the conditions described below, whereas the majority of the total nucleated cells had a density greater than this. Accordingly, a simplified procedure was adopted in which a washed spleen cell pellet was resuspended in a BSA solution, $\rho = 1.082$, at a final cell concentration of 5–20 × 10^7 nucleated cells/ml. Before centrifugation, 0.7 ml of BSA, $\rho = 1.060$, was layered over the column to prevent cell clumping in the pellicle which subsequently forms. The columns were spun to equilibrium at 10,000 *g* avg. for 30 min at 4°C. The cell fractions (pellicle, BSA column, pellet) were then harvested with curved Pasteur pipettes and washed in medium 199 before counting and cultivation. BSA solutions were prepared from BSA powder, Armour fraction V (Armour Pharmaceutical Co., Chicago, Ill.) For every 10 g of BSA solubilized, a standard amount of 0.31 *N*-sodium hydroxide (from 8.0 to 9.0 ml, depending on the lot of BSA) was mixed with an appropriate amount of phosphate saline (0.31 osmol/l, pH 7.4) to give a final pH of 7.35 ± 0.05. The final density was adjusted with phosphate saline to 1.082 using a refractometer and a standard curve relating refractive index to density. The solution was finally millipored and kept at 4°C before use.

Tests for Endocytosis.—In vitro tests for endocytic activity were performed on adherent cell preparations usually enriched in typical macrophages by previous collagenase digestion. The macrophages served as a positive control of active endocytic cells. The various endocytic markers were administered for 1 h at 37°C after which the cells were washed, fixed, stained with diaminobenzidine where appropriate, and then examined by phase contrast or electron microscopy. The numbers of positive macrophages demonstrating detectable interiorization of the marker, and negative macrophages and dendritic cells were enumerated in the light microscopic studies. The markers and loads used were: soluble horseradish peroxidase (Sigma, Type II), 1 mg/ml (18); particulate HRP-anti-HRP immune complexes precipitated at equivalence with hyperimmune rabbit or mouse anti-HRP as previously described (19), 2 μg of HRP as a complex being added to 1.5 ml culture medium in 30-mm culture dishes; sheep red blood cells (SRBC, Colorado Serum Co., Denver, Colo.) opsonized with amboceptor (Baltimore Biological Laboratories, Baltimore, Md.) according to the method of Bianco et al. (2), 10–25 SRBC per adherent cell; polystyrene particles, 1.1 μ in diameter (Dow Chemical Co., Midland, Mich.), 25–50 particles per adherent cell; and heat killed (*Staphylococcus albus*, 25–50 particles per adherent cell. Tests for endocytic capacity were also

performed in vivo using three markers administered i.v. at varying time periods before sacrifice. The markers were colloidal carbon (Pellikan India Ink), 50 μl 1, 3, or 18 h before; colloidal thorium dioxide (Fellows Testagar, Detroit, Mich.), 50 μl 18 h before; and soluble HRP, 5 mg in 0.2 ml saline, 1 h before.

Surface Markers on Spleen Cells.—A variety of antisera were used to screen dendritic and other nucleated spleen cells for established mouse surface markers. E. A. Boyse, U. Hämmerling, and C. Iritani of the Sloan Kettering Institute, New York, most generously supplied us with: anti-θ C3H, prepared by injection of ASL1 spontaneous leukemia cells into congenic θ-AKR (A-Thy-1^a) mice; anti-μ and anti-κ (actually anti-F(ab)'$_2$ of $\kappa_2\gamma_2$ myeloma) both prepared in rabbits by immunization with myeloma fragments; antiplasma cell (anti-PC-1), prepared as described by Takahasu et al. (22); and antihistocompatibility (*H-2*), prepared by injection of histoincompatible leukemia cells into appropriate mice. An anti-mouse peritoneal macrophage membrane reagent, prepared according to Gallily and Gornostansky (5), was given to us by Dr. S. Gordon. Though highly active towards macrophages, it reacted with mouse L cells as well.

Two types of assay were used to detect binding of the antiserum to cells: (*a*) Cytotoxicity assays were first performed on spleen cells in suspension using the following reagents diluted in 2% FCS-medium 199: spleen cells, 5×10^6/ml; diluted antiserum; rabbit serum diluted 1:15 as a source of complement. 50 μl of each reagent were incubated 45 min at 37°C, 100 μl of freshly mixed 0.16% trypan blue was then added, and the percentage of trypan blue positive cells scored in a hemocytometer. Appropriately diluted antisera and complement were then added to similar numbers of adherent spleen cells after which the cells were fixed and the number of morphologically normal dendritic cells were counted and compared to controls. Trypan blue could not be used in adherent cell preparations without loss of a majority of the cells. (*b*) Mixed hemagglutination assays were performed on adherent cells which were incubated in the presence of antiserum for ½ h at 4°C, washed, and then treated with suitable indicator cells for ½ h at 4°C to visualize surface binding of the antiserum. The indicator SRBC were prepared with reagents kindly supplied by Dr. S. Gordon. To detect binding of an alloantiserum, the SRBC were coated successively with mouse anti-SRBC and pepsin-digested rabbit antimouse Ig. For heteroantisera, the reagents were rabbit anti-SRBC and F(ab)'$_2$ of a goat antirabbit Ig. After rosetting, the number of nonrosetted dendritic cells were counted and compared with controls not treated with antiserum.

The presence of the surface receptor for the activated third component of complement was assessed in adherent cells using a rosette assay as adapted from Bianco et al. (2). Again we counted the number of nonrosetted dendritic cells compared to controls treated with SRBC ± antibody in the absence of complement.

Tests for Binding of Antigen and/or Antibody by Dendritic Cells.—Two systems were used in these experiments. In the first, DBA/2 mice were immunized with 0.2 ml of 5% SRBC i.v. At varying time points, splenic adherent cell preparations were made. The latter were then exposed for 1 h to: (*a*) antigen (SRBC at an approximate load of 25 SRBC/nucleated adherent cell); (*b*) antigen and antibody (SRBC opsonized with 10^0, 10^{-1}, 10^{-2}, and 10^{-3} dilution of the animal's antiserum required to give a positive hemagglutination test, using the same final concentration of SRBC as that present in the culture dishes); (*c*) antigen, antibody, and complement (SRBC opsonized as above with antibody and then a 1:20 final dilution of fresh, nonimmune mouse serum as a source of complement); or (*d*) indicator, SRBC, coated with mouse anti-SRBC and pepsin-treated rabbit antimouse Ig, to detect Ig on the surface of dendritic cells. The number of dendritic cells not binding SRBC following each of the above treatments was compared with the number of dendritic cells in control preparations. A second system to detect antigen binding utilized outbred NCS mice primed i.p. with 100 μg of soluble HRP in complete Freund's adjuvant, and boosted 3–6 mo later with 250 μg soluble HRP in saline i.v. (after protection against anaphylaxis with 0.2 mg of

tripelennamine i.v.). At varying time points, adherent spleen cells were exposed for 1 h to 0, 10, 100, or 1,000 μg/ml soluble HRP or to particulate HRP-anti-HRP complexes at equivalence. Positive-binding cells were sought using the diaminobenzidine technique at the light microscope level.

Assay for Spleen Colony-Forming Units.—10^6 DBA/2 spleen cells in 0.2-ml suspension minimal essential medium (MEM) were injected i.v. into lethally-irradiated syngeneic recipients (900 rad Co^{60}). 9 days later, the recipient spleens were fixed in Bouin's reagent and the number of surface colonies counted. The donor spleen cells were either whole spleen, or nonadherent spleen cells depleted of dendritic cells by a 1-h incubation on 100 mm glass Petri dishes, using 6 ml of cells at a concentration of 5×10^6/ml 10% FCS-medium 199. At this cell concentration and volume, all dendritic cells appear to adhere to glass during a single plating step. If the Petri dishes are pretreated with serum before use, nonspecific cell sticking is greatly reduced such that the number of viable nucleated spleen cells adherent by this technique, as detected by total cell counts before and after adherence, is 0–10% of the total applied.

Incorporation of [^3H]Proline into Macromolecular [^3H]Hydroxyproline (Collagen).—[^{3}H]-Proline 3 Ci/mmol, 100 μc/ml, Schwartz/Mann, Div. Becton, Dickinson and Co., Orangeburg, N.Y. was administered for 4 h in 10% FCS-MEM supplemented with 25 mM sodium ascorbate, to three cell populations: monolayers of mouse L cells, a continuous fibroblast-like line given to us by Dr. A. Hubbard and tested at the onset of the stationary phase of growth; spleen cells in suspension, enriched 15-fold in dendritic cells by centrifugation in BSA columns; adherent spleen cells from BSA column pellicles such that 60% of the adherent cells were dendritic cells. After 4 h at 37°C, EDTA was added to a concentration of 50 mM; the cells were chilled on ice, and both culture medium and cells (after scraping where necessary) were transferred to dialysis bags. Dialysis was run against several changes of 50 mM EDTA in 0.01% acetic acid for 4 days. The nondialyzable material was then lyophilized, hydrolyzed in 6N HCl for 48 h at 110°C, and then separated on a Jeolco 5 AH aminoacid analyzer (13) by Dr. R. Trelstad, The Massachusetts General Hospital, Boston, Mass. The number of counts in the proline and hydroxyproline peaks were measured and the relative amounts of collagen-like material synthesized by the cells expressed as the percentage Hypro/Hypro + Pro × 100.

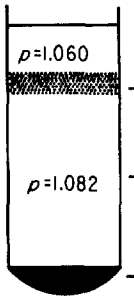
^3H-Thymidine Labeling.—Adherent cell populations were exposed for 1 h (during the adherence step) or for 6 h in vitro to [*methyl-^3H*]thymidine (Schwartz/Mann, 6.0 Ci/mmol, 0.1 μc/ml). The cells were washed, fixed in glutaraldehyde, air dried, dipped in Ilford L4 emulsion, and exposed for 3–4 wk at 4°C in the presence of desiccant. Following development, the preparations were examined under phase contrast. It was possible both to identify dendritic cells and macrophages after this procedure, and to distinguish the spherical phase dense mitochondria in dendritic cells from the smaller, brown, supranuclear silver grains.

RESULTS

Enrichment of Dendritic Cells by Isopycnic Centrifugation.—Lymphoid cell (spleen, lymph node) suspensions can be enriched in dendritic cells by a relatively simple equilibrium density procedure. When cells are suspended in BSA (ρ = 1.082, pH 7.35, tonicity = 0.31 osmole/1) and centrifuged to equilibrium, three fractions of cells can be harvested (Table I): pellicle, pellet, and the BSA column itself. An additional band floats above the P = 1.060 layer but contains only a mixture of cell debris, isolated nuclei, and platelets. For each of the three main fractions, we determined: the total nucleated cell number, the percentage of trypan blue positive cells, the cell types as evident

TABLE I

Enrichment of Dendritic Cells by Floatation in BSA



Fraction	% of total cells applied	% of total dendritic cells	Relative conc. Dendritic cells
Total	100	100	1
Pellicle	5 - 14	> 90	7 - 20
Column	2 - 3	< 5	<< 1
Pellet	75 - 95	< 5	<< 1

Single cell suspensions of spleen or lymph node were made in medium 199, pelleted by centrifugation, and resuspended in BSA, ρ = 1.082. A dilute solution of BSA (ρ = 1.060) was layered over the column to facilitate collection of the pellicle fraction. The cells were centrifuged to equilibrium at 4°C and 10,000 *g* avg. for 30 min. Three fractions were collected; pellicle, column, and pellet. The total cell recovery ranges from 80–100%. The percentage of cells in the pellicle varies from 5 to 14%, the higher values occurring in lymph node. Almost all of the dendritic cells are recovered in the pellicle, thus providing an enrichment in the concentration of dendritic cells of 7–20-fold.

in Wright-Giemsa-stained smears, and the number of adherent dendritic cells and mononuclear phagocytes identified by previously defined phase microscopic criteria (20).

The most interesting result of the procedure is that the pellicle fraction contains most, and sometimes all, of the spleen or node dendritic cells originally placed in the column. This pertains at loads of 5–20 × 10 nucleated cells/ml BSA. The pellicle fraction contains a variable (5–14%) proportion of the total cells spun to equilibrium, resulting in a variable but significant (7–20-fold) enrichment of dendritic cells. As a result, monolayers can be prepared from the pellicle fraction in which 50% or more of the adherent cells can be classified as dendritic, and in which the latter are distributed at a relatively high density (Fig. 1 *a* and *b*). In our previous attempts to increase the number of dendritic cells per unit area, by increasing the concentration of unfractionated spleen cells adhering to glass, we observed that the number of dendritic cells reached a plateau at a relatively sparse level (0–2 per × 800 field) and then began to decrease (20).

In addition to dendritic cells, the pellicle fraction was relatively enriched (Table I) in certain classes of lymphocytes (large lymphocytes, dividing cells,

plasma cells, and plaque-forming cells in mice immunized to SRBC). It was depleted of mature granulocytes, red cells, and trypan blue positive cells. Typical mononuclear phagocytes (macrophages, monocytes, promonocytes) were found in both pellicle and pellet fractions. To render endocytic cells more dense, animals were pretreated with colloidal thorium dioxide, 50 μl i.v. 18 h before sacrifice. The pretreatment depleted the pellicle of large phagocytic macrophages without altering the apparent density of dendritic cells. However, a portion of the less active endocytic monocytes and promonocytes still floated on the albumin column.

The dendritic cells recovered in the pellicle have the same morphological features recognized in unfractionated lymphoid populations (Fig. 1 *a* and *b*), i.e., they adhere to glass, exhibit a number of cell processes of varying size and shape, contain numerous phase dense, spherical mitochondria, and possess a large, often contorted, refractile nucleus. The availability of these enriched pellicle preparations enabled us to corroborate our previous characterization (20) of the adherent dendritic cell at the ultrastructural level (Fig. 2). Dendritic cells all have large nuclei with a dense rim of heterochromatin and small nucleoli. The cytoplasm contains scattered mitochondria, short slips of rough endoplasmic reticulum, and free polysomes. There are none of the morphological counterparts of active endocytic cells, i.e., there are few dense membrane-bound lysosomes or presumptive pinocytic vesicles, the Golgi apparatus is small, and the cell surface smooth and nonruffled.

Tissue Culture Characteristics on Glass or Plastic Surfaces.—Dendritic cells are remarkably short-lived when cultivated in 10% FCS-medium 199, in contrast to typical mononuclear phagocytes in the same cultures. Few if any normal dendritic cells could be identified 12–16 h after adherence to glass or plastic surfaces. This loss of dendritic cells occurred as well if cells were cultivated in suspension and then adhered to glass at varying time points, or if small (½–1 mm) explants of spleen were cultivated and then dissected at later intervals. It appeared that the dendritic cells were dying in these cultures rather than being converted to another cell type. At no time in unfractionated or BSA column-enriched adherent cell preparations, did our quantitative or morphological observations suggest that dendritic cells were transforming into macrophages. We were also unable to recover dendritic or reticular cells among cells floating off the glass surface, under phase contrast or following Giemsa staining. Finally, presumptive degenerative changes were frequently observed in the dendritic cells adherent to a surface, i.e., cell processes became narrow and irregular in shape, mitochondria decreased in number and their size and shape became quite variable.

Several alterations in the culture medium, and a 10–20-fold increase in the concentration of dendritic cells in the monolayer (using BSA column-enriched preparations) did not improve dendritic cell viability (Table II). We then examined dendritic cells cultivated in plasma clots, since De Bruyn (3) reported

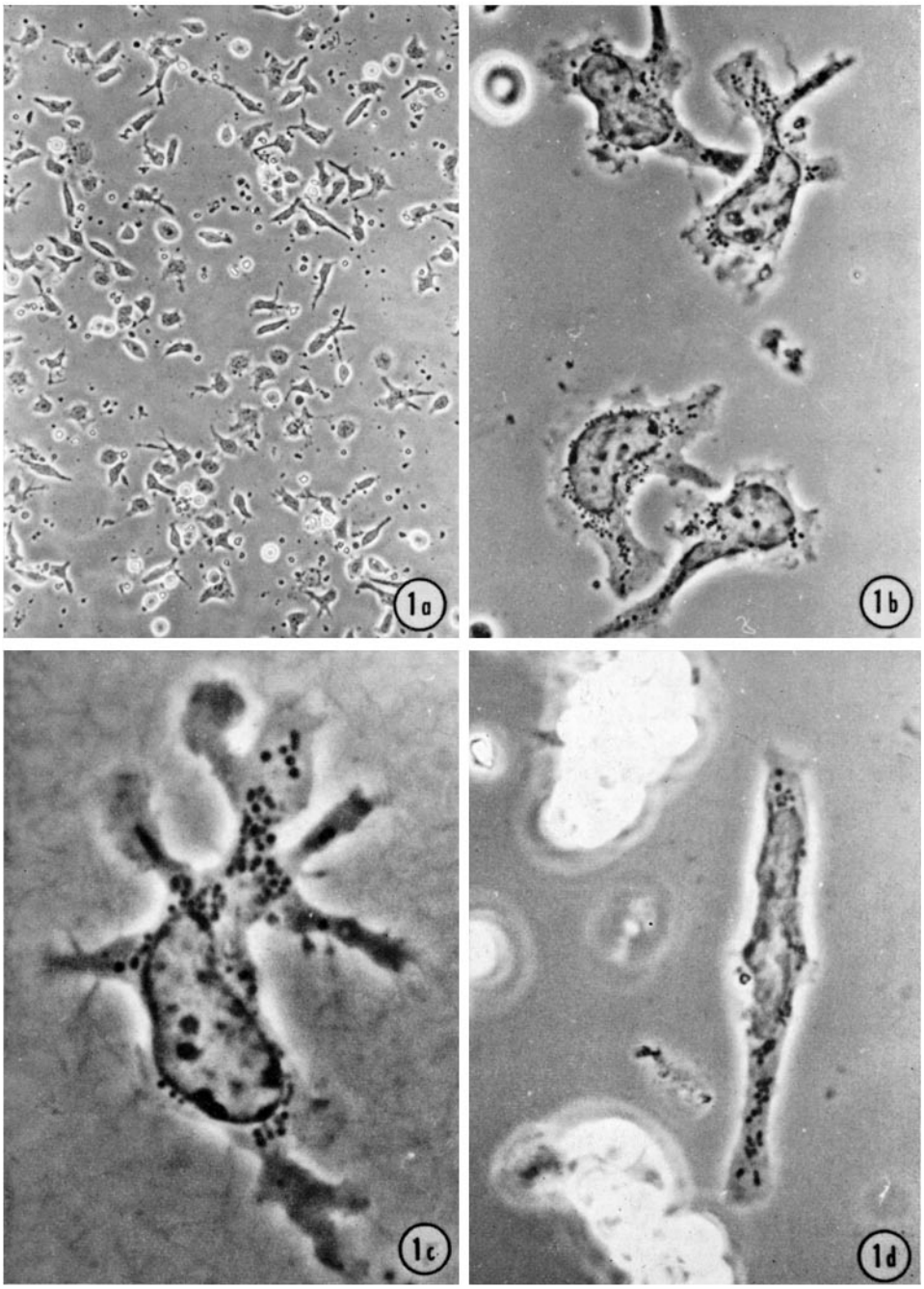


FIG. 1. Phase contrast micrographs of glutaraldehyde-fixed splenic adherent cells. (*a*) Low power view of a pellicle fraction obtained following centrifugation to equilibrium in BSA, $\rho = 1.082$. Most of the elongate or stellate cells have the cytological features of dendritic cells at high power. × 510. (*b*) High power view of a pellicle fraction. These dendritic cells have the same morphological characteristics as are present before isopycnic centrifugation. The nucleus is large, irregular in shape, and refractile in quality. The cytoplasmic processes contain many spherical phase dense mitochondria. × 2,800. (*c*) A dendritic cell cultivated 24 h in a plasma clot. The cytological features of these "long-lived" dendritic cells are similar to those seen on initial cultivation. Macrophage-like surface ruffles, pinocytic vesicles, and phagolysosomes are not acquired in vitro. × 5,300. (*d*) Splenic adherent cells exposed at 4°C to an anti-κ antiserum followed by suitable indicator cells to detect antiserum binding. The dendritic cell does not bind the antibody whereas other adherent cells form rosettes in this assay. × 4,000.

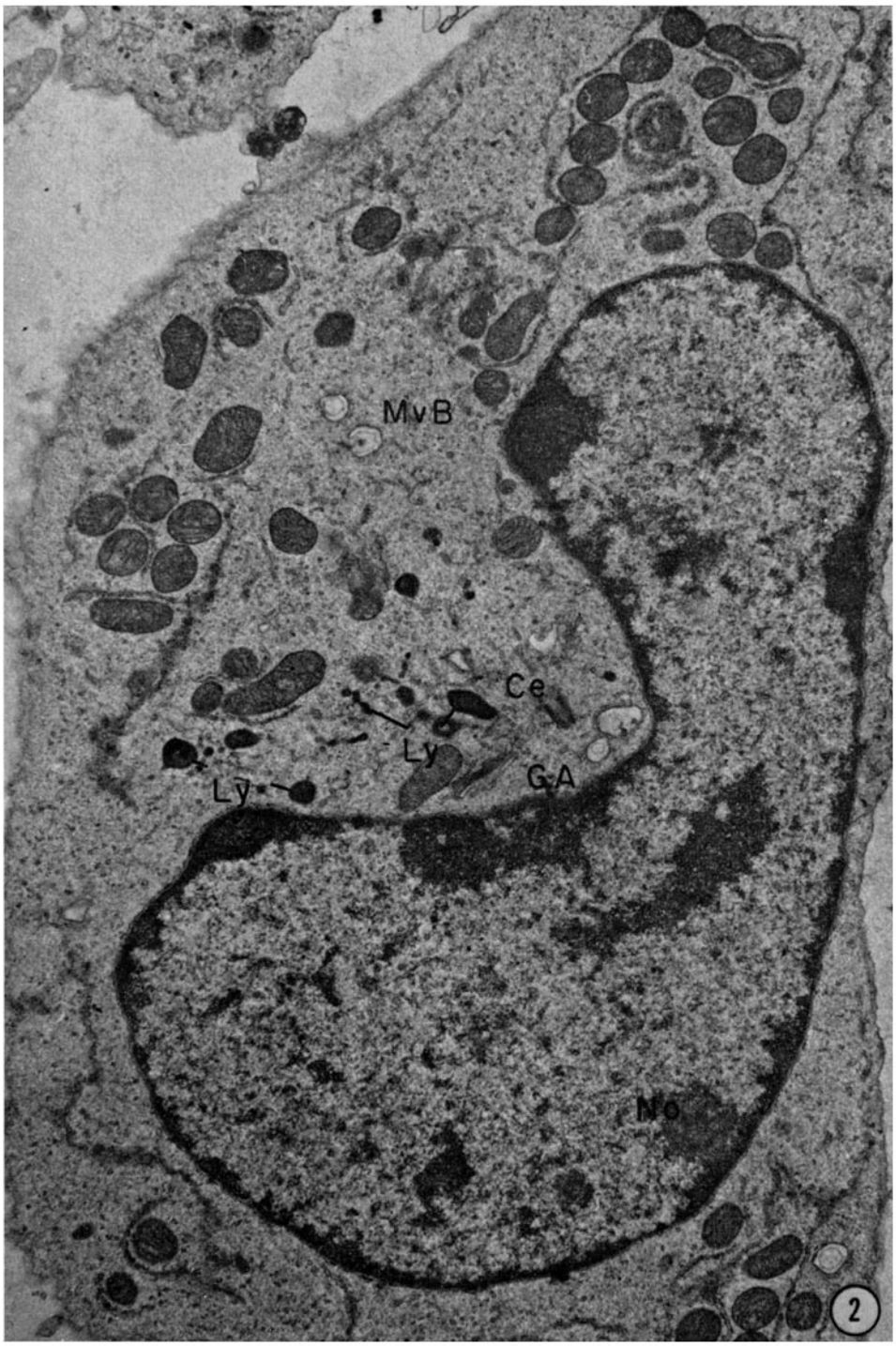


FIG. 2. An electron micrograph of a typical dendritic cell taken from a BSA pellicle fraction. The large nucleus has a peripheral rim of chromatin and a small nucleolus (*No*). The cytoplasm has many mitochondria with well developed cristae. Short slips of rough endoplasmic reticulum are often close to the mitochondria and small numbers of free polysomes are also present. The Golgi apparatus (*GA*) is associated with typical centrioles (*Ce*), several multivesicular bodies (*MVB*), and a few small, membrane-bound, dense granules and lysosomes (*Ly*). This particular cell is from a preparation exposed to 1 mg/ml soluble HRP in vitro. The enzyme is visualized cytochemically in lysosomes though the uptake is small relative to that observed in typical macrophages (Fig. 3 *b*). × 10,000.

TABLE II

Culture Conditions Employed to Maintain Spleen Dendritic Cells In Vitro

Liquid media
Prepared media: MEM, 199, RPMI 1640, NCTC, Dulbecco's, Trowell's
Natural products: FCS (10–40%), NBCS (10–40%), mouse serum (10–40%), chick and beef embryo extracts, egg ultrafiltrate, human ascites fluid, beef heart infusion broth
Buffers: HEPES, bicarbonate-CO_2
Conditioned media: L cell, macrophage, chick embryo fibroblast (30% v/v)
Other: mercaptoethanol, low oxygen content (10%), cocultivation with peritoneal macrophages
Increased concentration of dendritic cells
Adherent cells from BSA column-enriched preparations
Semisolid media
Plasma clots
Collagen (rat tail tendon)
Agar
Methyl cellulose

Adherent spleen cells, in some cases enriched in dendritic cells by BSA columns, were cultured in a variety of tissue culture media and supplements after attachment to glass and plastic surfaces. In all instances, typical splenic macrophages survived for prolonged (1 or more days) periods of time. In contrast, most dendritic cells disappeared within 12–16 h of culture, except when cultivated in plasma clots where survival was maintained 1 or more days.

the emigration of a cell type possibly identical to the mouse lymphoid dendritic cell, from 1–2-day old plasma clot organ cultures of rabbit spleen and lymph node. Formation of a clot of mouse plasma on coverslips containing adherent cells did enhance dendritic cell survival with some 50% of the original cells remaining after one day in culture, and some cells surviving 3–4 days. The morphological features of the dendritic cells were essentially unchanged during this period (Fig. 1 *c*), whereas macrophages underwent a profound increase in their size and number of cytoplasmic organelles. Cultivation of adherent cells in other gels—collagen (4), methyl cellulose (26), and agar (15)—did not enhance dendritic cell survival.

Tests of Endocytic Capacity.—The dendritic cell was first identified as a potentially distinct cell type because it was a large glass adherent cell that lacked the morphological features of actively endocytic macrophages. In keeping with these observations, dendritic cells were in fact incapable of interiorizing a variety of marker substances in vitro. Typical macrophages in the same cultures ingested large amounts of each test substance (Table III). The markers tested included a soluble protein (HRP, Fig. 3 *b*), nonantibody coated particles (polystyrene, heat-killed *S. albus*) and antibody-coated objects (SRBC, HRP-anti-HRP aggregates, Fig. 3 *c*). In some cases the differences between macrophages and dendritic cells were quantitative rather than

TABLE III

Absence of Endocytosis by Dendritic Cells In Vitro

Endocytic marker	% positive cells	
	Dendritic	Macrophages
Soluble horseradish peroxidase	<5	>90
Heat-killed *S. albus*	<5	>90
Polystyrene particles (1.1 μ)	<5	>90
Antibody-coated SRBC	<5	>90
HRP-anti-HRP immune aggregates	<5	>90

Adherent cell preparations were made from spleen, usually following collagenase treatment to increase the yield of typical macrophages. Various kinds of markers were then administered for 1 h, and the number of positive dendritic cells or macrophages capable of active endocytosis was determined by light microscopy. Macrophages rapidly interiorized appropriate pinocytic and phagocytic markers, especially antibody coated materials. Endocytosis by dendritic cells is limited in extent, but can be observed by electron microscopy (Figs. 2 and 4) in the case of nonantibody-coated markers.

qualitative, i.e., though interiorization of the marker was not clearly evident at the light microscope level, some markers, e.g., HRP, could be identified at the ultrastructural level within the relatively small numbers of dendritic perinuclear lysosomes (Fig. 2). However, dendritic cells appeared totally incapable of binding and interiorizing antibody-coated materials.

Three endocytic markers were administered in vivo to animals before sacrifice: soluble HRP, colloidal carbon, and colloidal thorium dioxide. By light microscopy, few of the dendritic cells took up the markers in contrast to typical macrophages (Fig. 3 *d*). Again, in the electron microscope, the differences appeared quantitative in that small amounts of all three markers were detected in dendritic cell lysosomes (Fig. 4 *a* and *b*).

Cell Surface Markers.—Dendritic cells exhibit the *H-2* specificity of the animal from which they are harvested in both the cytotoxicity and mixed hemagglutination assays we employed (Table IV). However, they lack a number of established surface markers of lymphocytes or their subpopulations including θ-antigen, PC-1 antigen, immunoglobulin (κ-and-μ chains) and EAC receptor (Fig. 1 *d*). Dendritic cells can also be distinguished from splenic macrophages in that they lack determinants detected by high dilutions of a heteroantiserum to a mouse peritoneal macrophage membrane preparation.

Absence of Binding of Antigen or Immune Complexes by Splenic Dendritic Cells.—It has been postulated that secondary lymphoid follicles or germinal centers contain a type of dendritic cell capable of binding antigen and specific antibody extracellularly on its cell surface (reviewed in 11). We next determined if the dendritic cell identified in vitro exhibited this antigen-binding capacity. In addition to lacking detectable surface Ig (see above), dendritic cells from normal and immunized animals (days 1, 4, 7, 14, 28 of a primary response to

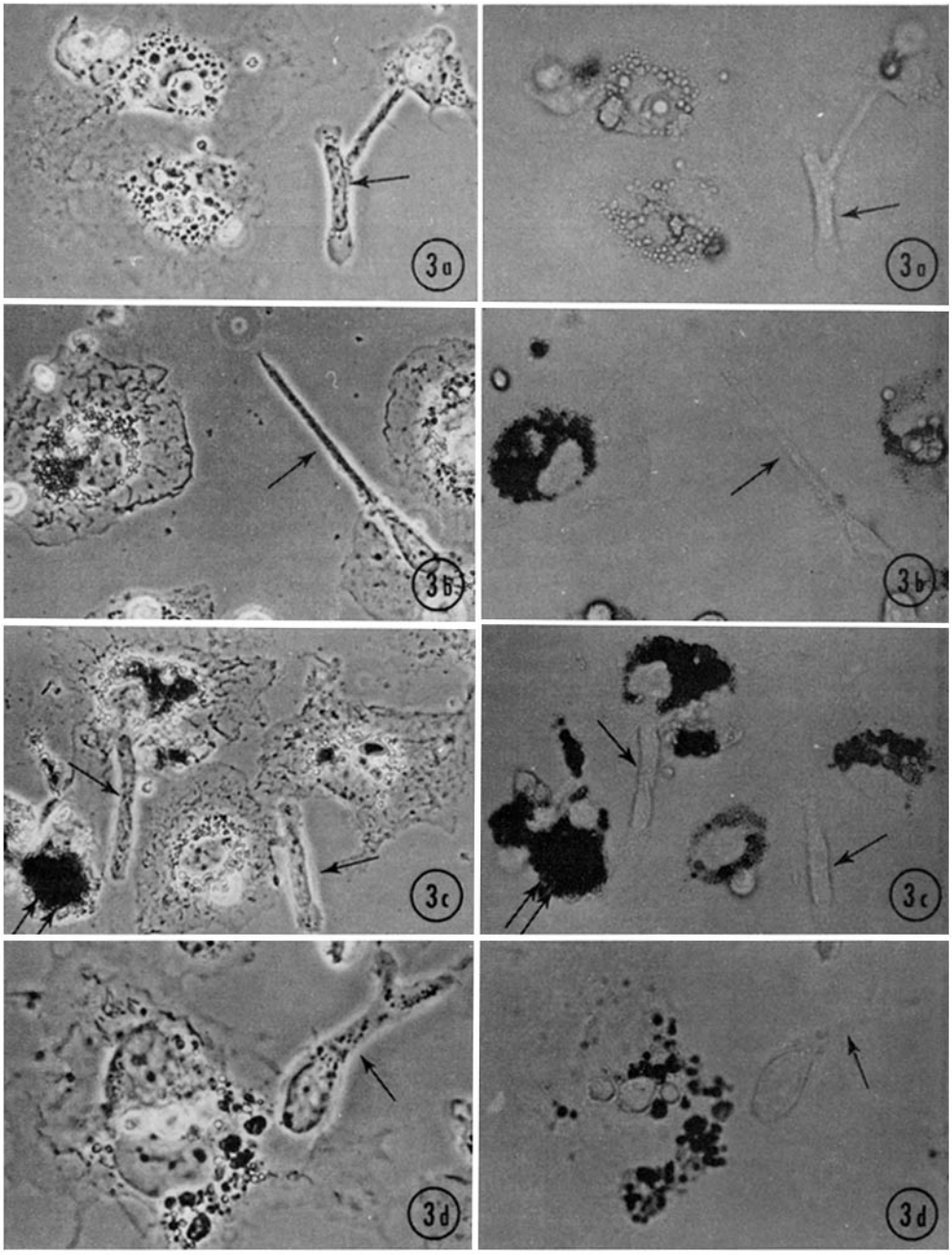


FIG. 3. Various endocytic markers were given to adherent cells isolated after collagenase digestion of spleen. Phase contrast (left) is used to identify the cell types and bright field microscopy (right) to ascertain if uptake has occurred. Dendritic cells are marked with arrows. (*a*) Control preparation from collagenase-treated spleen. The macrophages contain numerous refractile inclusions which have little color when viewed under bright field, even following staining with the diaminobenzidine-H_2O_2 substrate mixture for peroxidase. × 1,500. (*b*) Cells exposed for 1 h to 1 mg/ml soluble HRP in vitro. The diaminobenzidine-H_2O cytochemical procedure reveals extensive uptake of HRP by macrophages. × 1,500. (*c*) Particulate HRP-anti-HRP complexes are avidly interiorized by macrophages, one of which reveals surface binding of the aggregates (double arrows). Dendritic cells neither bind nor phagocytose antibody-coated materials. × 1,500. (*d*) Dendritic cells interiorize relatively little colloidal carbon following in situ administration i.v., again in contrast to macrophages. × 2,400.

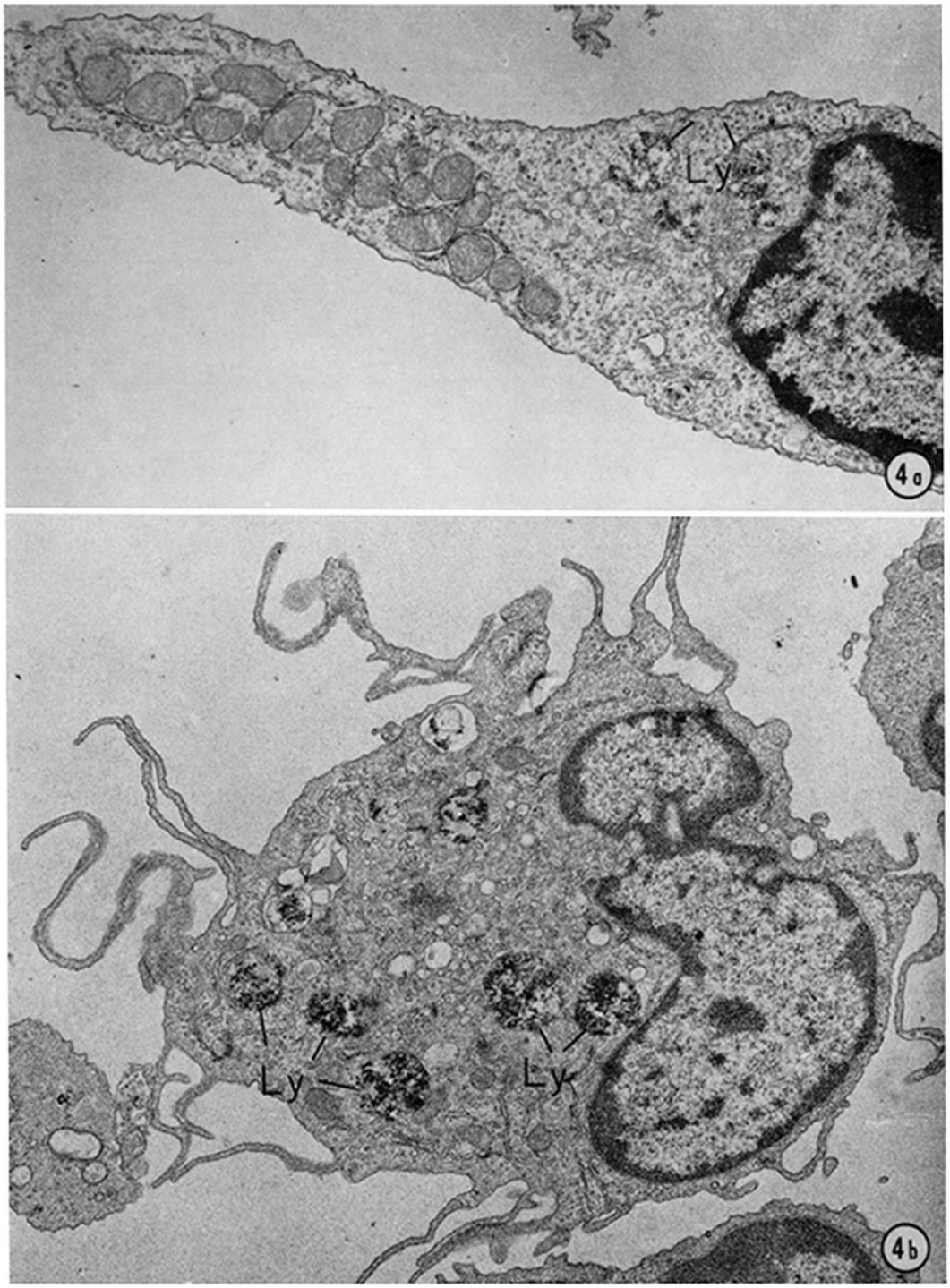


FIG. 4. Electron micrographs illustrating the distribution of intracellular colloidal carbon following administration in vivo. The cells were obtained from a BSA pellicle fraction. (*a*) A dendritic cell with a smooth surface and typical cytoplasmic organelles. A few perinuclear lysosomes (arrows) contain the colloid. Frequently the uptake of carbon by dendritic cells cannot be visualized in the light microscope (Fig. 3 *d*), because the number and size of their lysosomes are too small. × 11,500. (*b*) A macrophage containing abundant surface ruffles and, membrane-bound, carbon-laden, phagolysomes (Ly). × 11,200.

TABLE IV

Surface Markers of Spleen Cells

Surface marker	Assays	% positive dendritic cells	Positive control	
H-2	Cyto, M. H.	90–100	Nucleated spleen cells	>90%
θ	" " "	<10	" " "	20–35%
Immunoglobulin-μ	" " "	<10	" " "	30–45%
Immunoglobulin-κ	" " "	<10	" " "	35–55%
PC-1	" " "	<10	Not done	
EAC receptor	Rosette	<10	Nucleated spleen cells	25–40%
"Macrophage" antigen	M. H.	20	Macrophages	>95%

Cytotoxicity (Cyto) or mixed hemadsorption (M. H.) assays were used to try to detect a variety of surface markers on dendritic and other spleen cells. In each case, cells were exposed for 30–45 min to antiserum, with complement (Cyto), or antiserum followed by indicator red cells (M. H.). The dendritic cell surface exhibits appropriate histocompatibility antigens by either assay, but does not manifest other markers such as θ, PC-1, immunoglobulin, activated C3 receptor, or antigens detected by an antimacrophage antiserum.

SRBC i.v., and days 1, 4, 7 after a boosting dose) were incapable of binding antigen, or antigen coated with the animal's serum antibody, with or without complement. Histological examination of the spleens revealed that germinal center development was prominent at several of the time points measured. If binding to SRBC or opsonized SRBC was performed in suspension, and then the cells were adhered to glass, normal numbers of free dendritic cells were still recovered. We were also unable to demonstrate by cytochemical techniques the binding of HRP, or HRP-anti-HRP aggregates, to dendritic cells taken from animals undergoing a secondary response to HRP (days 0, 1, 2, 4, 7). In both SRBC and HRP-immunized animals, binding and interiorization of antibody-coated materials was evident in typical macrophages.

Colony-Forming Units in Nonadherent Spleen Cells.—Dendritic cells have the morphological features of reticular cells, i.e., when stained with basic dyes, the nucleus is large and pale with little nucleolar development and the cytoplasm is arranged in weakly basophilic processes. The work of Maximow (14) suggested that reticular cells may be primitive elements capable of giving rise to mature erythroid, myeloid, and lymphocytic cells. We therefore asked if spleen cells, depleted of dendritic cells by glass adherence at relatively low plating densities (20), give rise to hematopoietic colonies when adoptively transferred to lethally irradiated mice. In fact, nonadherent spleen cells do generate normal numbers of surface colonies in recipient spleens (Table V), so that if so-called "primitive" reticular cells exist in spleen, they do not correspond to the dendritic cell we have described.

Absence of "Collagen" Formation by Dendritic Cells.—The connective tissue of lymphoid organs contain large amounts of an extracellular material, reticulin (24). Lymphoid reticulin has not been characterized chemically, but in

kidney, material with the tinctorial properties of reticulin contains proteins with an amino acid composition similar to collagen (17, 25). In situ, elongate cells, associated with extracellular deposits of amorphous reticulin and typically striated collagen fibrils, are morphologically distinguishable from dendritic cells. The former contain well-developed rough endoplasmic reticulum, bands of cytoplasmic microfilaments, and a distinctive nuclear chromatin pattern (R. M. Steinman, unpublished observations). In order to test directly that dendritic cells are incapable of synthesizing collagen-like proteins, we pulsed BSA column-enriched populations of spleen cells with [^{3}H]proline and determined if macromolecular [^{3}H]hydroxyproline was synthesized, as occurs during collagen formation (8, 9). In populations consisting of more than 50% dendritic cells, proline was actively incorporated into a nondialyzable form, but was only converted to hydroxyproline in trace amounts (Table VI). In

TABLE V

Inability of Dendritic Cells to Serve as Colony-Forming Units

Cells transferred	Surface colonies/spleen ± SD
Whole spleen	28 ± 6.4
Nonglass adherent spleen	29 ± 4.7

10^6 whole spleen, or nonglass adherent spleen cells depleted of dendritic cells, were adoptively transferred intravenously to syngeneic, irradiated (900 rads Co^{60}) recipients. 9 days later, the recipient spleens (7 mice/group) were fixed in Bouin's fluid and the number of surface colonies counted. Spleen cells depleted of dendritic cells by glass adherence contain normal numbers of colony-forming units.

TABLE VI

Inability of Dendritic Cells to Synthesize "Collagen-Like" Material

Cells	Type of culture	Total nondialysable cpm/100 μg cell protein	Total nondialysable [H^3]Hypro/100 μg cell protein	% collagen
Mouse L	Monolayer	190,000	9,500	4.8
Spleen pellicle BSA columns	Suspension	91,000	80	0.09
Spleen adherent BSA pellicle	Monolayer	645,000	450	0.07

Three cell populations were cultivated for 4 h in MEM supplemented with 10% FCS, 0.25 mM ascorbic acid, and [^{3}H]proline (100 μc/ml, 3 Ci/mmol). The combined culture medium and cells were then processed to determine the total number of nondialyzable counts synthesized by the cells, as well as the number of counts chromatographing as hydroxyproline. The relative synthesis of collagen-like protein is indicated by the ratio of hydroxyproline counts divided by the total incorporated counts. Mouse L cells synthesize large amounts of collagen under these conditions, whereas spleen cells enriched in dendritic cells by BSA columns, ± glass adherence, convert only minute amounts of the [^{3}H]proline tracer into nondialyzable [^{3}H]Hydroxyproline.

comparison, hydroxyproline accounts for 4.8% of the total counts incorporated by a fibroblast monolayer (mouse L cells) as has been frequently observed by others (e.g., 10).

[^3H]*Thymidine Incorporation.*—When adherent spleen cells are exposed to [^{3}H]thymidine for 1 or 6 h in vitro, only 1.5–2.5% of the dendritic cells exhibit nuclear labeling. The 6-h exposure data are more difficult to interpret since ⅓–½ of the starting dendritic cells may be lost during the labeling period. In all instances, positive dendritic cells are heavily (80 + grains) labeled. An average of 1 in 200 macrophages were labeled in these same cultures and the grain counts were lower (10–30 grains).

DISCUSSION

In this paper, we show that the dendritic cell, previously distinguished on morphological grounds (20) can be functionally differentiated from other cell types in lymphoid organs. Two properties of dendritic cells were especially useful in carrying out these functional tests. The first is that dendritic cells adhere to glass and distribute themselves randomly over the surface. The number of dendritic cells in a given cell suspension can then be quantitated with considerable reliability, the standard deviation of multiple counts being less than 10% (20). This assay can then be used to quantitate the number of dendritic cells, e.g., exhibiting a given surface antigen or interiorizing an appropriate endocytic marker. A second property is that the density of dendritic cells, under the conditions employed, is less than that exhibited by 90% of the other nucleated cells in spleen and lymph node. If lymphoid cells are suspended in BSA, (0.31 osmol/l, pH 7.35, $P = 1.082$), and centrifuged to equilibrium, a pellicle of floating cells is obtained which is greatly enriched (7–20-fold) in typical dendritic cells. This cell fraction can then be used to verify and extend data obtained on unfractioned cell suspensions, in which dendritic cells comprise 1% or less of the total nucleated cells.

Most of the cells in lymphoid organs are lymphocytes. The dendritic cell does not have any of the currently accepted surface differentiation markers of lymphocytes. These include θ-markers, receptors for the activated third component of complement and immune complexes, and surface immunoglobulin. In addition, its labeling index following exposure to [^{3}H]thymidine in vitro is too low (1.5–2.5%) for the dendritic cell to be a large lymphocyte, as these latter cells have high labeling indices under similar conditions (6). The dendritic cell also would not seem to fall in the plasma cell lineage. It lacks the PC-1 surface antigen of antibody-secreting cells (22), and it adheres to glass and plastic surfaces. The precursors of antibody-secreting cells in mouse spleen appear to arise largely if not entirely from the nonadherent population (12). Finally, the dendritic cells we are studying in vitro probably do not correspond to the "null" lymphocyte population identified by Stobo et al. in murine lymphoid organs (21). Both populations lack the θ- and Ig-surface markers, but null lymphocytes can be distinguished from dendritic cells because of morpho-

logical dissimilarities, greater abundance (3–14% of spleen cells), and inability to adhere to glass.

Histological studies of lymphoid organs demonstrate an abundance of typical macrophages. Dendritic cells clearly lack the endocytic capacity of macrophages. The latter involves both qualitative and quantitative differences from other cell types. Qualitatively, macrophages avidly bind and interiorize antigens coated with specific antibody, especially of the IgG class. Dendritic cells lack this ability when tested with HRP-anti-HRP aggregates, or antibody-coated SRBC. Quantitatively, macrophages may differ from other cells in the rate at which they interiorize particles and solutes. For example, when HRP is used as a marker solute, macrophages pinocytose at 10–20 times the rate (per unit of cell protein) of fibroblasts in vitro.[2] Homogeneous populations of dendritic cells are not available for quantitative studies, but our morphological observations suggest that they pinocytose HRP and small colloidal particles at a much slower rate than spleen macrophages—a rate consistent with the "nonprofessional" fibroblast and other cultured cells.[2]

Dendritic cells can also be distinguished from less mature members of the mononuclear phagocyte family—the promonocyte and monocyte. Mouse promonocytes are proliferating cells and thus exhibit a high labeling index in vitro (23) unlike the 1.5–2.5% seen in dendritic cells. Promonocytes and monocytes characteristically differentiate into large macrophages in vitro (1, 23), whereas dendritic cells are rapidly lost from adherent cell monolayers, presumably by cell death. When viability is maintained for longer periods by cultivation in plasma clots, the dendritic cell does not assume the morphological features of macrophages.

Lymphoid organs are thought to contain other functionally distinct cell types, all of which are said to have the morphological features of reticular cells and/or are given the name reticular cell by various authors. The dendritic cell we have identified in vitro does not correspond to any of these cell types by direct functional tests. Thus, lymphoid organs contain elongate connective tissue cells. However, dendritic cells are incapable of converting exogeneous [^{3}H]proline into macromolecular [^{3}H]hydroxyproline, as occurs in connective tissue cells (8, 9). Lymphoid organs are thought to contain primitive reticular cells which give rise to mature erythroid, myeloid, and lymphocytic cells (14). However, spleen cells depleted of dendritic cells by glass adherence have a normal capacity to give rise to marrow colonies in irradiated recipient spleens. Finally, it has been proposed that there are dendritic reticular cells or dendritic "macrophages" which retain antigen as immune complexes on their cell surfaces during an immune response (reviewed in 11). Again, in normal or immune mice, the dendritic cell we have identified appears unable in vitro to bind specific antigen, antibody, or immune complexes.

It would seem then that the dendritic cell represents a novel form of cell, pre-

[2] Steinman, R. M., J. Silver, and Z. A. Cohn. Manuscript in preparation.

sumably differentiated, which is restricted in distribution to peripheral lymphoid organs. Until this point, we have concentrated on those morphological and functional features in vitro which help establish the distinctiveness of this cell type. In subsequent papers, we employ some of this in vitro data to gain further understanding of the properties and distribution of dendritic cell in situ.

SUMMARY

Dendritic cells are morphologically distinct cells isolated in vitro from peripheral lymphoid organs of mice. They have a buoyant density of less than 1.082 and can be enriched 7–20-fold by isopycnic centrifugation in albumin columns. Surface adherence of enriched populations may yield cultures containing 50% dendritic cells—preparations which can then be studied in more detail. By functional tests, dendritic cells do not represent morphological variants of either lymphocytes or macrophages. They lack lymphocyte surface differentiation markers and do not exhibit the endocytic capacities of macrophages. In tissue culture, they do not differentiate into macrophages. Dendritic cells have a low labeling index in vitro (1.5–2.5%) following administration of [^{3}H]thymidine, and this property distinguishes them from large lymphocytes and promonocytes. Dendritic cells also do not possess the functional properties of other types of reticular cells proposed to exist in lymphoid organs, i.e., they do not synthesize collagen-like macromolecules, they are not stem cells for erythroid and myeloid colony formation, and they do not retain antigens or immune complexes on their cell surface. Dendritic cells thus represent a novel cell type on both functional and morphological grounds.

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