

## STUDIES OF THE CELL SURFACE OF MOUSE DENDRITIC CELLS AND OTHER LEUKOCYTES\*

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Our laboratory has focused on the structure and function of the murine dendritic cell (DC; 1).<sup>1</sup> This lymphoid element has a characteristic structure and surface topography and represents a minor population of the cells (1% or less) released from dissociated spleen and lymph node. DC can be purified, and by cytologic criteria they are >90% pure. Purified DC exhibited unique functional properties. They are the most potent stimulators of the allogeneic and syngeneic mixed leukocyte reactions (2, 3) and act as accessory cells in proliferative and cytotoxic T cell responses (4–7).

Cell surface markers have contributed to the characterization of spleen DC. Initially it was noted that DC lacked surface immunoglobulin (Ig), brain antigen (Ag), Thy-1 Ag, and Fc and C3 receptors (8). These markers set DC apart from macrophages (M $\phi$ ) and lymphocytes, particularly when combined with such other differences as distinctive morphology, steroid and radiosensitivity, rapid turnover, weak endocytic activity, and distribution in situ (9). The lack of Fc receptors was useful in purifying DC from DC-M $\phi$  mixtures (10). Purified DC all expressed Ia Ag, and did not acquire the surface markers of M $\phi$  or lymphocytes when cultured in vitro for several days. Further analysis of the cell surface should facilitate the identification of DC in complex cell mixtures, help outline its lineage, and explain its functional capacities.

We have therefore performed a detailed study comparing the surface of DC with other bone marrow-derived elements. Monoclonal antibodies and lactoperoxidase-mediated surface iodination provided sensitive quantitative and biochemical information. The data show that spleen DC constitutively express high levels of Ia and H-2D alloantigens. DC carry the leukocyte common antigen, but can be distinguished from other leukocytes by several surface markers detected with monoclonal antibodies. Our findings indicate that spleen DC are part of a distinct, Ia-rich, leukocyte differentiation pathway.

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<sup>1</sup> *Abbreviations used in this paper:* BCG, bacille Calmette-Guérin; BPA, bovine plasma albumin; DC, dendritic cells; FCS, fetal calf serum; HSA, high salt buffer A; LC, leukocyte common; LODAC, low-density adherent cells; LPO, lactoperoxidase; LPS, lipopolysaccharide; MHC, major histocompatibility complex; M $\phi$ , macrophages; PBS, phosphate-buffered saline; PLL, poly-L-lysine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPF, specific pathogen-free.

## Materials and Methods

*Mice.* B6D2F<sub>1</sub>, C57BL/6, BALB/c, A, and DBA/2 specific pathogen-free (SPF) mice were obtained from the Trudeau Institute, Saranac Lake, N. Y.; CBA/J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine; Swiss mice were obtained from The Rockefeller University. AKR.H-2b mice were the generous gift of E. A. Boyse (Sloan-Kettering Institute). Males and females 6 wk to 6 mo old were used.

*Antibodies (Ab).* A large group of rat anti-mouse monoclonal Ab (Table I) were obtained using the strategy of Kohler and Milstein (11). In our laboratory, P3U1 was used as the mouse myeloma carrier. The 2.4G2, 1.21J, 2.6, and 2D2C Ab were obtained from rats immunized with the M $\phi$  cell lines J774 and P388D<sub>1</sub> (12). 2.4G2 recognizes the Fc $\gamma$  receptor (13); 1.21J is specific for an Ag that is similar if not identical to Mac-1 (14; see also Results); 2.6 and 2D2C recognize distinct polypeptides that are abundant on M $\phi$  (12). 2D2C recognizes an alloantigen because it binds to peritoneal M $\phi$  and spleen cells from DBA/2, BALB/c, and CBA, but not from A, B10, B10.D2, AKR, or Swiss mice. Hybridoma Ab specific for the 2.6, 2.D2C, and 1.21J Ag were frequently obtained when animals were immunized with M $\phi$  or M $\phi$  cell lines. B5-3 (anti-Thy-1.2), B21-2 (anti-I-A<sup>bd</sup>), and B25-1 (anti-H-2D<sup>d</sup>) were obtained from a rat immunized with DC-T cell mixtures (4). 2E8, another anti-Ia Ab, was the product of a fusion in which the rat was immunized with  $5 \times 10^6$  DC intravenously 30 and 3 d before fusion. 2E8 immunoprecipitates typical class II polypeptides and, in sequential studies, 2E8 removes the Ia Ag precipitable with B21-2. However, 2E8 binds to all haplotypes tested (H-2b, d, k, and Swiss). For all of the above Ab, ascites were prepared in irradiated mice, and an IgG fraction was obtained by chromatography on DEAE (Whatman, Ltd, Maidstone, England) developed with 25 mM Tris, pH 8. When the purified Ab were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), contaminating proteins were absent and the light chain region contained one or two sharp bands characteristic of rat P3U1 hybridoma Ab (13).

Another group of monoclonals was provided by other investigators. F4/80 is a clone obtained from rats immunized with peritoneal exudate cells (15). It recognizes a distinct M $\phi$  Ag and was generously provided by Dr. S. Gordon and Dr. J. Austyn (Oxford University); 53-7.31 (anti-Lyt-1) and 53-6.72 (anti-Lyt-2) were produced by Ledbetter et al. (16) and distributed by The Salk Institute; I3/2 (anti-leukocyte common Ag) was kindly provided by Dr. Ian Trowbridge The Salk Institute (17). All of these Ab were purified by affinity chromatography on columns of affinity-purified goat anti-rat Ig (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Cytotoxic monoclonal anti-Lyt-1.1 (clone W3) and Lyt-2.2 (clone 19.178) were gifts of U. Hammerling, Sloan-Kettering Institute (18, 19). A summary of the monoclonal Ab used in this study and the products they detect is provided in Table I.

Rabbit anti-rat Ig (N. L. Cappel Laboratories) was purified on columns of rat Ig coupled to CNBr-activated Sepharose 4B. The antibody was digested with 1% wt:wt pepsin (P-7012; Sigma Chemical Co., St. Louis, Mo.) for 15 h at 37°C in 0.1 M acetate buffer, pH 4.5. F(ab)<sub>2</sub> fragments were retrieved by a second affinity purification on rat Ig-Sepharose.

Monoclonal Ab and rabbit anti-rat F(ab)<sub>2</sub> were iodinated with 1,3,4,6-tetrachloro-3 $\alpha$ -6 $\alpha$ -diphenylglycouril (Iodogen, Pierce Chemical Co., Rockford, Ill.) and carrier-free Na <sup>125</sup>I (New England Nuclear, Boston, Mass.) to 2–20  $\mu$ Ci/ $\mu$ g (20). Iodinations were carried out at 4°C with 50  $\mu$ g of Ab and 0.5–1 mCi of Na <sup>125</sup>I in 50–100  $\mu$ l of phosphate-buffered saline (PBS), pH 7.4. Free <sup>125</sup>I was removed by passing the reaction mixture over 0.4-ml columns of DOWEX-1 (Sigma Chemical Co.). Iodinated Ab were stored at 50  $\mu$ g/ml in PBS plus 1 mg/ml bovine plasma albumin (BPA) plus 0.02% sodium azide at 4°C for up to 1 mo. Specific activities were calculated from trichloroacetic acid precipitable counts assuming total recovery of antibody from the columns. Specific activities were not absolute because our preparations contained hybrid Ab molecules, only some of which are active.

*Cells.* Spleens and thymuses were teased with forceps and further disrupted on stainless steel sieves. B lymphocyte-enriched fractions were retrieved by “panning” on anti-Ig-coated plates using the method of Wysocki and Sato (21). Lipopolysaccharide (LPS)-induced B lymphoblasts were obtained by a three-step procedure involving transformation for 24–48 h

with 50 g/ml LPS (LPS-W *Escherichia coli* O55:B5; Difco Laboratories, Detroit, Mich.), flotation on dense BPA columns (22), and binding to anti-Ig-coated petri dishes (21).

DC were prepared from low-density adherent cells (LODAC) that were depleted of M $\phi$  by rosetting with opsonized erythrocytes (10).

Spleen M $\phi$  for autoradiography and binding experiments were obtained from spleen cells cultured overnight. Cells that pelleted in  $\rho = 1.080$  BPA (which are usually discarded in the first step of DC purification), were cultured overnight and refloated in  $\rho = 1.080$  BPA. Adherence of this low density fraction reproducibly yielded concentrated monolayers of M $\phi$  that were free of DC, because DC do not adhere firmly to glass after overnight culture. An advantage of this method is that the M $\phi$  have been through the same BPA gradients as the purified DC. In some biosynthetic labeling experiments, spleen M $\phi$  were also obtained from LODAC of collagenase-treated spleens. Spleens were exposed to 0.5 mg/ml collagenase (clostridial collagenase III; Worthington Biochemical Corp.) and 30  $\mu$ g/ml DNase (DN-100; Sigma Chemical Co.) incubated at 37°C for 30 min, and further disrupted on stainless steel sieves. LODAC from collagenase-treated spleen contain large numbers of hemosiderin-laden M $\phi$  (23) that remain adherent after overnight culture.

Peritoneal M $\phi$  were obtained by glass or plastic adherence of resident peritoneal cells.

Monocytes were enriched by adherence of Ficoll-purified peripheral blood leukocytes (4).

The culture medium was RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf serum (FCS; Flow Laboratories, Inc., Rockville, Md., and North American Biologicals, Inc., Miami, Fla.), 20  $\mu$ g/ml gentamycin sulfate (Schering Corp., Kenilworth, N. J.),  $5 \times 10^{-5}$  M 2-mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.), and 2 mM glutamine (Grand Island Biological Co.).

*Quantitative Binding Studies.* Binding of iodinated Ab was measured on cells attached to 16-mm-diameter tissue culture wells (Linbro 76-33-05, Linbro Chemical Co., Hamden, Conn., and Costar 3524, Costar, Data Packaging, Cambridge, Mass). M $\phi$  from the spleen and the peritoneal cavity were adhered to the wells by a 1-3-h culture at 37°C, whereas other cell types were attached by centrifugation onto wells coated with 40  $\mu$ g/ml poly-L-lysine (PLL; type VII, Sigma Chemical Co.).

Saturating concentrations of the monoclonal Ab, with the exception of I3/2, were incubated with the cells on ice for 45 min in RPMI-1640 plus 5% newborn calf serum (North American Biologicals) plus 0.02% azide. I3/2 was used at 10  $\mu$ g/ml, which yields  $\sim 1/2$  the maximum binding. Excess Ab was rinsed off with PBS, and binding was determined by counting the cells solubilized in 0.1 N NaOH in a gamma scintillation counter. All binding assays except those for 2.4G2 were done in the presence of saturating concentrations of unlabeled anti-Fc receptor Ab to block Fc-mediated binding. F(ab) fragments of 2.4G2 were used to determine the numbers of Fc receptors on test populations. Background binding was 50-150 cpm on PLL-coated surfaces without cells. Ab binding to cells attached to PLL was equivalent to binding in suspension. The data are expressed as bound Ab molecules/cell-background. The number of cells in M $\phi$  preparations was determined by counting cells in 100 1,000-magnification fields, and checked by protein determinations using the method of Lowry (24). Spleen M $\phi$  and DC had 1.5-2  $\mu$ g of protein/ $10^5$  cells; peritoneal M $\phi$  had 2-3  $\mu$ g/ $10^6$  cells; spleen and thymus had 1  $\mu$ g/ $10^6$  cells.

*Autoradiography.* Cells were adhered or attached to 12-mm-diameter coverslips coated with 50  $\mu$ g/ml PLL. Binding of  $^{125}$ I-Ab was as described for the quantitative studies above. Coverslips were blow dried, fixed in methanol, and dipped in Ilford L-4 emulsion (Ilford Ltd., Basildon, Essex, England). All cell populations exposed to a given Ab were dipped and developed simultaneously. After exposures of 1-6 d, slides were developed in Kodak D19 (Eastman Kodak Co.) and stained with Giemsa.

*Cytotoxic Assays.* Clones B21-2, B5-3, W3, and 19.178 secrete cytotoxic Ab. Cytotoxicity assays were performed using equal volumes of DC, spleen, or thymocytes at  $5 \times 10^6$ /ml, saturating levels of Ab, and 1:5 selected rabbit complement. Killing was assessed by trypan blue exclusion.

*Cell Surface Iodination.* Cells were iodinated in suspension by the method of Hubbard and Cohn (25). Briefly,  $0.1-1 \times 10^7$  cells were incubated with 0.2-1 mCi/ml Na  $^{125}$ I, 50 mU/ml glucose oxidase (Sigma G-6500, type V), and 15  $\mu$ g/ml lactoperoxidase (LPO; 427466;

Calbiochem-Behring Corp., San Diego, Calif.) for 15 min on ice. Cells adherent to culture surfaces were iodinated as described by Muller et al. (26). LPO coupled to latex beads was centrifuged onto the adherent cells and 0.24 mU/ml glucose oxidase, 0.2–1 mCi/ml Na <sup>125</sup>I, and 10 mM glucose were added in PBS. The reaction was allowed to proceed for 15 min on ice and was terminated by aspirating the reaction mixture and washing with RPMI-1640. The efficiency of surface iodination of M $\phi$  is increased at least 10-fold using solid-phase LPO, and the distribution of iodinated peptides is identical to that obtained when the iodination is performed in suspension (26).

Viability following iodination was always >95%, and the surface iodination patterns differed markedly from patterns obtained from iodinated cell lysates.

After iodination, cells were extracted with 0.5% Nonidet-P40 (Bethesda Research Labs, Bethesda, Md.), 100 U/ml aprotinin (Sigma Chemical Co.), and 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) in PBS (lysis buffer). Lysates were cleared by centrifugation for 20 min at 20,000 *g*. Samples used for comparing cell surface patterns were prepared for electrophoresis by mixing lysates 1:1 with 4% SDS (Sigma Chemical Co.), 24% sucrose, 50 mM dithiothreitol, 0.05% bromphenol blue, and 100 mM Na<sub>2</sub>CO<sub>3</sub>, pH 8.5, and boiling for 2 min. For immunoprecipitation, the samples were either processed immediately or stored for 1–2 days at –70°C.

**Biosynthetic Labeling.** Cells were exposed to [<sup>35</sup>S]methionine (Amersham Corp., Arlington Heights, Ill.; 1,000 Ci/mM, 10–50  $\mu$ Ci/ml culture medium) in methionine-free culture medium for 6–18 h. Cells were then washed, extracted with lysis buffer, and processed for immunoprecipitation as described below.

**Immunoprecipitation.** Lysates were incubated with either 1  $\mu$ g of Ab in solution or its equivalent immobilized on CNBr-activated Sepharose 4B for 1 h at 4°C. When immune complexes were formed in solution, they were retrieved by incubating for 1 h at 4°C with affinity-purified rabbit anti-rat Ig immobilized on protein A-Sepharose (Pharmacia Fine Chemicals). Solid-phase immune complexes were washed three times with 0.6 M NaCl, 0.0125 M potassium phosphate, pH 7.4, 0.02% NaN<sub>3</sub> (high salt buffer A [HSA]); then complexes were washed twice with 0.1% SDS, 0.05% Nonidet-P40, 0.6 M 10 mM Tris HCl, pH 8.6, and once again with HSA. Ag was released by boiling in 2% SDS, 50 mM Na<sub>2</sub>CO<sub>3</sub>, 25 mM dithiothreitol, 12% sucrose, and 0.01% bromphenol blue, pH 8, for 2 min.

Iodinated polypeptides from immunoprecipitates were quantitated by cutting the bands out of dried gels and counting in a gamma scintillation monitor. To compare the amount of specific Ag from different cell types, the immune precipitable counts were normalized on the basis of shared polypeptides (e.g., clone 2.6, Fig. 6).

**SDS-PAGE.** Samples were electrophoresed in 1-mm 4–11% polyacrylamide gradient slab gels at 40 mA constant current according to the method of Neville (27). For <sup>125</sup>I-labeled samples, Coomassie Blue-stained gels were dried onto filter paper, and autoradiograms were developed at –70°C using Kodak XR-1 (Eastman Kodak Co.) or Dupont Cronex (Dupont Co., Wilmington, Del.) film with image intensifier screens (Lightening Plus, Dupont Co.). Gels with [<sup>35</sup>S]methionine-labeled samples were fluorographed as described by Bonner and Laskey (28).

**Mixed Leukocyte Reactions.** Nylon wool purified responders were cultured at 4  $\times$  10<sup>5</sup>/6-mm well with varying doses of x-irradiated (1,500 rad) stimulators in 0.2 ml complete RPMI-1640. Cultures were pulsed with 2.5  $\mu$ Ci/ml [<sup>3</sup>H]TdR (6 Ci/mM; Schwarz/Mann Div., Becton, Dickinson & Co., Oxnard, Calif.) for 18 h after 72 h of culture.

## Results

**Cells and Assays.** Our interest was to characterize the DC surface and to compare it with other leukocytes, especially M $\phi$ . DC were purified from spleen-adherent cells as previously described (10). Mononuclear phagocytes were enriched from blood, peritoneal cavity, and spleen by adherence techniques. Lymphocytes were studied in unfractionated spleen and thymus suspensions, and in some cases, purified B and T cells were prepared. Granulocytes and platelets were examined as constituents of blood and spleen.

Monoclonal Ab were used to study the expression of specific Ag (Table I), whereas LPO-mediated iodination displayed a broad spectrum of exteriorly disposed polypeptides. Although the fluorescence-activated cell sorter has been used in most previous studies with monoclonal Ab, we were unable to use it because adherence techniques were required to purify mononuclear phagocytes. Instead we used  $^{125}\text{I}$ -Ab (2–20  $\mu\text{Ci}/\mu\text{g}$ ). Quantitative binding studies were performed with saturating concentrations of  $^{125}\text{I}$ -Ab in the presence of unlabeled 2.4G2 Ab to block Fc receptors. This assay readily detected  $0.6\text{--}6 \times 10^3$  bound molecules/cell using test populations of  $1 \times 10^5\text{--}4 \times 10^5$  cells. The specificity and sensitivity of this assay was illustrated with three Ab directed to alloantigens on BALB/c but not B10.BR mice (Table II). The binding of  $^{125}\text{I}$ -Ab

TABLE I  
*Monoclonal Ab to Leukocyte Ag*

Clone	Ag recognized	Molecular weight of Ag	Ab subclass	Saturating concentration
				$\mu\text{g}/\text{ml}$
I3/2	LC	220,000–180,00	IgG2b	50
53-7.3	Lyt-1	70,000	IgG2a	3
53-6.7	Lyt-2	34,000	IgG2a	3
W3	Lyt-1.1	70,000	—	—
19.178	Lyt-2.2	34,000	—	—
B5-3	Thy-1.2	25,000	IgG2b	3
1.21J	Mac-1	180,000, 95,000	IgG2a	1
2.4G2	Fc $\gamma$ receptor	70,000–48,000	ND*	0.3
2.D2C	—	90,000	IgG2a	1
2.6	—	21,000	ND	30
F4/80	—	160,00	IgG2b	1
B21-2	I-A <sup>b,d</sup>	34,000–25,000	ND	1
B25-1	H-2D <sup>d</sup>	45,000, 12.5	IgG2a	3
2E8	I-A	34,000–25,000	IgG2a	1

\* Ab subclass not determined at this time.

TABLE II  
*Specificity of  $^{125}\text{I}$ -Ab Binding Assay*

Clone	Strain of test M $\phi$	Ab bound with		
		$^{125}\text{I}$ -Ab only	$^{125}\text{I}$ -Ab + specific unlabeled Ab	$^{125}\text{I}$ -Ab + nonspecific unlabeled Ab
		<i>cpm</i>	<i>cpm</i>	<i>cpm</i>
B21-2	BALB/c	11,603 (0.68)	1,713 (0.50)	12,442 (0.69)
	B10.Br	90	95	176
B25-1	BALB/c	28,000 (2.8)	5,968 (2.9)	30,342 (3.1)
	B10.Br	0	107	172
2D2C	BALB/c	20,190 (1.8)	2,389 (1.4)	21,180 (1.9)
	B10.Br	296	288	0

Three criteria for the specificity of the  $^{125}\text{I}$ -Ab binding assay. For each Ab,  $10^5$  M $\phi$  were exposed to either  $^{125}\text{I}$ -Ab at saturation, or  $^{125}\text{I}$ -Ab plus excess unlabeled Ab, or  $^{125}\text{I}$ -Ab plus excess nonspecific unlabeled Ab. Ab concentrations were: 1  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -B21-2  $\pm$  4  $\mu\text{g}/\text{ml}$  B21-2  $\pm$  4  $\mu\text{g}/\text{ml}$  2.6; 5  $\mu\text{g}/\text{ml}$   $^{125}\text{I}$ -B25-1  $\pm$  20  $\mu\text{g}/\text{ml}$  B25-1  $\pm$  20  $\mu\text{g}/\text{ml}$  2.6; 3  $\mu\text{g}/\text{ml}$  2.D2C  $\pm$  26  $\mu\text{g}/\text{ml}$  2.D2C  $\pm$  26  $\mu\text{g}/\text{ml}$  2.6. BALB/c mice express the B21-2, B25-1, and 2.D2C alloantigens and B10.Br mice do not. 2.6 is expressed by both strains. The amount of 1.21J binding to the two types of M $\phi$  was identical in this experiment, so the numbers of cells were similar for both strains.

was quantitatively inhibited by an excess of specific nonlabeled Ab, but the total amount of bound Ab was similar (compare columns A and B). Nonspecific Ab did not compete (compare columns A and C). Finally, binding above background was negligible in nonreactive strains (B10.BR) and not competed with unlabeled Ab. We conclude that binding assays with  $^{125}\text{I}$ -Ab can be used to quantitate the levels of specific cell surface Ag on relatively small numbers of test cells, including M $\phi$ .

Autoradiography was employed to visualize binding of  $^{125}\text{I}$ -Ab at the single cell level (Figs. 1-3; Table V). Grain development was adjusted to a maximum of 200 grains/cell, a level beyond which grain numbers do not vary linearly with the amount of bound label (29). Background was always 10 grains or less per cell. Specificity controls for autoradiography were the same as for quantitative studies. Autoradiography allowed examination of cell types that were not available for the binding studies, e.g., spleen granulocytes and blood monocytes. In many cases, immunoprecipitation was used to establish Ag synthesis (Fig. 5) and to show that the amount of membrane Ag corresponded to that determined by binding assays (Fig. 6).

These three assays were used to evaluate several cell populations and Ab simultaneously. The data for different cell types is provided in a composite form in many of the tables and figures, but for ease of presentation, we will describe our findings as they pertain to the two main cells being studied, DC and mononuclear phagocytes.

*Surface Ag of DC.* All DC expressed the leukocyte common Ag (LC; clone I3/2; Fig. 1). I3/2 binding was similar for all leukocytes tested (Table III), and by autoradiography, all leukocytes were labeled similarly. Erythrocytes and platelets were unlabeled. LC synthesis was detected in [ $^{35}\text{S}$ ]methionine-labeled DC (not shown).

Purified DC preparations bound only small amounts (<300 molecules/cell) of  $^{125}\text{I}$ -anti-Thy-1.2 (clone B5-3) and anti-Lyt-1 (clone 53-7.3). Only 1-2% of the DC were labeled by autoradiography with either Ab (Fig. 1). This labeling presumably represents the level of T lymphocyte contamination. In contrast, 30-40% of splenocytes had 100-200 grains/cell with both Ab (data not shown). We conclude that DC are

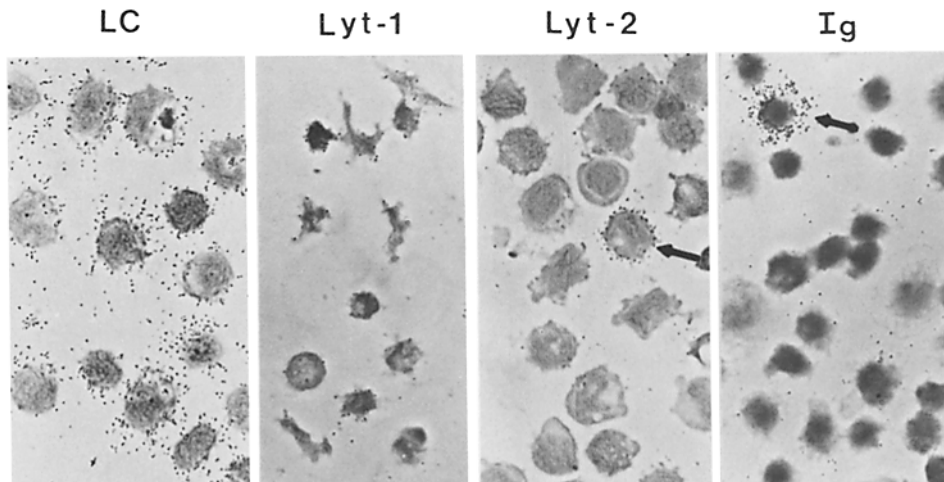


FIG. 1. Autoradiograms of purified spleen DC exposed to  $^{125}\text{I}$ -Ab specific for lymphocyte antigens. All DC express the LC Ag. Purified DC do not express Lyt-1, but a small subpopulation expresses Lyt-2 (arrow). 1-2% of the purified DC bind anti-Ig (arrow).  $\times 600$ .

TABLE III  
Quantitation of Lymphocyte, MHC, and M $\phi$  Ag

Monoclonal antibody	Antibody molecules bound/cell $\times 10^{-3}$ on				
	Spleen M $\phi$	Peritoneal M $\phi$	DC	Spleen	Thymus
B5-3 (Thy-1.2)	6.3 $\pm$ 0.6 (3)	0.085 $\pm$ 0.1 (2)	2.8 $\pm$ 0 (2)	30 $\pm$ 3 (4)	146 $\pm$ 23 (4)
53-7.3 (Lyt-1)	1.0 $\pm$ 1 (3)	1.2 $\pm$ 1.5 (2)	1.5 $\pm$ 1.6 (3)	7.8 $\pm$ 3 (3)	22 $\pm$ 0.05 (2)
53-6.7 (Lyt-2)	1.8 $\pm$ 1.2 (2)	1.0 $\pm$ 1.2 (2)	2.7 $\pm$ 0.4 (2)	3.2 $\pm$ 0.4 (2)	15 $\pm$ 1.4 (2)
I3/2 (T200)	85 $\pm$ 0 (2)	ND*	82 $\pm$ 25 (2)	63 $\pm$ 4 (2)	107 $\pm$ 18 (2)
B21-2 (Ia)	128 $\pm$ 80 (4)	40 $\pm$ 30 (3)	200 $\pm$ 40 (5)	23 $\pm$ 8 (4)	6.8 $\pm$ 2 (4)
B25-1 (H-2)	61 $\pm$ 8 (3)	91 $\pm$ 10 (3)	116 $\pm$ 14 (3)	20 $\pm$ 14 (3)	6.4 $\pm$ 4 (3)
1.21J (Mac-1)	51 $\pm$ 12 (6)	142 $\pm$ 60 (5)	3.5 $\pm$ 1.6 (7)	1.3 $\pm$ 0.16 (4)	0.2 $\pm$ 0.16 (4)
2.4G2 (FcR)	94 $\pm$ 8 (3)	99 $\pm$ 30 (2)	5.2 $\pm$ 5.2 (3)	8.4 $\pm$ 1.2 (3)	1.1 $\pm$ 1.0 (3)
F4/80	12.4 $\pm$ 0.4 (2)	104 $\pm$ 30 (2)	0.85 $\pm$ 0.4 (2)	3.0 $\pm$ 1 (2)	0.28 $\pm$ 0.3 (2)
2.D2C	80 $\pm$ 32 (5)	23 $\pm$ 4 (4)	6.4 $\pm$ 2.4 (5)	4.0 $\pm$ 2.4 (4)	1.2 $\pm$ 0.8 (4)
2.6	143 $\pm$ 20 (3)	165 $\pm$ 50 (3)	135 $\pm$ 32 (3)	24 $\pm$ 8 (2)	6.2 $\pm$ 4 (2)

Quantitative binding of eleven monoclonal Ab on selected cell types. All Ab were used at saturation. The data are expressed as thousands of Ab molecules bound per cell and are the means of an indicated number of experiments in parenthesis. DC, spleen M $\phi$ , and peritoneal M $\phi$  were purified preparations whereas spleen cells and thymocytes were unfractionated. Cells were prepared from C  $\times$  B6F<sub>1</sub>, BALB/c, C57BL/6, CBA/J, and B6D2F<sub>1</sub> mice.

\* Not done.

Thy-1 and Lyt-1 negative and that the low levels of binding of these Ab is due to a 1–2% T cell contamination.

The level of Lyt-2 (clone 53-6.7) on DC approached that of spleen (Table III). In five autoradiographic experiments, discrete labeling was observed on 5–12% of the purified DC (Fig. 1) and on 15% of spleen cells. The Lyt-2-positive cells in the DC preparations had  $27 \times 10^3$ – $54 \times 10^3$  Lyt-2 molecules/cell vs.  $22 \times 10^3$  for spleen T cells. Cytotoxic assays with additional anti-Lyt-1 and Lyt-2 monoclonal Ab confirmed the binding studies. 5–12% of DC were killed with clone 19.178, a cytolytic anti-Lyt-2.2, in four consecutive experiments (Table IV). However, killing was not detected with anti-Lyt-1 (clone W3) or anti-Thy-1.2 (clone B5-3). More than 95% of DC were lysed with anti-Ia and complement (Table IV), and were reactive with  $^{125}\text{I}$ -anti-Ia by autoradiography (see below). Treating DC with anti-Lyt-2 and complement did not alter their ability to stimulate either syngeneic or allogeneic mixed leukocyte reactions (Table IV). Thus a small percentage of the cells in DC preparations are Ia<sup>+</sup>, Lyt-2<sup>+</sup>, Lyt-1<sup>-</sup>, and Thy-1<sup>-</sup>.

DC lacked surface Ig by autoradiography with  $^{125}\text{I}$ -affinity-purified rabbit anti-Ig. Only 1–2% of the cells in DC preparations were labeled (Fig. 1) under conditions in which 50–60% of spleen cells had 50–200 grains. Ig synthesis was weak or undetectable in [ $^{35}\text{S}$ ]methionine-labeled DC (Fig. 5 A).

DC did not express the 2.4G2 Ag of the murine, trypsin-resistant Fc receptor (13). In quantitative studies, the level of 2.4G2 on DC was  $\frac{1}{20}$  the level of peritoneal and spleen M $\phi$ . By autoradiography, 5% or less of the cells in DC preparations were labeled under conditions in which labeling of M $\phi$ , monocytes, and spleen lymphocytes was readily detected (Fig. 2; Table V).

DC also lacked two Ag previously shown to be expressed on most peritoneal M $\phi$  but not peritoneal or spleen lymphocytes. These are the Mac-1 Ag of Springer et al. (our clone 1.21J; 14), and the F4/80 Ag of Austyn et al. (15). The level of 1.21J and F4/80 on DC was 1–6% of that on spleen and peritoneal M $\phi$  (Table III). By autoradiography, light labeling with 1.21J was evident on 5–10% of DC, whereas

TABLE IV  
*Cytotoxicity Experiments with Anti-Lyt-2*

Experiment 1				
Final Ab concentration	Percent killing of			
	DC	Spleen	Thymus	
0	0	0	0	
Complement	0	0	0	
B21-2, 10 µg/ml	98	57	0	
Anti-Ig, 1:100	0	49	0	
Lyt-2, 1:100	12	22	90	
B5-3, 10 µg/ml	2	40	99	

Experiment 2				
DC added per culture	Allogeneic T cells		Syngeneic T cells	
	Complement	Lyt-2 + complement	Complement	Lyt-2 + complement
3 × 10 <sup>4</sup>	109,480	106,376	32,695	27,154
10 <sup>4</sup>	76,236	71,537	21,725	18,550
3 × 10 <sup>3</sup>	40,114	40,473	9,734	7,899
0		1,294		705

In experiment 1, C × B6F<sub>1</sub> spleen and thymus cells were freshly prepared on the day of assay and DC were purified by rosetting with opsonized erythrocytes. Viability was assessed by trypan blue exclusion after a 1 h incubation at 37°C. Rabbit anti-mouse Ig was from N. L. Cappel Laboratories and αLyt-2 was clone 19.178. In experiment 2, 4 × 10<sup>5</sup> A × B6 (syngeneic) or B6 (allogeneic) NyT responders were cultured with A × B6 DC for 3 d in 5% FCS supplemented with RPMI-1640. Triplicate cultures were pulsed with 2.5 µCi/ml [<sup>3</sup>H]TdR for 18 h, and the results are the means of the three. SD = <10% for all variables. 10% of the DC were killed with Lyt-2.2 in this experiment.

F4/80 labeled cells were rare (Fig. 2; Table V). Additional data on the tissue distribution of 1.21J and F4/80 are given in the next section.

Clones B25-1 (anti-H-2D<sup>d</sup>), B21-2 (anti-I-A<sup>b,d</sup>), and 2E8 (anti-I-A) were used to monitor class I and II major histocompatibility complex (MHC) Ag. DC expressed 5–30 times more Ia and H-2D than spleen or thymus cells (Table III). Mφ Ia levels were variable and will be discussed below. By autoradiography, all DC were heavily labeled even under conditions where small lymphocyte labeling was weak or undetectable (Fig. 3). The amount of Ia on DC was similar for all groups of mice tested, including SPF and germ-free mice. The grain counts on DC purified after a day of culture were comparable to the grain counts of DC in the initial low density adherent population (data not shown).

More detailed binding studies were then performed to compare the expression of Ia on purified DC, Mφ, B cells, and B blasts. A constant amount of <sup>125</sup>I-anti-Ia and various doses of unlabeled Ab were added to the four populations. Three experiments were performed with two different monoclonal Ab on cells from SPF mice (Fig. 4). The amount of Ia expressed by DC was 10–20-fold greater than by B cells, and 1–3-fold greater than LPS blasts. Ia on spleen Mφ was equivalent to that on B cells. Saturation was reached at 0.5 µg/ml of Ab for all cell types. In additional experiments, a 48-h exposure to LPS did not affect DC or spleen Mφ Ia.

We conclude that DC are Fc-receptor negative leukocytes rich in MHC antigens.



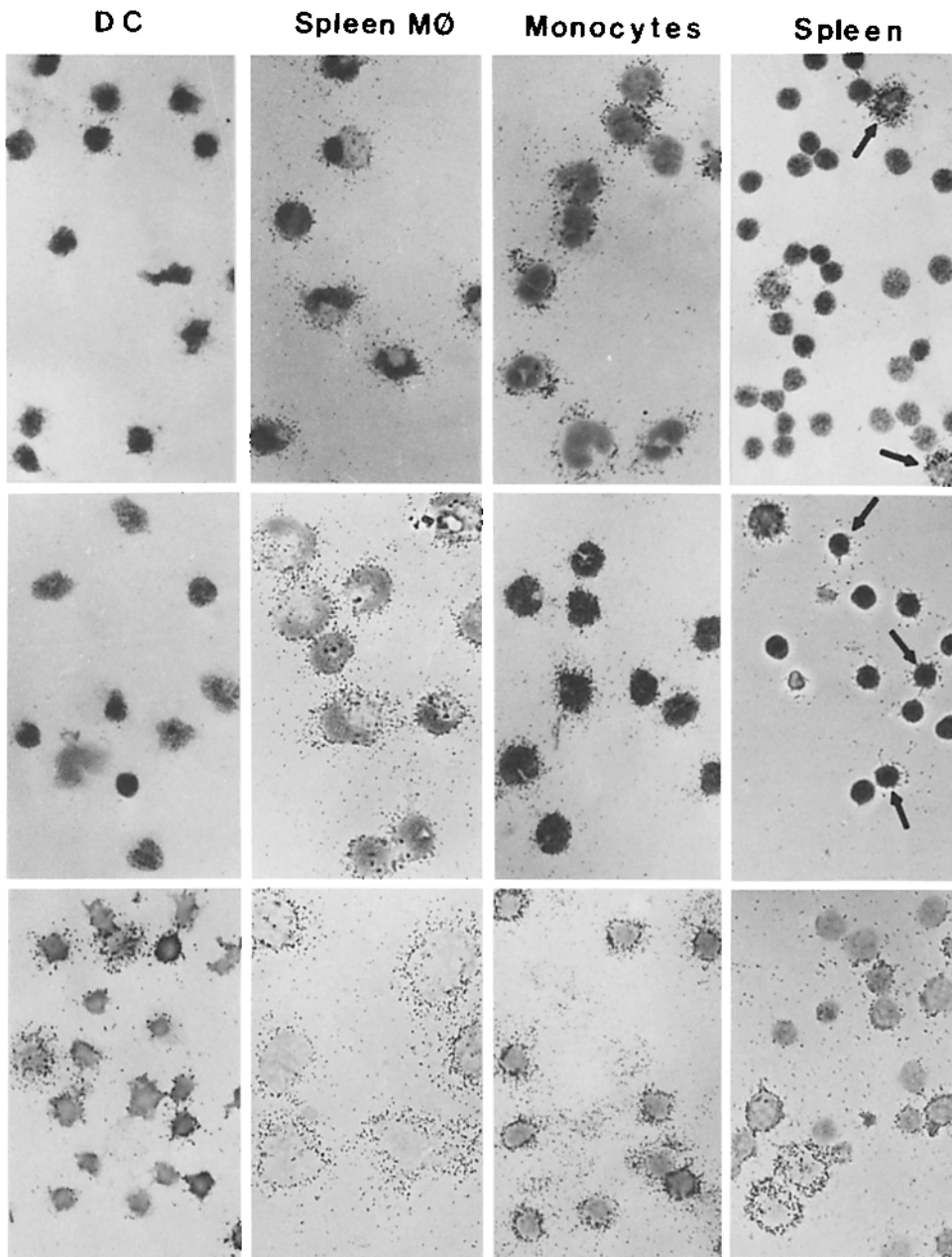


FIG. 2. Autoradiograms of four test populations exposed to  $^{125}\text{I}$ -Ab specific for antigens that are rich on M $\phi$ . These three clones, 1.21J (top), 2.4G2 (middle), and 2D2C (bottom), bind strongly (50–200 grains/cell) and uniformly to all spleen M $\phi$  and blood monocytes. DC do not bind 2.4G2 or 1.21J, whereas expression of the 2D2C antigen is heterogeneous. Many spleen lymphocytes are labeled (10–30 grains) with 2.4G2 (arrows) and with 2D2C, but not with 1.21J. Large cells in spleen, primarily granulocytes and M $\phi$ , label heavily with 2.4G2 (data not shown), 1.21J (arrows), and 2D2C (arrows).  $\times 600$ .

TABLE V  
*Autoradiographic Binding Assay with <sup>125</sup>I-Monoclonal Ab*

Ab	Cell type	Percentage of cells having grain counts of				
		Background	10-50	50-100	100-200	>200
1.21J	Spleen	94	—	—	6	—
	Spleen Mφ	7	17	76	26	—
	DC	96	4	—	—	—
	Monocytes	9	71	20	—	—
2.4G2	Spleen	40	50	10	—	—
	Spleen Mφ	3	11	47	40	—
	DC	95	—	5	—	—
	Monocytes	17	55	27	—	—
F480	Spleen	97	3	0.5	—	—
	Spleen Mφ	16	35	41	8	2
	DC	98	2	—	—	—
	Monocytes	43	39	16	—	1
2D2C	Spleen	48	32	13	7	—
	Spleen Mφ	2	—	—	—	98
	DC	36	41	23	—	—
	Monocytes	2	—	—	98	—
2.6	Spleen	85	—	—	15	—
	Spleen Mφ	2	—	—	98	—
	DC	—	95	5	—	—

Distribution of grains in four test populations. All cell types were processed, dipped, exposed, and developed simultaneously. Exposure times were adjusted so that the most reactive cells had 100-200 grains; beyond this level grain development no longer varies linearly with the amount of radiolabel (29). Background binding was 10 grains or less per cell.

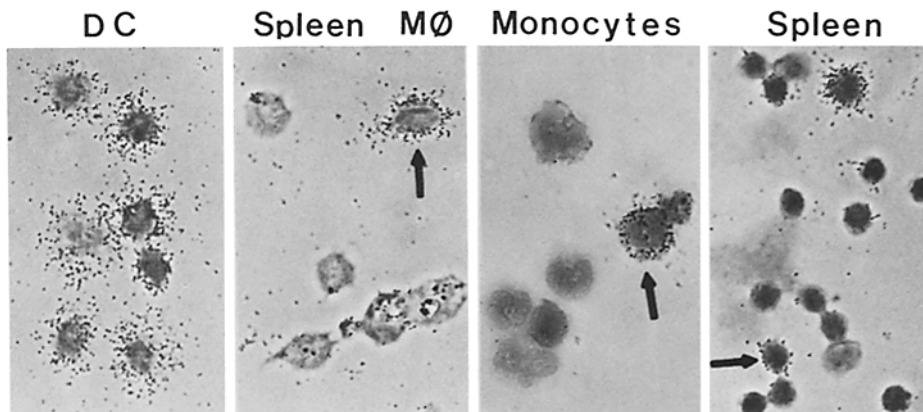
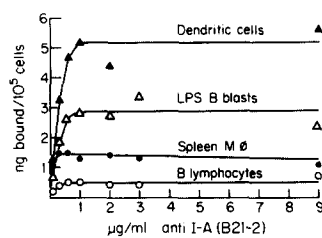


FIG. 3. Binding of <sup>125</sup>I-anti-I-A to four cell populations from SPF mice. Clones B21-2 and 2E8 gave identical results. All DC were heavily labeled. Subpopulations of Mφ and monocytes were labeled (arrows) with grain counts ranging from 20 to 200. Small spleen lymphocytes, presumably B cells, were lightly labeled under these exposure conditions (10-20 grains/reactive cell; arrows). × 600.



Ab	Molecules of Ab bound per cell			
	DC	B blast	B cell	Spleen Mφ
21-2	$1.9 \times 10^5$	$1.5 \times 10^5$	$0.13 \times 10^5$	$0.2 \times 10^5$
21-2	$2.5 \times 10^5$	$1.2 \times 10^5$	$0.1 \times 10^5$	$0.4 \times 10^5$
2E8	$2.6 \times 10^5$	$0.8 \times 10^5$	$0.18 \times 10^5$	$0.5 \times 10^5$

FIG. 4. Quantitation of anti-Ia binding to spleen Mφ, DC, LPS blasts, and B cells from SPF animals.  $0.1 \mu\text{g/ml}$  of  $^{125}\text{I}$  Ab plus increasing concentrations of unlabeled Ab were added to the four test populations. Saturation was reached at similar Ab concentrations for all cell types. Similar results were obtained for two different Abs for three experiments (summarized at bottom of figure). All experiments were with C  $\times$  B6F<sub>1</sub> mice.

The critical differentiation antigens of Mφ and lymphocytes are either scarce or absent on DC.

*Surface Ag of Mononuclear Phagocytes.* Mφ from spleen, peritoneal cavity, and blood were enriched by adherence techniques. Cells with the cytologic properties of either DC or lymphocytes were rare in all Mφ preparations. Little or no binding of anti-Lyt-1, Lyt-2, and Thy-1 to Mφ was detected in quantitative studies (Table III). Autoradiography with each of these monoclonal Ab established that T lymphocyte contamination was 1% or less. Small amounts of Ig were visualized with  $^{125}\text{I}$ -F(ab)<sub>2</sub> anti-Ig on most Mφ. This Ig was probably adsorbed to the Mφ because biosynthesis of Ig was weak or undetectable (Fig. 5A).

Comparable levels of LC and H-2D were expressed by DC and Mφ from either the peritoneal cavity or the spleen. By autoradiography, monocytes were all reactive with anti-LC. Biosynthesis of the LC (data not shown) and class I MHC polypeptides (Fig. 5B) was established for purified spleen Mφ.

Mφ expressed high levels of the 2.4G2 Ag (Table III). Spleen and peritoneal Mφ had  $10^5$  binding sites/cell and, by autoradiography, the Ag was abundant on most cells (Fig. 2; Table V). Because of limited cell numbers, quantitative binding studies were not performed on blood monocytes. By autoradiography, most monocytes were labeled with  $^{125}\text{I}$ -2.4G2, but the grain counts were  $\frac{1}{3}$  that of spleen Mφ (Fig. 2; Table V).

A group of four monoclonal Ab were evaluated as possible Mφ-specific reagent. In preliminary studies, three of the clones, 1.21J (Mac-1), F4/80, and 2.6, were observed to bind to Mφ cell lines, but not to a large group of lymphoid lines. A fourth clone, 2D2C, bound to lymphoid lines but was included because binding to DC appeared weak or absent by indirect immunofluorescence. In quantitative binding studies with all four Ab, peritoneal and spleen Mφ expressed  $2 \times 10^4$ – $10 \times 10^4$  binding sites/cell (Table III). Binding of these Ab to DC, spleen, and thymus was  $\frac{1}{10}$ – $\frac{1}{100}$  of that on Mφ, except for clone 2.6, which reacted similarly with spleen Mφ and DC. Each of the four clones was then evaluated by autoradiography and immunoprecipitation.

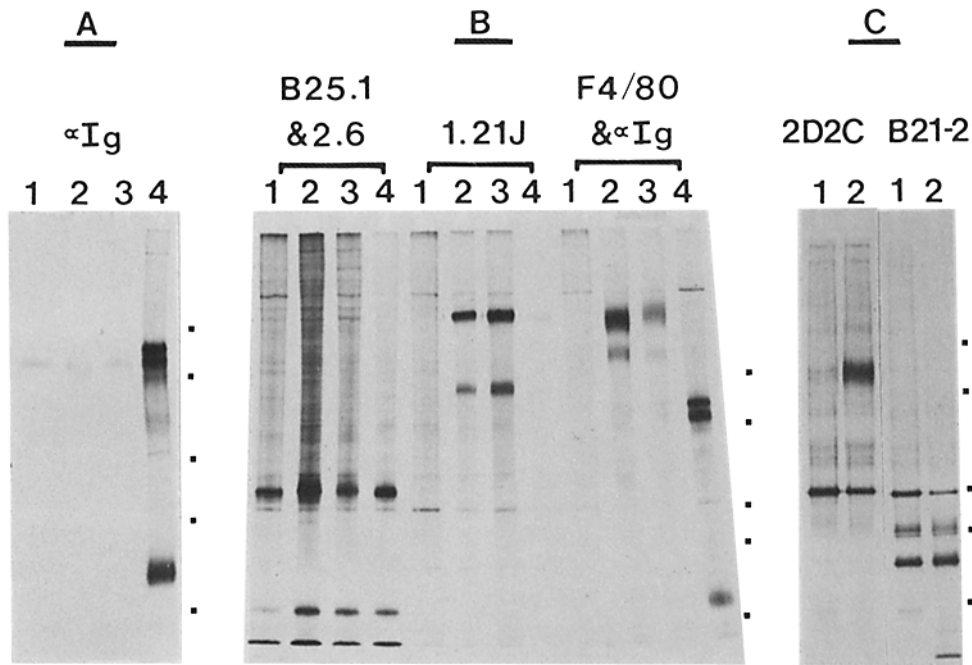


FIG. 5. (A) After 18 h of culture, four populations were prepared from the spleens of B6D2F<sub>1</sub> mice: (1), DC; (2), reloaded pellet Mφ; (3), collagenase Mφ; and (4), unfractionated spleen. The cells were then exposed to 50 μCi/ml [<sup>35</sup>S]methionine for 6 h. Ig synthesis was abundant in spleen, and just detectable in purified DC and Mφ. Molecular weight standards are on the right (94,000, 67,000, 43,000, 30,000, and 14,000). (B) The same lysates as in A were used, but aliquots were precipitated with solid-phase monoclonal Ab, or in the case of F4/80, the immune complexes were retrieved with anti-Ig on protein A-Sepharose. H-2D (B25-1) and 2.6 antigens were synthesized by all populations. Labeled 1.21J is abundant in Mφ, just detectable in spleen, and absent in DC. The results with F4/80 were similar to 1.21J. This Ag is synthesized by Mφ but not by DC or lymphocytes. In addition, Ig synthesis is evident in the spleen lane of F4/80 because an anti-Ig was used to retrieve the complexes. (C) Collagenase LODAC were labeled during overnight culture and then separated into DC (1) and Mφ (2). Mφ remained adherent, and DC were purified by sheep erythrocyte rosetting from the nonadherent fraction. Labeling of the 90,000 mol wt 2D2C antigen was active in Mφ but not DC, whereas labeling of Ia was equivalent in both lysates. The alpha and beta regions of Ia are similar in both cell types.

All spleen Mφ and 90% of the cells in monocyte preparations were heavily and uniformly labeled with 1.21J. 5–10% of the DC and 6–10% of the spleen cells were labeled (Fig. 2; Table V). In the spleen, many of the 1.21J<sup>+</sup> cells were neutrophils as judged by Giemsa or diaminobenzidine staining. Platelets and small lymphocytes were not labeled. Immunoprecipitation confirmed the binding studies. Biosynthesis of 1.21J was readily detected in [<sup>35</sup>S]methionine-labeled Mφ; however, it was weak in unfractionated spleen and undetectable in DC (Fig. 5B). Cell surface iodinated Mφ had abundant labeled 1.21J (e.g., 11,229 cpm; Fig. 6), whereas DC labeling was not above the background (280 cpm in Fig. 6). In both <sup>125</sup>I and [<sup>35</sup>S]methionine studies, the labeling of different cell types was normalized through a shared Ag such as H-2D and 2.6 (Figs. 5 and 6).

Labeling of cultured spleen Mφ and fresh monocytes was heterogeneous with F4/80. 90% of the spleen Mφ and 50–60% of the blood monocytes were labeled, and the number of grains ranged from 20 to 200/cell (Table V). 3% of the spleen cells

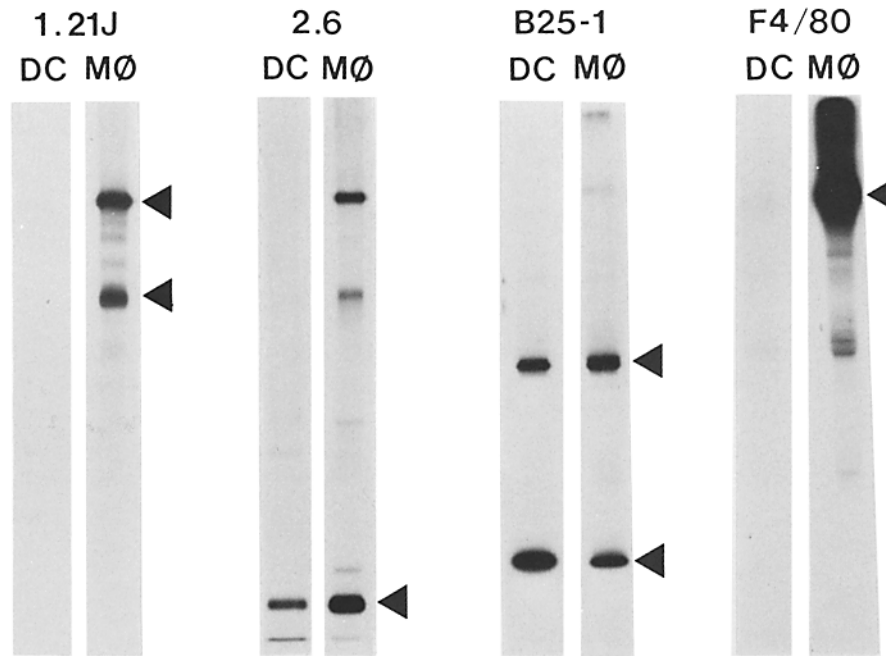


FIG. 6. Immunoprecipitations of surface iodinated peritoneal M $\phi$  and spleen DC. Similar numbers of trichloroacetic acid-precipitable counts were exposed to four monoclonal Ab, and the labeled Ag were visualized by SDS-PAGE and autoradiography. The major polypeptides recognized by each Ab are denoted with arrows on the M $\phi$  lane. Lysates were first precipitated with 1.21J, which retrieved an abundant 95,000–180,000 mol wt complex from M $\phi$  but not DC. The supernates were then exposed sequentially to 2.6, which precipitated a 21,000 mol wt polypeptide from both M $\phi$  and DC. Small amounts of residual 1.21J Ag were visualized in the M $\phi$  lane because of Ag carry-over. Supernates from the 2.6 precipitation were divided in two. Half were exposed to B25-1. The other half was precipitated with F4/80, which identified a 160,000 mol wt component on M $\phi$  but not DC. M $\phi$  preferentially express abundant 1.21J and F4/80 under conditions in which the iodination of DC and M $\phi$  was normalized. M $\phi$  had 11,000 cpm of 1.21J and DC were background.

reacted with F4/80, including some lightly labeled cells with donut-shaped nuclei, probably granulocytes. Lymphocytes, DC, and platelets were not labeled. A 160,000 mol wt polypeptide was the predominant species precipitated by F4/80 from iodinated M $\phi$  (Fig. 6), whereas a 160–125,000 mol wt complex was retrieved from [ $^{35}$ S]-methionine-labeled cells (Fig. 5B). F4/80 labeling was not detectable in  $^{125}$ I- or [ $^{35}$ S]-methionine-labeled DC (Figs. 5 and 6).

All spleen M $\phi$  and blood monocytes were heavily labeled with  $^{125}$ I-2D2C. 20–30% of the DC were heavily labeled (50–100 grains/cell), whereas the remainder were lightly labeled (Table V). 50% of spleen lymphocytes were lightly labeled with 2D2C and spleen granulocytes and blood platelets were strongly reactive. The 90,000 mol wt 2D2C polypeptide was precipitated from  $^{125}$ I- and [ $^{35}$ S]-methionine-labeled M $\phi$ , but 2D2C labeling was weak or absent on DC (Fig. 5C). Thus 2D2C binds to an alloantigen (see Materials and Methods and Table II) that is rich on phagocytes and platelets but present in smaller quantities on other cell types.

Spleen M $\phi$ , granulocytes, and DC bound  $^{125}$ I-2.6 (Table V), but lymphocytes were not labeled. Platelets were the most heavily labeled cells. Monocyte labeling appeared weak but was difficult to evaluate because platelets in the preparation were so heavily

labeled. 2.6 immunoprecipitated a single 21,000 mol wt polypeptide in internally and externally labeled DC and Mφ (Figs. 5 and 6). The 2.6 Ag is thus abundant on most nonlymphocytic elements.

The amount of Ia on Mφ was variable. Mφ from animals maintained under SPF conditions had up to 20% of the Ia on DC (Fig. 4), whereas Mφ from mice maintained in a non-SPF facility (Table III) or injected with bacille Calmette-Guérin (BCG; 4), could have as much Ia as DC. By autoradiography (Fig. 3), fresh monocytes and overnight-cultured spleen Mφ from SPF mice were 3-5% and 25-30% Ia-positive, respectively. Overnight-cultured DC from the same mice were all Ia<sup>+</sup>. We previously noted that all spleen Mφ and monocytes from BCG-infected mice were Ia<sup>+</sup>, whereas DC Ia levels were unaltered by immunization (4).

Clone B21-2 was used to immunoprecipitate Ia from [<sup>35</sup>S]methionine- and <sup>125</sup>I-labeled Mφ and DC. Purity of the preparations was monitored using markers rich on Mφ (either clone 1.21J, F4/80, or 2D2C) and surface Ig. Purified Mφ and DC synthesized typical class II MHC products. By one-dimensional SDS-PAGE, the Ia molecules from both cell types appeared identical (Fig. 5C).

We conclude that monoclonal Ab can be used to distinguish Mφ from DC and

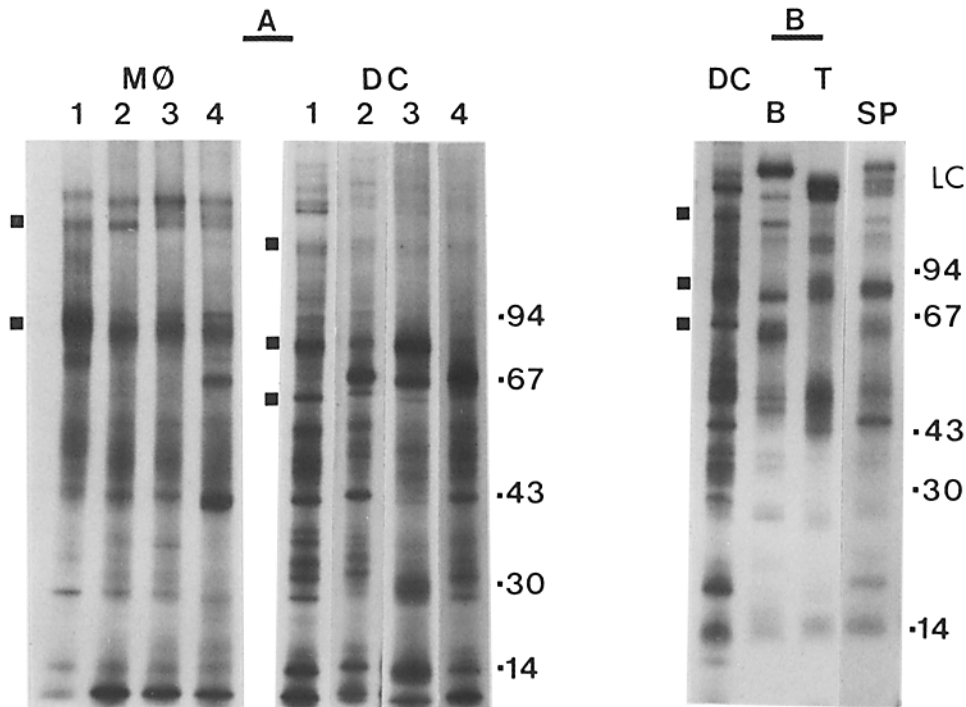


FIG. 7. (A) Surface iodination patterns of the J774 Mφ cell line (lane 1), B6 × D2F<sub>1</sub> peritoneal Mφ (lane 2), spleen Mφ (lane 3), and monocytes (lane 4). DC were from B6 × D2F<sub>1</sub> mice (lanes 1 and 2), C × B6 mice (lane 3), and Swiss mice (lane 4). Molecular weight standards are on the right. Differences in intensities of bands at 180,000 and 90,000 mol wt were noted on Mφ (dots on left). Bands that appear to be unique to DC are at 155,000, 85,000, and 62,000 mol wt (dots on left). Albumin 69,000 is variable on DC depending on washing procedures. (B) Surface iodination patterns of DC, anti-Ig purified B cells, nylon wool-purified T cells, and unfractionated spleen. Molecular weights are on the right; DC bands on the left are the same as in Fig. 5A.

lymphocytes. M $\phi$  express 2.4G2, 1.21J, and F4/80, and DC do not. The 2.6 and 2D2C Ag are shared by M $\phi$ , DC, granulocytes, and platelets. Expression of Ia on M $\phi$  may be under separate controls because tissue M $\phi$  and blood monocytes were frequently Ia<sup>-</sup>, whereas DC and B cells were always Ia<sup>+</sup>. Many of the surface Ag we have studied are expressed on mononuclear phagocytes from the spleen, the peritoneal cavity, and blood as well as granulocytes.

*Cell Surface Iodination.* The plasma membrane of purified M $\phi$ , DC, and lymphocytes were compared using LPO-mediated surface iodination. First, M $\phi$  from the spleen, the peritoneal cavity, blood, and the J774 cell line were compared with four different preparations of DC (Fig. 7A). All types of M $\phi$  exhibited similar surface patterns. Differences in the intensity of labeling of bands running at 200,000, 180,000 and 90,000 mol wt were noted and may represent differences in the amounts of surface LC, 1.21J, and 2D2C, respectively. The pattern of iodinated polypeptides on DC differed from M $\phi$ . DC always lacked the band corresponding to the higher molecular weight component of 1.21J or Mac-1. DC exhibited unique bands running at 155,000, 85,000 and 62,000 mol wt (Fig. 7A) and had variable quantities of BPA (69,000 mol wt) on their surfaces.

The DC and M $\phi$  patterns were also distinguishable from purified B and T lymphocytes (Fig. 7B). The purity of the B and T cells was evident on the 180,000–220,000 mol wt region, corresponding to known differences in LC molecular weights (17), and in the 60,000–85,000 Ig region. Again, polypeptides running at 155,000, 90,000 and 62,000 appeared to be DC restricted (Fig. 7B). We conclude that the composition of the DC plasma membrane differs substantially from other cells.

### Discussion

Lymphocyte subsets have been identified with specific surface markers, whereas DC and M $\phi$  have been differentiated using the combined approaches of morphology, endocytic tracers, surface adherence, and buoyant density. Information was therefore needed that would compare and quantitate the expression of surface antigens on these cells. In this report, we have initiated the quantitative comparison of surface antigens on DC, M $\phi$ , and other lymphoid cells. This analysis was facilitated by using three groups of monoclonal Ab obtained from rats immunized with M $\phi$ , lymphocytes, or DC-lymphocyte mixtures. These Ab were tested on several cell populations that varied in homogeneity. In the case of DC, M $\phi$ , and monocytes, most preparations were >90% pure.

It has been difficult to obtain monoclonal Ab specific for either mouse mononuclear phagocytes or DC. In the case of M $\phi$ , clones 1.21J and F4/80 were the most restricted probes. 1.21J or anti-Mac-1 reacted with M $\phi$  in peritoneal cavity spleen and blood and bound weakly if at all to DC, lymphocytes, and platelets. However, Mac-1 has been found on granulocytes and natural killer cells (14). F4/80 reacted with most tissue M $\phi$  but not lymphocytes, DC, or platelets. However, the F4/80 Ag was difficult to detect on some monocytes, and seemed to be present on some granulocytes as well (Table V). One difficulty in obtaining M $\phi$ -specific reagents was that Mac-1, and other less restricted Ag such as 2D2C and 2.6, were dominant immunogens. Immunization and/or screening procedures must be devised, as suggested by Springer (30), to eliminate these clones so that putative cell-specific reagents can be obtained. A similar problem arises in the case of DC, where Ia was immunodominant. We have

estimated (1) that several hundred anti-Ia hybrids are obtained from a single immune rat spleen. Many initially appeared to be DC specific because DC had so much more Ia than other cells.

Because of the difficulties in identifying cell-specific Ag, we iodinated the surface of purified DC, M $\phi$ , and lymphocytes to visualize a spectrum of plasma membrane polypeptides. Several bands visualized by SDS-PAGE appeared to be DC or M $\phi$  restricted. LPO-mediated iodination is less rigorous and direct than monoclonal Ab in identifying specific surface components. However, the iodination data provided the first positive evidence for DC-restricted polypeptides.

The monoclonal Ab we studied were not specific for DC and M $\phi$ , but they did provide a substantial amount of new comparative and quantitative information about the surface of the two cell types. DC expressed the LC Ag at levels similar to other cell types. This Ag is found on all leukocytes, but not on primary erythroid cells, fibroblasts, several solid tissues, or platelets (17). Because DC are bone marrow-derived (1), express LC, and are now being identified in peripheral blood, it seems appropriate to refer to DC as leukocytes.

Direct and sensitive binding assays with <sup>125</sup>I-Ab showed that DC lacked the major surface markers of lymphocytes, especially Ig, Lyt-1, and Thy-1. The methods for purifying DC depleted lymphocytes so efficiently that <2-3% of the enriched preparations were identified as lymphocytes. An unexpected finding was the presence of 5-12% Lyt-2 cells in DC preparations. This subpopulation had a distinctive phenotype, i.e., Ia<sup>+</sup>, Lyt-2<sup>+</sup>, Thy-1<sup>-</sup>, and Lyt-1<sup>-</sup>. It shared the adherence and buoyant density properties of DC and did not have the cytologic features of small or large lymphocytes after Giemsa staining. The nature of the Lyt-2 cells is unclear. Positive selection techniques should distinguish between the two main possibilities, viz., the Lyt-2 cells represent a state of DC differentiation or activation, or they are distinct cells copurifying with DC.

The 2.4G2 Fc receptor Ag was identified on a variety of cell types, but not on DC (Tables III and V). Autoradiographic assays with <sup>125</sup>I, Fab 2.4G2 were sensitive enough to detect the Fc receptor on many lymphocytes and most monocytes (Fig. 2). When indirect immunofluorescence was used, we were unable to visualize the small amounts of 2.4G2 on lymphocytes (~10<sup>4</sup> binding sites/cell). It remains possible that Fc or C3 receptors can be expressed on DC at some stage of their life history, or that immunoglobulin binding sites distinct from the 2.4G2 binding molecule will be found.

A group of four anti-M $\phi$  reagents, clones 1.21J, F4/80, 2D2C, and 2.6, were considered. Each had a different tissue distribution (Table V). All of the Ab detected Ag that were abundant (5 × 10<sup>4</sup>-10 × 10<sup>4</sup> binding sites/cell) and uniform (Table V; Fig. 2) on M $\phi$  from the peritoneal cavity and the spleen. The exception was F4/80, where Ag expression on cultured spleen M $\phi$  was weak and heterogeneous. The latter might be attributed to the day of culture that preceded spleen M $\phi$  purification; Ezekowitz et al. (31) found that the level of F4/80 on peritoneal M $\phi$  fell 80-90% after overnight culture. All the M $\phi$  Ag were detected on blood monocytes. 1.21J, 2.4G2, and 2D2C were abundant and uniform (Fig. 2), whereas labeling with <sup>125</sup>I-F4/80 and 2.6 was weaker and heterogeneous. In contrast to M $\phi$  and monocytes, 1.21J, 2.4G2, and F4/80 Ag were scarce or absent on DC. 2D2C was heterogeneous (Fig. 2), whereas 2.6 was abundant but expressed on platelets as well as DC. In addition, DC expressed high levels of Ia, whereas most monocytes and M $\phi$  were Ia<sup>-</sup> (see below and Fig. 3).



These differences in surface Ag, combined with differences in the functional capacities of DC and mononuclear phagocytes (2-4), indicate that DC are not part of the monocyte-M $\phi$  system. For DC to be monocyte derived, there would have to be two distinct pathways of monocyte differentiation. In one, monocytes would retain a variety of surface and functional features while maturing into tissue M $\phi$ ; in the other, surface and functional properties would have to be dramatically altered for the monocyte to become a DC. The latter is unlikely and has not been observed in colonies of mononuclear phagocytes developing *in vitro*.

A group of anti-MHC Ab were employed to quantitate, visualize, and immunoprecipitate class I and II products from several different cell types. These clones provided the most interesting new information about DC. First, DC expressed high levels of both Ia and H-2D. The average amount of anti-Ia bound per DC was  $2 \times 10^5$  molecules. This may be an underestimation of the level of Ia, if significant bivalent binding occurred. Ia was 5-20 times more abundant on DC on a per cell or protein basis than on B cells or M $\phi$  from SPF mice. There is evidence that irregularly shaped cells at other sites express abundant Ia, e.g., Langerhans cells in epidermis, veiled cells in afferent lymph, and interdigitating cells in lymphoid tissue sections. All these cells resemble spleen DC (9). Surface markers that distinguish between DC and M $\phi$  might be used to further classify these cells.

A second feature of DC Ia is that it is present in constant levels on DC from all groups of mice studied: SPF, germ free, conventionally reared (this paper), BCG infected (4), and nude (unpublished data). B cell Ia increases following transformation with LPS, as expected from previous work (32), whereas DC and M $\phi$  Ia was unaffected by LPS. M $\phi$  Ia undergoes dramatic alterations. Most monocytes and M $\phi$  were Ia<sup>-</sup> in SPF mice. However, entire populations of Ia<sup>+</sup> cells have been visualized during BCG infection (4) after exposure to spleen cell factors *in vitro* (33), and even in some conventionally reared mice (Table III). DC and B cell Ia were not altered under similar conditions. We propose that a high level of Ia is a constitutive feature of DC, whereas Ia on other cells can be altered by environmental and immunologic factors. Large amounts of Ia are likely to contribute to the functional effects of DC on mixed leukocyte and accessory cell assays *in vitro*. DC may also provide accessory function *in situ* because they are isolated from white vs. red pulp, express high levels of Ia, and have been identified in the periarteriolar lymphoid tissue (1) where responding lymphocytes can first be identified in many immune responses.

DC lack the critical differentiation markers of lymphocytes and phagocytes. DC fail to express surface Ig, Thy-1 and Lyt-1, which are all expressed early during the development of B and T cells. 1.21J and 2.4G2 are also not detected on DC but are found on most monocytes as well as M $\phi$  and granulocytes. These findings suggest that DC diverge from both myeloid and lymphoid lineages early in development. We propose that spleen DC are part of a new Ia-rich leukocyte differentiation pathway that functions in the afferent or sensitization phase of the immune response.

### Summary

The surface of dendritic cells (DC) has been analyzed by means of monoclonal antibodies (Ab) and lactoperoxidase (LPO)-mediated radioiodination. Antigens and other exteriorly disposed polypeptides of purified spleen DC were compared with

those of tissue macrophages (M $\phi$ ), monocytes, and other bone marrow-derived elements.

Quantitative binding studies and autoradiography with <sup>125</sup>I-Ab established that DC expressed high levels of I-A and H-2D,  $2 \times 10^5$  and  $1 \times 10^5$  Ab binding sites per cell, respectively. DC from conventional, germ-free, and specific pathogen-free mice were all rich in Ia. Expression of Ia on B cells was 5–10% of that on DC and increased fivefold during lipopolysaccharide mitogenesis. More than 70–90% of purified M $\phi$  and monocytes from specific pathogen-free mice were Ia negative, but increased levels of Ia were noted on cells from mice reared under conventional conditions. Thus large amounts of Ia on DC is a constitutive trait, whereas the expression of Ia by other cell types may be governed by the environmental and immunological status of the host.

The 2.4G2 Fc receptor Ag was not detected on DC. Peritoneal and spleen M $\phi$  had  $10^5$  2.4G2 binding sites/cell, whereas monocytes and lymphocytes were less reactive ( $1 \times 10^4$ – $3 \times 10^4$  binding sites/cell).

Four other M $\phi$ -related antigens were evaluated. Each had a distinctive tissue distribution and none bound exclusively to M $\phi$  and monocytes. Neither 1.21J (Mac-1) nor F4/80 reacted with DC. Immunoprecipitation studies of externally (<sup>125</sup>I) and biosynthetically ([<sup>35</sup>S]methionine)-labeled cells confirmed the binding data.

Sensitive binding assays with <sup>125</sup>I-Ab confirmed previous observations that DC lack Ig and Thy-1. Lyt-1 was also not found on DC, but 5–12% of the cells in purified DC preparations expressed both Lyt-2 and Ia. All DC expressed the leukocyte common antigens at levels similar to other leukocytes.

The spectrum of surface polypeptides labeled by LPO-mediated iodination was different on M $\phi$ , DC, and lymphocytes. Polypeptides migrating at molecular weights of 155,000, 85,000, and 62,000 appeared to be restricted to DC.

These observations establish that the cell surface of DC differs considerably from other leukocytes, including the blood monocyte, and suggest that the DC is part of a unique Ia-rich leukocyte differentiation pathway.

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