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Ralph M. Steinman

Gilla Kaplan

Margit D. Witmer

Zanvil A. Cohn

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

V. Purification of Spleen Dendritic Cells, New Surface Markers, and Maintenance In Vitro*

BY RALPH M. STEINMAN,‡ GILLA KAPLAN,§ MARGARET D. WITMER, AND ZANVIL A. COHN

From The Rockefeller University, New York 10021

We have previously demonstrated that mouse peripheral lymphoid organs contain a new cell type which we termed lymphoid "dendritic cells" (DCs).¹ These cells adhere to tissue culture surfaces (1) and exhibit distinctive morphologic features (1). They lack many of the distinguishing surface and functional properties of other lymphoid cell types, e.g., the capacity to actively endocytose suitable tracers for pinocytosis and phagocytosis, expression of surface immunoglobulin (Ig) and T-cell antigens, and ability to rosette erythrocytes coated with antibody and/or complement (2). Spleen DCs probably are derived from a thymus-independent, bone marrow precursor (3). DCs occur in low frequency, accounting for 1% or less of lymphoid cell suspensions (1). Cells with a similar appearance occur in small numbers in sections of splenic white pulp (4).

A major difficulty with our earlier work was that purified populations of DCs could not be obtained. In this paper, we report on the purification of DCs from mouse spleen in good yield using many of the criteria outlined above. Purification depended upon the observations that adherent spleen cells consist of two cell types, DCs and immature macrophages, both of which elute from glass surfaces after overnight cultivation. The availability of purified DCs has enabled us to document two striking new surface properties, i.e., topography in scanning electron microscopy (EM) and expression of Ia alloantigens, and to show that DCs can be maintained essentially unchanged in vitro for several days.

Materials and Methods

Mice. DBA/2, C57B1/6, BALB/c, B6D2F₁ (DBA/2 × C57B1), C3H, CBA, and A strain mice were obtained from The Trudeau Institute, Saranac Lake, N. Y., and The Jackson Laboratory, Bar Harbor, Maine. CD2F₁ (DBA/2 × Balb/c) mice were from Flow Laboratories, Rockville, Md., and NCS mice from The Rockefeller University colony (New York).

Purification of Dendritic Cells. An adherent low density subpopulation of spleen was prepared

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¹ *Abbreviations used in this paper:* BPA, bovine plasma albumin; DC, dendritic cells; EA-IgG, sheep erythrocytes coated with IgG antibody; EA-IgMC, sheep erythrocytes coated with IgM antibody and complement; EM, electron microscopy; FCS, fetal calf serum; MHC, major histocompatibility complex; PLL, poly-L-lysine; SRBC, sheep erythrocytes.

as previously described (2). First, single-cell suspensions of spleen in dense bovine plasma albumin (BPA; Armour Pharmaceutical Company, Chicago, Ill.), $\rho = 1.080$, were floated to equilibrium. The floating subpopulation (5–15% of total spleen cells) was adhered to glass, generally by plating $5\text{--}10 \times 10^6$ cells in 0.5 ml medium on 32-mm circular coverslips (#R 2005-32, Rochester Scientific Co., Rochester, N. Y.) for 1–3 h. Nonadherent low density cells were removed by gentle Pasteur pipetting, and the coverslips were maintained overnight in RPMI 1640 medium supplemented with penicillin, 5×10^{-5} M 2-mercaptoethanol, and 1–5% heat-inactivated mouse or fetal calf serum. During this culture period, most of the adherent cells either eluted from the glass or could be easily dislodged, yielding a cell suspension that was >90% trypan blue negative and in excellent yield relative to the initial number of adherent cells. The eluted cells were comprised of DCs and macrophages. The latter selectively and efficiently rosetted with sheep erythrocytes, heavily opsonized with subagglutinating doses of rabbit hyperimmune antiserum. Erythrocytes and leukocytes, in a ratio of 30 to 1, were spun together by centrifugation, rosetted in the cold for 30 min, resuspended, layered onto dense BPA columns, ($P = 1.088$), and spun to equilibrium. The floating fraction consisted almost entirely of DCs, whereas the pelleted fraction contained rosetted macrophages, some nonrosetted DCs, and dead cells (see Results).

Morphologic Techniques. To prepare uniformly distributed populations for counting and examination by phase contrast microscopy, $2\text{--}5 \times 10^5$ cells were spun onto 12-mm circular coverslips in 13-mm diameter glass cylinders as described (5). To look at adherent cells, the coverslips were maintained for 1–3 h at 37°C , the nonadherent cells were removed by pipetting or by shaking the cylinders, and the coverslips were fixed in 1.25% buffered glutaraldehyde. To look at nonadherent cells, the latter were spun onto coverslips previously coated for 20 min with poly-L-lysine (PLL, type VII, Sigma Chemical Co., St. Louis, Mo.), 25 $\mu\text{g}/\text{ml}$ phosphate-buffered saline. Cells on untreated and PLL-coated coverslips could be tested for Ig receptors by spinning 10^7 opsonized erythrocytes onto the coverslips. Binding and/or ingestion were allowed to occur over a 30 to 60-min period, the nonbound cells were removed by shaking, and the coverslips were examined after glutaraldehyde fixation.

For scanning EM, adherent cells or cells in suspension were fixed in buffered 1.25% glutaraldehyde. Suspension cells were cleared of fixative by washing in saline, spun onto PLL coverslips, and then put back in glutaraldehyde. Further processing of the coverslips included dehydration in graded alcohols, transfer to amyl acetate, critical point drying in liquid CO_2 in a Sorval C.P.D. System (Ivan Sorval, Inc., Norwalk, Conn.) and coating with gold (Edwards 5150 sputter coater, Edwards High Vacuum Inc., Grand Island, N.Y.). Specimens were scanned in an ETEC autoscan at a tilt of 45° .

For transmission EM, cells were fixed for 1 h at 4°C in mixed fixative i.e., two parts of 1% osmium to one part 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. Some specimens were stained en bloc with 0.5% uranyl acetate, whereas others were treated with 0.3% tannic acid as described by Simionescu and Simionescu (6). Embedding was in Epon (Shell Chemical Co., Houston, Tex.), and sections were stained with uranyl acetate and lead citrate.

Cell Surface Markers. Specific antisera and antibody-coated erythrocytes were used to test whether purified DCs exhibit surface markers characteristic of other lymphoid cell types. The antisera and results obtained on splenocytes and thymocytes are tabulated (Table I).

Cytotoxicity assays were usually one-stage reactions in which we mixed equal volumes of: cells in 2% fetal calf serum (Flow Labs, Rockville, Md.) (FCS)-RPMI 1640-100 $\mu\text{g}/\text{ml}$ deoxyribonuclease (DNAase I, Sigma Chemical Co.) at $5\text{--}10 \times 10^6/\text{ml}$; antibody diluted in RPMI 1640; and guinea pig complement (Cordis Laboratories, Miami, Fla.) diluted 1 to 5.

For indirect immunofluorescence, cells were stained 30 min in the cold with the primary reagent and washed through 5% FCS-RPMI 1640. Rabbit antisera were visualized with a rhodamine-labeled, IgG fraction of a goat anti-rabbit IgG serum (10340, N. L. Cappel Laboratories Inc., Cochranville, Pa.), whereas mouse alloantisera were visualized by successive treatments with a rabbit anti-mouse γG reagent (#8403-12, Litton Bionetics, Kensington, Md.), followed by the rhodamine goat anti-rabbit. After staining and washing, the cells were spun onto PLL-coated coverslips and fixed in cold 96% ethanol-1% acetic acid for 15–30 min, rinsed in PBS, and mounted in phosphate-buffered glycerol.

Receptors for Ig were detected by rosetting assays using opsonized sheep erythrocytes

TABLE I
Antisera Used on Purified DCs

Serum	Cells killed	
	Spleen	Thymus
	%	
Rabbit anti-mouse κ	45-60	<5
Rabbit anti-mouse brain	25-35	>95
Mouse anti-thy 1.2	20-25	>95
Mouse anti-Ia.2, +	50-55 H-2k; <5 H-2b, d	<5
Ia.4, +	<5 H-2 b, d, k	>5
Ia.7	50-55 H-2 d, k; <5 H-2b	>5
Ia.8, +	50-55 H-2d; 70-80 H-2b; <5 H-2 k	>5
Mouse anti-H-2 (D-2)	>95 H-2b; <5 H-2d, k	>95
(D-33)	>95 H-2b; <5 H-2k, d	>95
(D-32)	>95 H-2k; <5 H-2b, d	>95

Rabbit anti-mouse κ was prepared by immunizing rabbits with an F(ab')₂ fragment of a $\kappa\gamma$ myeloma in Freund's adjuvant (5). Rabbit anti-mouse brain serum was obtained from Accurate Chemical, & Scientific Corp., Hicksville, N. Y., and all the alloantisera were from the Research Resources Branch, National Institutes of Health.

H-2b mice were C57Bl/6; H-2d were DBA/2 and Balb/c; and H-2k were C3H and CBA.

(SRBC). Both a hyperimmune rabbit anti-SRBC serum, as well as purified IgG and IgM antibodies (Cordis Laboratories), were used. To detect complement receptors, we used IgM antibody-coated erythrocytes that were exposed to 10% fresh mouse serum for 10 min at 37°C. Rosetting assays were performed at 4° and 37°C for 20-60 min at ratios of 30 erythrocytes to 1 leukocyte. Under these conditions, mononuclear phagocytes rosette heavily with IgG-coated erythrocytes (EA-IgG) and IgM and complement-coated erythrocytes (EA-IgMC) and interiorize variable numbers of EA-IgG. B lymphocytes bind just zero to three EA-IgG under the conditions used but rosette large numbers of EA-IgMC.

Tissue Cultures. Populations enriched in DCs or macrophages were maintained in RPMI 1640 supplemented with penicillin, 5×10^{-5} M 2-mercaptoethanol, and serum—usually 5% heat-inactivated FCS or isologous mouse serum obtained by cardiac puncture. Flat-bottomed culture vessels were optimal. Circular glass coverslips, 5- and 12-mm diameter. (#R2005-5 and 12 Rochester Scientific), were placed at the bottom, to retrieve adherent cells when necessary.

Radiolabeling Experiments. To look for cell proliferation, we exposed cultures to [³H]thymidine (Schwarz/Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y., spec act 6.0 Ci/mM) either in a pulse (½ h in 1.0 μ Ci/ml) or continuously (24 h in 0.05 μ Ci/ml). The labeled cells were washed in medium supplemented with 200 μ M nonradioactive thymidine, spun onto 12-mm coverslips as described (5), dried with a hair dryer, fixed in methanol, dipped in NTB-3 emulsion (Eastman Kodak, Rochester, N. Y.), and exposed for 1-3 days. To radiolabel purified DCs so that they could be visualized after mixing with other nonlabeled cell types, we exposed DCs to [³H]uridine (Schwarz/Mann), 1.0 μ Ci/ml for 1 h, washed, and returned the cells to culture in medium supplemented with 200 μ M nonradioactive uridine to prevent reutilization of label. This radiolabeling protocol does not alter viability of purified DCs. To isolate viable cells in cultures containing labeled DCs, 2-4 $\times 10^7$ viable cells in 5 ml culture medium were layered onto 5 ml Lympholyte-M (Accurate Chemical & Scientific Corp., Hicksville, N. Y.) and spun to equilibrium. Viable cells were selectively recovered in the floating fraction in excellent yield.

Results

Purification of Spleen DCs. The purification of DCs was monitored by morphology and surface markers. Cytologic criteria seen by phase contrast and EM have been

TABLE II
Purification of DCs from Spleen

Populations at successive stages of purification	Total cells/spleen ($\times 10^{-6}$)	DCs	Cells killed with complement and		Cells rosetting with	
			Anti-Ig	Anti-T	EA-IgG	EA-IgMC
		%	%	%	%	%
Starting spleen cells	100	0.2-0.5	45-60	20-35	5-10	25-40
Low density cells from dense albumin gradient	5-15	2-5	45-60	20-35	5-10	25-40
Glass adherent low density cells	0.2-1.0	40-70	NT*	NT*	30-60	30-60
Cells eluted from glass after overnight culture	0.2-0.8	40-70	<5	<5	30-60	30-60
Low density cells after removal of EA-IgG rosetting cells	0.1-0.3	>95	<5	<5	<2	<2

Typical yields of total spleen cells and enrichment of DCs at successive steps in the purification procedure provided in the text. The percentages of cells expressing four surface makers that were absent on DCs are also given. The anti-Ig serum was an anti- κ reagent and the anti-T-cell serum either an antibrain or anti-thy-1 reagent (Table I).

* NT, not tested.

described (1, 2). Useful surface makers that are not expressed on DCs (2) include: surface Ig, T-cell antigens, binding and interiorization of EA-IgG, and binding of EA-IgMC.

Four steps were required to purify DCs (Table II), the first two of which have been published (2). A low density subpopulation of spleen cells were first floated on dense BPA columns, and contained >90% of the DCs and 5-15% of the total spleen cells. The low density population was then allowed to adhere to glass. 80-90% of the DCs and only 3-8% of the total low density cells attached. After 1-2 h on glass, two types of adherent cells could be distinguished by phase contrast. The DCs had irregular spinous shapes with rare surface ruffles; refractile, often contorted nuclei; and large, spherical, phase-dense mitochondria (Fig. 1). In contrast, most other adherent cells

FIG. 1. Monitoring the purification of spleen DCs by phase contrast and EA-IgG rosetting criteria. (a) Adherent low density spleen cells are comprised of well-spread DCs and round immature macrophages. The refractile, often irregular nucleus as well as phase-dense mitochondria are noted in the DCs. The immature macrophages are so termed on the basis of EA-IgG rosetting and phagocytosis, scanning EM, and subsequent behavior *in vitro* (see text, and Figs. 1b, 4a and b, 5d). $\times 1,280$. (b) Cultured adherent low density cells after elution from the glass surface. The eluted cells were spun onto PLL-coated coverslips and then rosetted with EA-IgG on ice for 20 min. Two populations of cells are evident. The DCs display their irregular shapes after flattening on PLL, as well as their refractile irregular nuclei and phase-dense mitochondria. The macrophages heavily rosette EA-IgG. The number of DCs identified by phase contrast in specimens not exposed to EA-IgG is similar to the number of nonrosetted cells. $\times 760$. (c) The purified DC fraction after removal of EA-IgG rosetted macrophages. Almost all the cells are irregularly shaped DCs with contorted nuclei and phase-dense mitochondria. In this preparation, the DCs extend processes in several planes of focus so that many of the organelles cannot be visualized in a single micrograph. $\times 760$. (d) Viable, cultured, high density, spleen cells after isolation on Lympholyte-M columns, spinning onto PLL-coated coverslips, and EA-IgG rosetting on ice as in Fig. 1b. Cells with the cytologic features of DCs are rare, and absent in this field. Most of the cells are small round lymphocytes, some of which (presumptive B cells, arrows) rosette one to three EA-IgG. Three larger cells are heavily rosetted. Although not evident in the presence of rosettes, these large cells are ruffled and have many granular inclusions, as is typical for macrophages. $\times 840$.

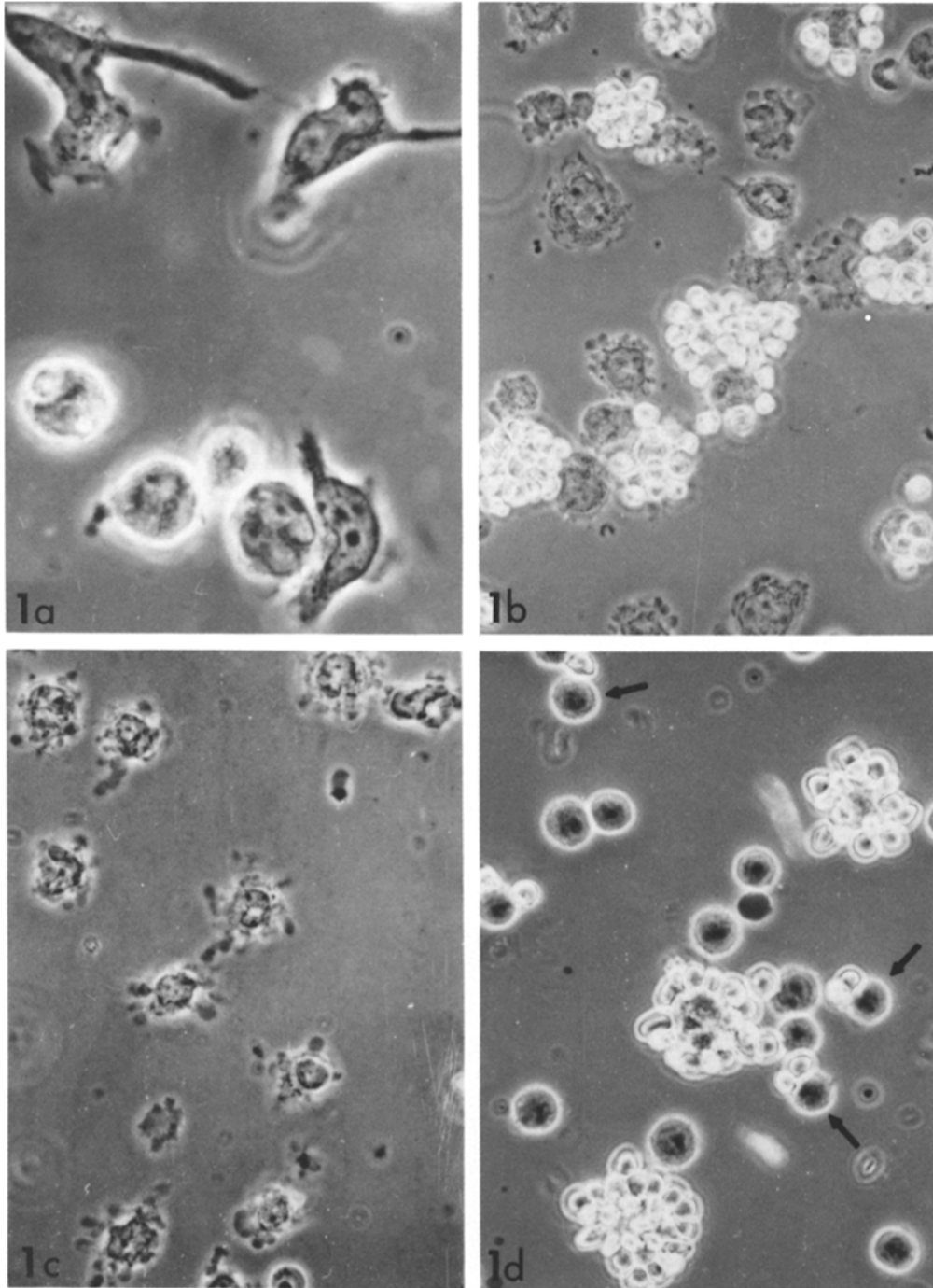


FIG. 1.

were small and round (Fig. 1a) with only an occasional surface ruffle. These round cells, however, selectively rosetted large numbers of EA-IgG or EA-IgMC and interiorized one to three EA-IgG. The term "immature" macrophage or mononuclear phagocyte is applied to these round cells, because in addition to phagocytosing EA-IgG, they look like macrophages in scanning EM, and they become larger, more actively phagocytic, macrophages with further cultivation (see below). Typical well-spread, mature macrophages with many ruffles, vesicles, and lysosomes were present in the starting adherent cell preparation from most spleens, but they accounted for <5% of the adherent cells.

The third step for purifying DCs was to culture the adherent cells overnight. DCs and most macrophages eluted from the dish surface or could easily be dislodged by gentle pipetting. More than 90% of the initially adherent cells were recovered in suspension as trypan blue-negative, viable cells. Cytotoxicity assays were then applied to show that the eluted cells lacked surface Ig and T-cell antigens (Table II). The eluted cells from most spleens would not readhere spontaneously to glass or plastic and were therefore examined after centrifugation onto PLL-coated coverslips. Immediately after flattening on coverslips, the presumptive DCs had irregular shapes and displayed typical morphologic properties (Fig. 1b). The mononuclear phagocyte population was generally circumferentially spread and ruffled, and would bind and phagocytose EA-IgG, even when attached to PLL (Fig. 1b).

The final step in the purification was to rosette the eluted low density cells with EA-IgG, and to remove the rosettes by centrifugation on dense BPA. The floating fraction consisted almost entirely of DCs by phase contrast (Fig. 1c), whereas the rosetted pellet contained macrophages, dead cells, and 10–20% of the DCs. The pelleted DCs lacked surface EA-IgG. They presumably were trapped with macrophages during rosetting.

Other cultured spleen cell populations were examined with similar light microscope techniques. High density cells from the albumin gradient (Fig. 1d) contained only rare DCs after flattening on PLL-coated coverslips, whereas nonadherent low density cells contained 1–5% DCs. 5–15% of the viable recovered cells were typical macrophages by cytologic criteria. These cells bound and ingested EA-IgG (Fig. 1d), and many would adhere to a fresh glass or plastic surface. 80% or more of the cultured cells were small, round lymphocytes, some of which rosetted one to three EA-IgG (Fig. 1d). Generally $\frac{2}{3}$ of the lymphocytes were killed by anti-Ig sera and complement, and $\frac{1}{3}$ with anti-T-cell reagents.

Transmission EM of purified DCs revealed a homogeneous population with the same cytologic features as those previously described for freshly isolated DCs (Fig. 2). The nucleus was extremely irregular in shape with a peripheral ridge of heterochromatin, few nuclear pores, and small nucleoli. The cytoplasm contained well-developed mitochondria, small thin pieces of rough endoplasmic reticulum, and scattered smooth vesicles. The Golgi region lacked typical membrane-bound lysosomes and secretory granules, but contained a variety of multivesicular bodies comprised of large vacuoles surrounded by, or containing, smaller vesicles. Thick filaments were prominent in the Golgi region.

We conclude that a population of morphologically distinct, irregularly shaped DCs can be obtained in high purity and good yield. Macrophages in particular were the major contaminant of the adherent spleen preparation and have been removed by

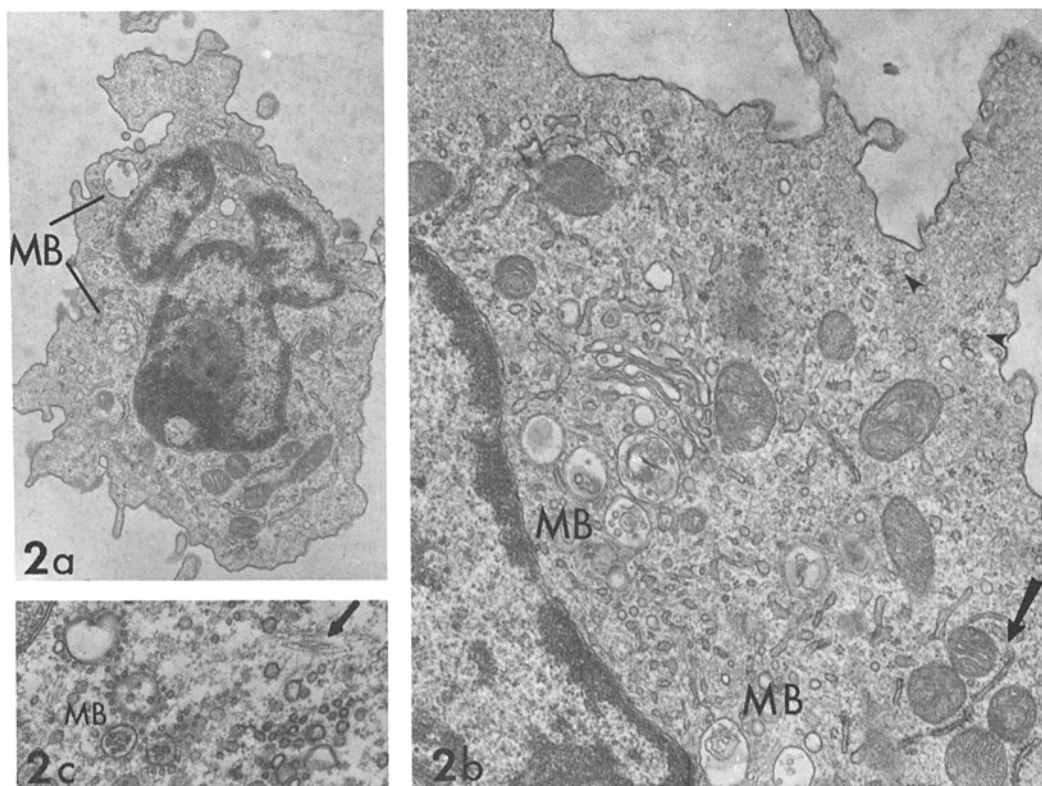


FIG. 2. EM features of suspensions of purified DCs. (a) Most profiles at low power show an irregularly shaped nucleus lined with a rim of heterochromatin. The cytoplasm contains scattered mitochondria and smooth vesicles of varying size, including multivesicular bodies (MB). These features are all similar to DCs freshly isolated from the animal and adherent to glass (1, 2). The surface of DCs in suspension shows bulbous protrusions and/or long thin flaps of cytoplasm, in contrast to the dendrites of varying size seen initially. $\times 8,700$. (b) At higher power, cell processes contain a filamentous mesh work excluding most organelles except for tiny smooth vesicles (arrowhead). Thin, short, pieces of rough endoplasmic reticulum are associated with the mitochondria (arrows), but ribosomes are otherwise scanty. The Golgi region has many multivesicular bodies (MB). $\times 23,500$. (c) The Golgi region in a specimen treated with the mordant, tannic acid (6). Multivesicular bodies in cultured DCs commonly are comprised of a large vacuole surrounded by many smaller vesicles (MB). Scattered thick filaments (arrow) are seen, primarily in the Golgi region. $\times 21,400$.

cytologic and EA-rosetting criteria. The purified DCs lack surface Ig and T-cell antigens, as do freshly harvested DCs (2). The presence of Ia antigens, surface topography in scanning EM, and tissue culture properties of the purified DCs will now be described.

Major Histocompatibility Complex-Encoded Alloantigens. Purified DCs were exposed to anti-Ia antisera and guinea pig complement in one-stage cytotoxicity assays. The major histocompatibility complex (MHC) haplotypes to which these Ia specificities are linked are detailed in the literature supplied by the Research Resources Branch, National Institutes of Health, and are summarized briefly in Table I. Anti-Ia.7 killed H-2d (DBA/2 and BALB/c) but not H-2b (C57B1/6) DCs, and anti-Ia.8⁺ killed DCs from both strains (Table III). Anti-Ia.7 and 8⁺ sera also killed adherent DCs freshly harvested from mouse spleens. Anti-Ia.2⁺ and 4⁺ served as negative controls for the

TABLE III
Ia and K-D Alloantigens on DCs

Treatment	Cells killed				
	Whole spleen		DCs		
	DBA/2	C57Bl/6	DBA/2	C57Bl/6	
Complement only	0	0	0	0	
Anti Ia.2+ 1/10	0	0	0	0	
Anti Ia.4+ 1/10	0	0	0	0	
Anti Ia.7 1/10	50	0	96	0	
	1/30	48	NT*	97	NT
	1/90	38	NT	50	NT
	1/270	0	NT	8	NT
Anti Ia.8+ 1/10	62	85	99	100	
	1/30	60	65	97	97
	1/90	38	30	55	84
	1/270	8	12	15	36
	1/810	0	0	0	0
D-2 1:30	0	100	0	96	
	1:90	NT	84	NT	72
	1:270	NT	19	NT	17
	1:810	NT	0	NT	0
D-32 1:30	0	0	0	0	
Anti-mouse 1:1,000	55	50	0	0	

Purified DCs and unfractionated spleen cells were brought to a concentration of 5×10^6 cells/ml 2% FCS-RPMI 1640-100 μ g/ml DNAase I. Equal volumes of cells, antiserum, and guinea pig complement (1:5) were incubated for 45 min at 37°C, and the number of trypan blue-negative cells counted. Means of two experiments are given. Unfractionated spleen cells could be obtained from freshly harvested spleen or cells cultured for 1 day. The large kill with anti-Ia.8+ on C57Bl/6 cells probably relates to K_b specificities in this serum. The antiserum concentration is the final dilution in the assay mixture. C3H and CBA(H-2k) mice were also tested with Ia antisera (see Table I) to provide further haplotype specificity controls (data not shown).

* NT, not tested.

H-2b and d haplotypes. In contrast, anti-Ia.2⁺ killed H-2k and k/d DCs (strains C3H, CBA, and A), but not H-2b and d populations (data not shown). The haplotype specificities of these anti-Ia sera toward DCs are thus identical to data on reactive spleen cells supplied by the National Institutes of Health. Anti-Ia sera were also used to stain the surface of DCs by indirect immunofluorescence. Specific staining was again seen on >95% of purified DCs (Fig. 3a and b). The cytotoxic (Table III) and staining titers against purified DCs were similar to those obtained on spleen cells, indicating that the expression of Ia on DCs and reactive spleen cells, primarily B lymphocytes, may be similar.

Anti-Ia sera were also used in cytotoxicity assays against populations rich in macrophages. As others have noted (7, 8), <10% of adherent mouse peritoneal macrophages were killed with anti-Ia and complement. A similar result was found on splenic macrophages in suspension isolated after EA-IgG rosetting.

Anti-H-2 sera directed to specificities expressed by K and D regions of the MHC (Table I) were also tested. Serum D-2, which recognizes specificity 2 linked to the D

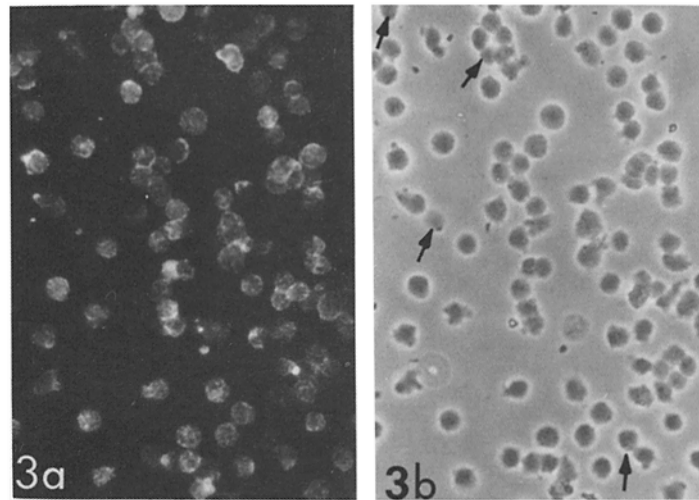


FIG. 3. Indirect immunofluorescent staining (a) and phase contrast (b) microscopy of purified DCs treated with anti-Ia serum as the primary reagent. In this case, DBA/2 (H-2d) DCs were stained with anti-Ia.7, whereas anti-Ia.2 and 4 served as negative antiserum controls (Table I). Negative cell controls were thymocytes and spleen, >99 and >40% negative, respectively. The alloantisera have been visualized by successive additions of rabbit anti-mouse-gamma followed by a rhodamine labeled, goat anti-rabbit reagent all applied at 4°C in the presence of 20 mM azide. More than 90% of the profiles in this micrograph show clear immunofluorescent staining of varying intensity. Some negative profiles are marked in Fig. 3b. \times 320.

end of the H-2b haplotype, killed purified DCs as well as spleen and peritoneal cells in C57B1/6 but not DBA/2 and CBA mice (Table III). The titers were similar in all three cell populations. Serum D-33, which recognizes specificities 33, 53, and 54 linked to the K end of H-2b mice, killed C57B1/6 but not DBA/2 and CBA cells, and again the titers were similar toward spleen, peritoneal cells, and DCs (data not shown). Finally, serum D-32, which recognizes specificity 32 linked to the D end of H-2k mice, killed CBA targets but not C57B1/6 and DBA/2 DCs.

Surface Topography of DCs. The surface of DCs was next examined by means of scanning EM. Immediately after isolation from spleen, adherent elongate DCs had smooth surfaces almost devoid of ruffles, microvilli, and blebs (Fig. 4a). This smooth surface stands in contrast to the topography of well-spread adherent macrophages, which in spleen as in other sites (9) exhibit ruffles, smaller ridges, and microvilli. DCs were not tightly adherent, but instead appeared to be lifted from the glass surface in many areas (Fig. 4a). The immature mononuclear phagocytes in the low density population were easily identified by scanning, because of their typical surface ridges and ruffles (Fig. 4a). For the most part, macrophages were circumferentially spread and tightly adherent to the glass surface.

After overnight culture and elution from the glass surface, both DCs and macrophages retained distinctive appearances. Most DCs exhibited an array of many bulbous protrusions of varying size and shape (Fig. 4b). About 10–20% of the purified DCs either lacked surface processes or had one or more large thin flaps of cytoplasm, several microns in length. The immature macrophages retained their distinctive surface ridges and ruffles (Fig. 4b). After removal of EA-rosetting cells from the eluted population, the nonrosetted cells all exhibited the unusual surface ascribed

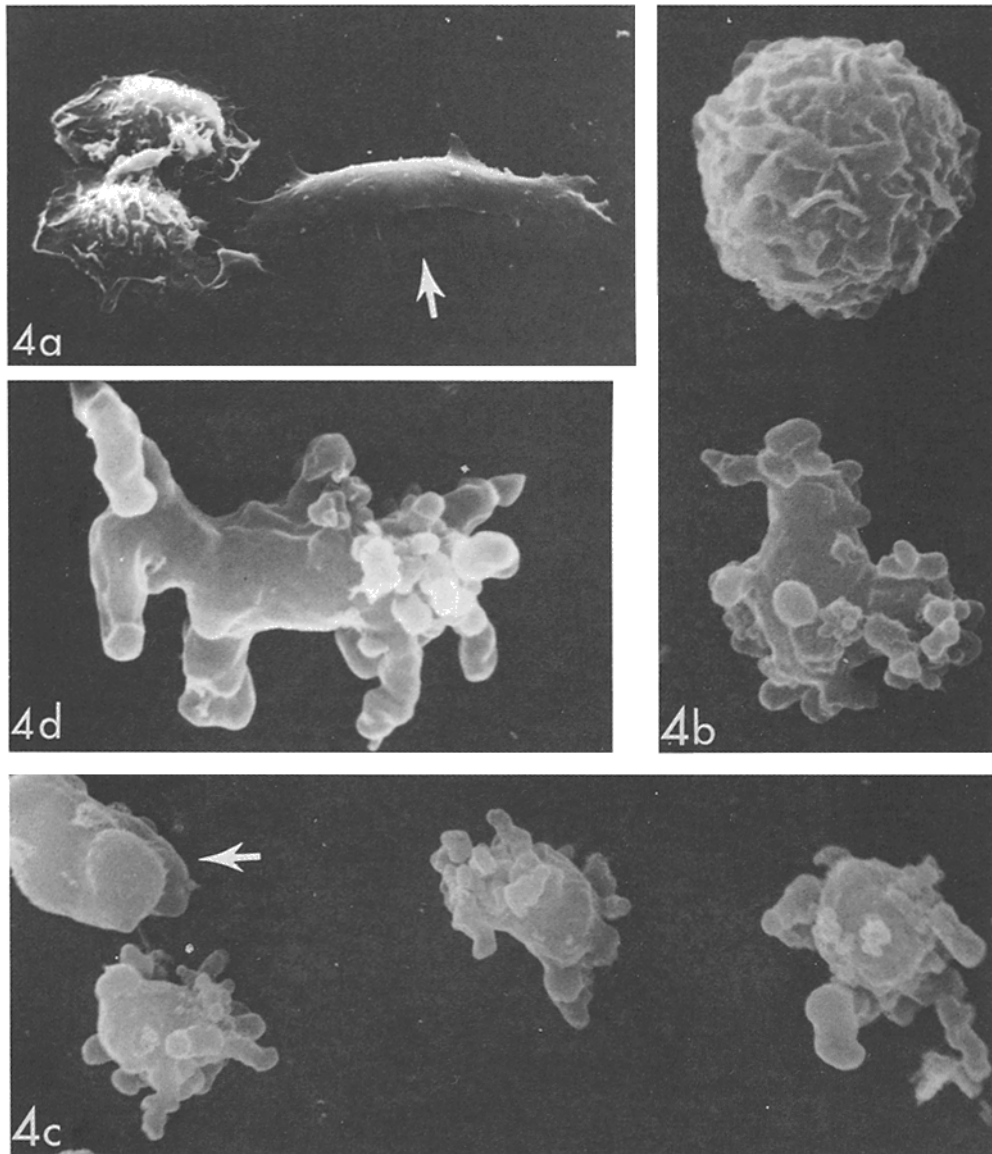


FIG. 4. Scanning EMs of adherent low density spleen cells at varying stages of purification and culture. (a) Initial adherent low density cells. The extended DC is remarkably smooth, lacking surface microvilli, ruffles, and ridges. DCs often appear lifted from the glass surface (arrow). Two immature macrophages on the left are readily identified by the ruffles and ridges characteristic of the mononuclear phagocyte lineage. $\times 2,500$. (b) The two types of adherent low density cells that elute from glass after overnight culture. After fixation in suspension, the cells were spun onto PLL-coverslips for further processing. The DC (bottom) is covered with bulbous protrusions of varying dimensions, whereas the macrophage is covered with typical ridges. $\times 4,500$. (c) Eluted low density cells after removal of cells rosetting EA-IgG. The purified fraction is comprised almost entirely of DCs. Most are covered with processes, but a few are still smooth (arrow). The ridged macrophages have been removed. $\times 3,200$. (d) A purified DC maintained in vitro an additional 2 days. The DC processes often are bent in unusual ways. $\times 6,000$.

to DCs, whereas ruffled macrophages were depleted (Fig. 4c).

We conclude from these cell surface studies that DCs purified according to previously described cytologic criteria lack most of the differentiation markers of lymphocytes and macrophages, as detected by cytotoxicity, staining, rosetting, and scanning EM assays. The striking exception is that all DCs exhibit MHC-linked alloantigens, including Ia antigens. In addition, their surface topography is unique.

Behavior of Cultured DCs. DCs could be cultured as highly viable cell suspensions as long as they were maintained at densities of 1×10^6 cells/cm² of culture surface. At these concentrations, and in mouse, fetal calf, or newborn calf serum, 70% or more purified DCs were recovered as viable cells after 2–3 days of culture. Decreasing the cell concentration by five fold or more lead to rapid death of the DCs.

Purified DCs were tagged with [³H]uridine in order to follow their fate when co-cultured with other spleen cells. They were either cultured for 2 days as pure DCs and then mixed with a 100-fold excess of cultured unfractionated spleen cells, or cultured continuously in the presence of spleen. Viable cells from both sets of cultures were then recovered on Lympholyte-M columns. By autoradiography in two experiments (Fig. 5a), 80% of the initial inoculum of labeled DCs, and 20–40% of the total spleen cells, were recovered in the viable cell fraction obtained from both culture conditions.

Purified DCs and DCs cultured with an excess of unfractionated spleen never reacquired the ability to readhere to glass or plastic surfaces once they had been eluted into suspension. However, purified DCs did associate with one another to form floating aggregates that were easily dissociated by pipetting. When purified DCs were co-cultured with macrophages from peritoneal cavity or spleen, the DCs attached efficiently to the macrophage surface (Fig. 5b). The presence of macrophages at a ratio of one or more per four DCs greatly reduced DC recoveries after an additional 1–3 days of culture.

DCs did not proliferate *in vitro*, even if the culture medium was supplemented with 10 µg/ml lipopolysaccharide or 3 µg/ml concanavalin A, known B- and T-lymphocyte mitogens. Autoradiography after [³H]thymidine pulse radiolabeling indicated that <1% of the cells were proliferating at any time during 3 days of culture (Fig. 5c).

The cytologic features of DCs by phase contrast, scanning, and transmission EM changed little with time in culture. By scanning, the verrucous surface of DCs persisted and even became more elaborate (Fig. 4d). Some of the cells had large thin flaps of cytoplasm, and transmission EM revealed similar flaps on DCs at the periphery of cell aggregates. Cultured DCs did not acquire the surface markers of other lymphoid cells, such as surface Ig, T-cell antigens, and ability to rosette opsonized erythrocytes. Surface Ia antigens persisted, at similar levels, because the cytotoxic titers of anti-Ia sera toward DCs did not change after an additional 3 days of culture.

Behavior of Immature Macrophages. Immature macrophages behaved quite differently. Whether or not subject to rosetting with opsonized erythrocytes, these cells gradually readhered and spread on glass or plastic over a 1- to 3-day culture period. Simultaneously, the cells enlarged into typical mature macrophages with abundant surface ruffles and cytoplasmic granules and vesicles of varying size (Fig. 5d). The mature cells interiorized large numbers (5–25) of EA-IgG, in contrast on the smaller numbers (1–3) taken up by their immature progenitors; and the concentration of antibody needed to opsonize the erythrocytes could be decreased 5- to 10-fold. The

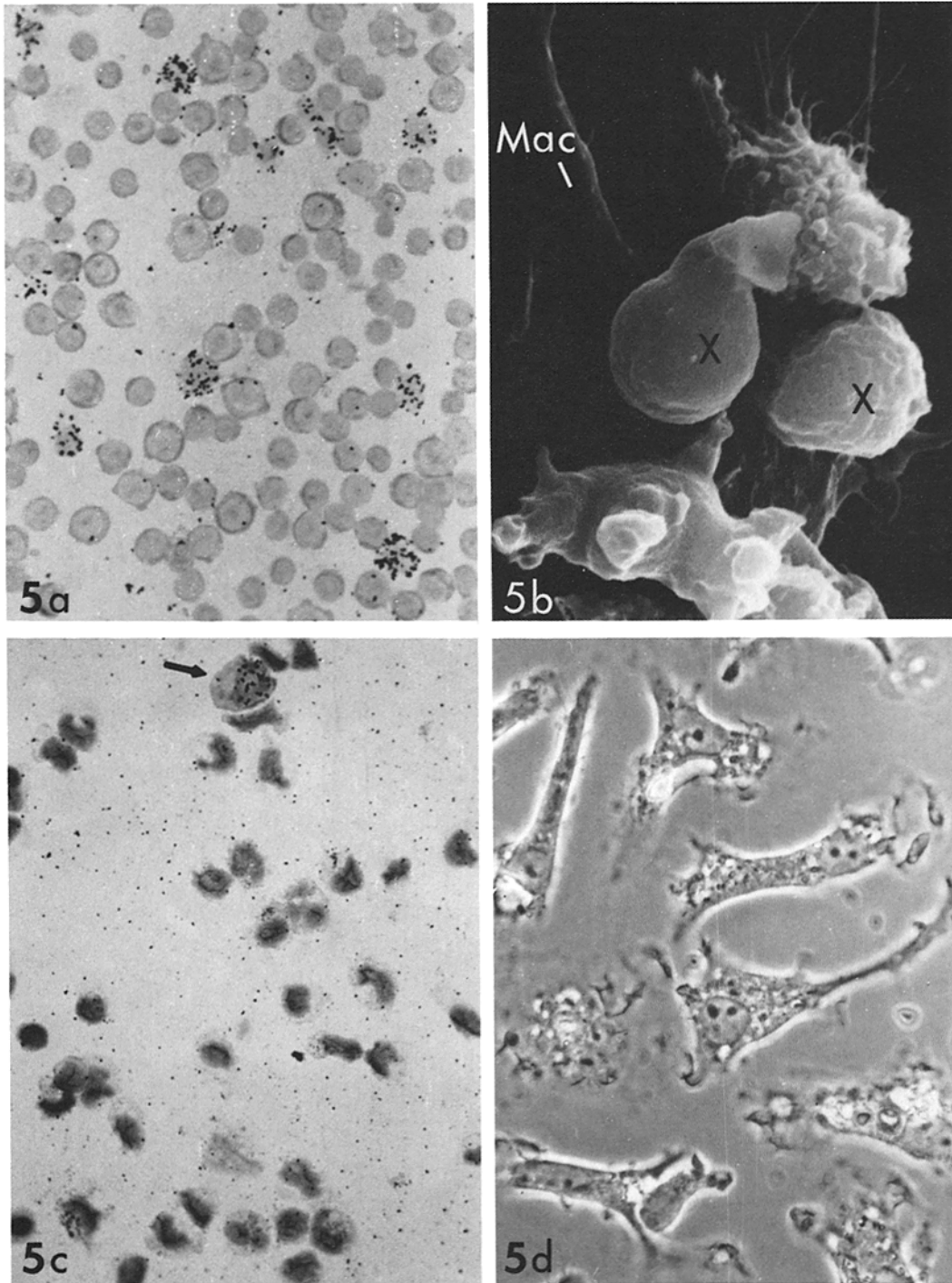


FIG. 5.

continuous presence of antiproliferative agents, i.e., high doses of nonradioactive thymidine (200 μM) or high levels of [^3H]thymidine (1–3 $\mu\text{Ci/ml}$), did not appear to alter the maturation or number of the typical macrophages. Finally, immature macrophages obtained from mice primed 2–3 wk to live *Bacillus Calmette-Guérin* mycobacteria i.p. or i.v. readhered and enlarged much more quickly than immature cells from mice obtained directly from the breeding facilities.

5- to 10-fold more large macrophages arose during culture of the nonadherent low density fraction of spleen. Macrophages also were readily detectable in cultures of high density spleen cells. Most of these macrophages were in suspension, but would adhere within an hour when plated on a fresh surface.

We conclude from these studies that DCs persist as nonproliferating, nonadherent viable cells with a distinctive morphology and set of surface markers, for at least 3–4 days *in vitro*. DCs do not acquire the features of macrophages or lymphocytes. In contrast, immature mononuclear phagocytes are present in adherent as well as nonadherent spleen cell populations, and these mature into typical large and actively phagocytic cells with time in culture. Depending on culture and other conditions, macrophages can exist as adherent or nonadherent cells.

Discussion

DCs, which represent <1% of total spleen cells, can be purified in good yield. A multistep procedure was required to achieve purification. Flootation on dense BPA and glass adherence provided an adherent cell preparation that contained most of the DCs, but was free of lymphocytic and marrow elements by cytology and surface markers. Further purification became possible when we realized that the contaminating adherent cells were immature macrophages. The latter designation was made because these cells had the surface topography of macrophages by scanning EM, were able to rosette and interiorize opsonized erythrocytes, and mature into large, actively phagocytic macrophages with time in culture. Because adherent low density cells (DCs and macrophages) eluted from glass with time in culture, it became possible to deplete the macrophages by rosetting with EA-IgG in suspension. The thrust of this paper is to show that DCs purified on the basis of cytologic criteria also have distinctive surface properties, and do not change into other cell types during at least 3 days of culture.

FIG. 5. Behavior of cultured adherent low density cells. (a) Autoradiographs used to enumerate viable DCs in whole spleen cultures. Purified DCs were tagged with [^3H]thymidine and added back to whole spleen cultures (cell ratio of 1 to 100) for 2 days. Viable cells were retrieved on Lympholyte-M columns and smears made for autoradiography. The field contains 10 labeled DCs, which otherwise stain only weakly with Giemsa (see 5c). The total number of labeled cells was 80% of that initially added to the culture. $\times 550$. (b) The interaction of DCs and macrophages is best visualized by scanning EM. In this case the macrophage (Mac) is a well-spread, tightly adherent cell. Four DCs adhere to the macrophage surface. It is noteworthy that most DCs attached to macrophages are smooth (X), lacking the bulbous protrusions usually seen in DCs. $\times 2,700$. (c) Autoradiograph of cultured purified DCs exposed continuously for 20 h to [^3H]thymidine, 0.05 $\mu\text{Ci/ml}$, after purification. Only a rare cell is radiolabeled (arrow), and we suspect these cells represent contaminating nonDCs. The DC cytoplasm does not stain with Giemsa, and usually just an irregularly shaped nucleus is evident. $\times 760$. (d) Behavior of EA-rosette-positive cells isolated from the adherent low density population. After 2–3 days in culture, most of the cells have attached firmly to the tissue culture surface, and have the typical cytologic features of mature macrophages, i.e., large cell size, active surface ruffling, abundant pinocytic vesicles, and lysosomal inclusions. $\times 760$.

The new surface properties of DCs were not previously appreciated because they were not looked for. By scanning EM, adherent DCs freshly isolated from spleen exhibit a smooth surface markedly different from the ridged and ruffled surface of well-spread adherent macrophages (e.g., 9). After elution from the glass surface and maintenance in suspension, most DCs have an array of bulbous protrusions unlike any other leukocyte described to date. The surface is so unique that we think scanning EM will be of great use in identifying DCs in heterogenous mouse lymphoid populations, and possibly in cells from other species. Aside from an unusual case of leukemia (reference 9, plate 209), the only cell type that forms such an unusual array of processes is the dendritic cell described in lymphoid follicles (10, 11). At this date, cytologic similarity is the only marker connecting the DC that we have studied *in vitro* and *in vivo*.

The presence and persistence of Ia antigens as a new surface marker for DCs is also clear cut. Most DCs express Ia, just like most B lymphocytes, but DCs lack surface Ig and C3 receptors, and do not proliferate or differentiate in response to mitogens in FCS and lipopolysaccharide preparations. We are now studying the synthesis and molecular properties of Ia antigens in DCs, because their presence suggests that DCs may be of importance in expressing Ir gene function.

The excellent viability of purified DCs *in vitro* is in contrast to our previous observation that these cells could not be maintained except in plasma clots (2). The main difficulty previously was that we were in effect keeping the cells in suspension at cell concentrations that were too low, i.e., $<1 \times 10^6/\text{cm}^2$ surface. We did not appreciate that DCs elute from glass surfaces intact, but must be quickly concentrated to remain viable. Further, we took no precautions to reduce the percentage of macrophages in the culture. In this paper we also found that the viability of DCs in heterogeneous spleen cell mixtures was excellent, by using selective [³H]uridine labeling of purified DCs followed by their addition to spleen cultures. This protocol should be useful in studying the distribution of DCs *in vitro* and *in vivo*, much as lymphocytes were tagged in Gowans' studies on lymphocyte distribution (12).

The differences between DCs and macrophages deserves further emphasis, because much of the work on accessory cell and Ir gene function is performed on spleen and assumes that these functions are mediated by macrophages. DCs and macrophages differ significantly in the three criteria that have been stressed by cellular immunologists i.e., adherence, phagocytosis, and expression of Ia antigens.

Glass adherence is still an empirical property that is best exemplified by, though not unique to, mouse peritoneal macrophages. Both resident and stimulated mouse peritoneal macrophages can be enriched to high purity and yield by glass adherence, and can be maintained with ease as adherent populations for several days *in vitro*. This also appears to be true of large, hemosiderin-filled, mouse spleen macrophages obtained by disruption with collagenase (2).² Certain mononuclear phagocyte populations do not adhere initially or do not remain glass adherent with current culture techniques, as is the case with most macrophages released by teasing mouse spleen. However, these cells can readhere to glass and remain adherent concomitant with maturation to a larger, more actively phagocytic cell. In contrast, most DCs from spleen adhere and spread actively on glass immediately after isolation from the mouse, but then elute from the surface and never readhere.

² Steinman, R. M. Unpublished observations.

The phagocytic capacity of macrophages has often been defined by their ability to take up latex spheres. This assay is not straightforward, because it is difficult to distinguish latex adherence from true uptake, especially at the enormous particle to cell ratios frequently used. Also, many "nonprofessional" cells phagocytose this particle (e.g., 13). We therefore think that the identification of splenic adherent cells as macrophages by latex phagocytosis may be misleading, and DCs clearly do not interiorize this particle at reasonable doses (100 particles/cell for 1–3 h). Phagocytosis in macrophages is best defined using opsonized particles, although this property varies quantitatively with the source and presumably the functional capacity of the cell. Freshly harvested resident peritoneal cells and most splenic macrophages obtained by mechanical means rosette large numbers of heavily opsonized erythrocytes (EA-IgG) and interiorize a few (1–5). With maturation *in vitro*, phagocytosis increases to 5–25 EA/cell, and lower doses of opsonin are needed. DCs, however, neither bind nor interiorize EA. Conceivably DCs will prove to have some sort of recognition capacity for immune complexes *in vitro*. We know that complexes *in situ* associate with the surface of a morphologically similar class of cells in spleen (11), but so far we have not identified a set of conditions to bring this about with DCs *in vitro*.

Finally, macrophages were initially thought to be a major cell type expressing Ia antigens (14). Currently available alloantisera (7, 8, and this paper) detect these antigens only on a small percentage of peritoneal macrophages at best, and we have found a similar result with most splenic macrophages. All DCs exhibit readily detectable Ia, by both fluorescence and cytotoxicity assays, and continue to express this antigen over a total of 4 days of tissue culture.

So DCs clearly differ from macrophages in all criteria available to date. Now the critical question is whether DCs express Ir gene-linked functions, especially some which have been ascribed to macrophages. The first indication of this is the finding that DCs are by far the most potent stimulator of the mixed leukocyte reaction in mouse spleen, whereas peritoneal macrophage populations lacking DCs stimulate weakly if at all (15).

Summary

Dendritic cells (DCs; 1) have been purified from mouse spleen in good yield. Spleen cell suspensions were floated on dense bovine plasma albumin (BPA) columns, and the low density fraction was adhered to glass (2). The adherent cells consisted of DCs and immature macrophages most of which eluted in a viable state from the culture dish after overnight incubation. The macrophages were then removed by selective rosetting with opsonized erythrocytes and recentrifugation on dense BPA.

This protocol resulted in a purified DC fraction, containing $1-3 \times 10^5$ DCs/spleen, which was homogeneous and distinctive in its properties. All cells exhibited the phase contrast and transmission electron microscopy (EM) cytologic features that were previously described for freshly isolated adherent DCs. By scanning EM, most purified DCs exhibited a remarkable array of bulbous protrusions of varying length and shape, unlike any other lymphoid cell. All DCs expressed surface Ia and other major histocompatibility complex (MHC)-linked alloantigens. DCs, however, lacked surface Ig and T-cell antigens, and did not bind or interiorize opsonized erythrocytes.

Purified DCs have been maintained *in vitro* for 3 days. Recovery of cultured purified cells was 70% or more of starting cell numbers. When [3 H]uridine-tagged

DCs were mixed with nonlabeled heterogeneous spleen cells, 70–80% of the labeled DCs were recovered as viable cells 2–3 days later. Purified DCs did not adhere to tissue culture surfaces and did not proliferate, even when cultured with mitogenic doses of concanavalin A and lipopolysaccharide. Finally, DCs did not change their cytologic or surface properties after 3 days of culture.

These observations extend the evidence that DCs are a novel cell type and provide useful properties and techniques for their further study.

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