SPECIFIC ANTIMONONUCLEAR PHAGOCYTE MONOCLONAL ANTIBODIES

Application to the Purification of Dendritic Cells and the Tissue Localization of Macrophages*

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The monoclonal antibody technique is providing new reagents for the study of mononuclear phagocytes. Among other applications, specific antibodies should make it possible to distinguish macrophages from other cell types in suspension and tissue sections. This potential has not been fully exploited, since the number of specific, panmacrophage reagents is limited. In mouse, a single monoclonal antibody, F4/80, appears to be truly macrophage restricted (1, 2). In rat, guinea pig, and rabbit, macrophage-specific reagents have yet to be described to our knowledge. In man, several antimonocyte antibodies have been produced (3–10). However, there has been little work using these reagents to distinguish macrophages from dendritic cells and to visualize macrophages in tissue sections.

The distinction between macrophages and dendritic cells is critical, since the two cell types differ so markedly in functional capacities. The dendritic cell lacks Fc receptors (2, 11, 12), does not exhibit active phagocytic or pinocytic activity (11), but is the principal accessory cell needed to stimulate lymphocyte responses (see 13). Recently it has been shown that enriched populations of dendritic cells from human blood are markedly more potent than monocytes in stimulating mixed leukocyte reactions and polyclonal T cell mitogenesis (14, 15).

Here we describe monoclonal antibodies directed to an antigen that is restricted to mononuclear phagocytes including monocytes in blood and macrophages in lung, skin, spleen, and lymph node. We then use the monoclonals in two types of experiments to further characterize human mononuclear phagocytes and dendritic cells. First, depletion of monocytes with antibody and complement can be used to prepare circulating dendritic cells in high purity and yield. Second, the monoclonals stain macrophages in tissue sections, localizing this cell type to the dermis of skin, red pulp of spleen, and lymphatic channels of lymph node. In contrast, presumptive dendritic cells are localized in entirely different anatomic regions: skin epidermis, splenic white pulp, and lymph node cortex. Therefore these monoclonal antibodies provide a clear distinction between macrophages and dendritic cells in blood and tissues.

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Materials and Methods

Cells. Blood was obtained from normal volunteers, and leukocyte-enriched buffy coats were purchased from the Greater New York Blood Center. Mononuclear cells were harvested from the interface of Ficoll-Hypaque (density = 1.077; Histopaque 1077-1; Sigma Chemical Co., St. Louis, MO) columns and cultured for 1–2 h on 100-mm plastic culture dishes. The medium was 5% heat-inactivated fetal calf serum (FCS)[1] (Sterile Systems, Logan, UT, and Kansas City Biologicals, Kansas City, MO)-RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 20 μg/ml gentamycin, 100 U/ml penicillin, 1 mM glutamine, and 5×10^{-5} M 2-mercaptoethanol. Nonadherent cells (~70% of the total) were removed with several washes and used to prepare T cells by rosetting with neuraminidase-treated sheep erythrocytes or by nylon wool filtration (14). Adherent cells were cultured overnight and separated into monocyte-, B cell-, and dendritic cell-enriched fractions as described (14). T lymphoblasts were prepared by stimulating nonadherent cells with 2 μg/ml phytohemagglutinin (Burroughs Wellcome & Co., Greenville, NC) for 4 d in 5% FCS-RPMI 1640 medium. B lymphoblasts were prepared by stimulating high density B cell-enriched fractions (14) with 3 μg/ml pokeweed mitogen (Difco Laboratories Inc., Detroit, MI) for 4 d in 5% FCS-RPMI-1640 medium. Granulocytes were kindly provided by Dr. H. B. Fleit (The Rockefeller University) who sedimented the granulocytes through Ficoll-Hypaque, removed most erythrocytes by dextran sedimentation, and depleted residual erythrocytes by hypotonic lysis (16). Platelets were present in mononuclear cell fractions and could be isolated because they did not pellet at 180 *g* for 10 min. To study the stability of cell surface antigens in culture, monocyte-enriched fractions were maintained for 1–3 wk either adherent to glass coverslips in 10% AB+ human serum-RPMI 1640, in plastic culture dishes in 5% FCS-RPMI 1640, or in teflon beakers as described (17; kindly provided by Dr. S. B. Wright). The cultured cells were >98% viable monocytes by morphology, nonspecific esterase reactivity, and binding of specific monoclonal antibodies. Dr. A. Fels and Dr. T. Nash (The Rockefeller University) provided alveolar macrophages by bronchial lavage of nonsmokers, followed by collection of mononuclear cells on Ficoll-Hypaque columns, and adherence to glass coverslips (18). Several human cell lines were provided by Dr. J. C. Unkeless (The Rockefeller University) and Dr. H. B. Fleit: HL60 (promyelocyte-like), K562 (multipotential blood cell precursor), U937 (immature myeloid), and Daudi (B cell). Several human lymphocyte lines were provided by Dr. L. Mayer (The Rockefeller University): T cell lines CEM T and Jurkat, and B cell lines SeHa D, 8866P, and 32a1.

Monoclonal Antibodies. A large panel of mouse anti-human monoclonal antibodies (Table I) was produced in our lab and obtained from outside sources. For the new reagents (3C10, 1D9, 1E8), a BALB/c mouse (The Trudeau Institute, Saranac Lake, NY) was injected intraperitoneally with 10^6 blood monocytes in saline three times at monthly intervals, and four times with $3–5 \times 10^6$ monocytes on each of the 4 d before fusion (19). For 9.3F10, which recognizes Ia-like or class II major histocompatibility products (see Results), exactly the same protocol was used except that dendritic cell-enriched (14) preparations were injected into mice. Spleen cells from these mice were fused to P3U1 myeloma cells at a ratio of one myeloma cell to five spleen cells as described (20). The products of these fusions were seeded at 5×10^4 myeloma cells per 0.32-mm^2 microwell (3596; Costar, Cambridge, MA) with 100 μl of hypoxanthine-aminopterine-thymidine (HAT) culture medium (Dulbecco's minimal essential medium, supplemented with HAT, horse serum, FCS, vitamins, and NCTC 109 medium as described [20]).

The immunization method of Staehli (19) that we used provided a large number of hybrids reactive with human leukocytes, so that a rapid radioimmunoassay was used to detect antibodies of interest. A large panel of test cells and cell lines (see above) were attached tc Terasaki microassay plates (3034; Falcon Labware, Oxnard, CA) that had been exposed to 100 μg/ml poly-L-lysine (70,000 average mol wt; P2636; Sigma Chemical Co.) in phosphate-buffered saline (PBS) for 20 min, rinsed in distilled water and dried.

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; HAT, hypoxanthine-aminopterin-thymidine; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

TABLE I

Monoclonal Mouse Antihuman Leukocyte Antibodies

Name	Ig subclass	Source	Molecular weight of polypeptide	Specificity	Reference
T29/33	IgG_{2b}	Dr. I. S. Trowbridge, Salk Inst., CA	200,000	All leukocytes (Leukocyte-common Ag)	21
OKIal	IgG_{2a}	Ortho Diagnostic Systems Inc., Westwood, MA	34,000, 29,000	HLA-class II Ag	22
OKMl	IgG_{2b}	Ortho Diagnostic Systems Inc.	170,000	Monocytes, natural killer cells granulocytes, Tγ	3
63D3	IgG_1	Bethesda Research Laboratories, Gaithersburg, MD	200,000	Monocytes, granulocytes	4
61D2	IgG_{2a}	Bethesda Research Laboratories	44,000	HLA-class I Ag	4
Leu 1	IgG_{2a}	Becton, Dickinson & Co., Oxnard, CA	67,000	All T cells	23
3G8	IgG_1	Dr. H. B. Fleit, The Rockefeller Univ.	66,000, 53,000	Anti-Fc γ receptor on human leukocytes	16
1B4	IgG_{2a}	Dr. S. D. Wright, The Rockefeller Univ.	182,000, 123,000, 105,000	Complement receptor (C_{3bi}) on human leukocytes	In preparation
BA-1	IgM	Hybritech, Inc., San Diego, CA	NA	B cells, granulocytes	24
OKT6	IgG_1	Ortho Diagnostic Systems Inc.	52,000	Thymocytes, Langerhans' cells	25
1D9	IgG_1	Authors	55,000	Monocytes	This paper
3C10	IgG_{2b}	Authors	55,000	Monocytes	This paper
1E8	IgG_1	Authors	ND	Monocytes, granulocytes	This paper
9.3F10	IgG_{2a}	Authors	29,000, 33,000	HLA-class II Ag	This paper

1–4 × 10^4 cells in 5–10 μl of RPMI 1640 with 0.02% NaN_3 (azine) were added to each well. The plate was centrifuged at 150 *g* for 5 min at 4°C and quenched in cold RPMI 1640 with 10% horse serum and azide. After 20 min, the fluid was aspirated and 10 μl of culture supernatant was added for 1 h on ice. The plates were rinsed by dipping in cold PBS with azide, aspirated, and exposed to 10 μl of 5 μg/ml ^{125}I-$F(ab')_2$ affinity-purified rabbit anti-mouse IgG (specific activity of 6 × 10^6 cpm/μg) in RPMI 1640 with 10% horse serum and azide on ice for 30 min. Finally the plates were rinsed, dried, and autoradiographed for 3–24 h at −70°C using Kodak XAR5 film (Eastman Kodak Co., Rochester, NY) and an intensifying screen (Lightening Plus; Dupont Co., Wilmington, DE).

Using this technique, we noted that hybrid wells 3C10 and 1D9 reacted with monocytes alone; 1E8 with monocytes, granulocytes, and the U937 and HL60 cell lines; and the commercially available reagent OKMI with granulocytes and monocytes. In a similar assay, 3C10, 1D9, and 1E8 did not show detectable binding to B cells, T cells, dendritic cells, and three B cell and two T cell lines, suggesting that the reagents were specific for myeloid elements. In contrast, 9.3F10 reacted with monocytes, B cells, dendritic cells, and three B cell lines, but not with granulocytes, T cells, two T cell lines, and the U937,

HL60, and K562 cell lines, indicating it was an anti-Ia or anti-class II reagent. Many additional hybrids were identified with similar reactivity patterns to 3C10, 1D9, 1E8, or 9.3F10. The latter were cloned in soft agar, and further characterized as described in Results. All hybridoma clones produced an Ig-rich ascites in DBA/2 × BALB/c F_1 mice.

Purification and Radiolabeling of Monoclonal Antibodies. Monoclonal antibodies 3C10, 1D9, 1E8, and 9.3F10 were purified from ascites using 45% (vol/vol) $(NH_4)_2SO_4$ precipitation followed by ion exchange chromatography on diethylaminoethyl cellulose (DE-52; Whatman Inc., Clifton, NJ). The Ig fraction gave a single band on cellulose acetate electrophoresis that co-migrated with the monoclonal band in the unseparated ascites. The subclass of the purified antibody was determined by Ouchterlony analysis using subclass-specific antisera prepared in goats (Meloy Laboratories Inc., Springfield, VA) and the results are presented in Table I. To prepare active (see Results) Fab fragments of the 3C10 antimonocyte and 9.3F10 anti-class II antibodies, Ig at 8 mg/ml and 5 mg/ml, respectively, was digested with 1% (wt/wt) papain (P-4762; Sigma Chemical Co.) in pH 5.5 0.1 M sodium acetate buffer with 2 mM EDTA and 10 mM 2-mercaptoethanol for 4 h at 37°C. The reaction was stopped by bringing the solution to 20 mM iodoacetamide. The mixtures were dialyzed into 25 mM Tris pH 8.0 and passed over DE-52. Following electrophoresis on 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26) under reducing conditions, the papain digests contained four to five polypeptides, including the candidate Fc, Fd, and light chain portions; the flow-through fractions were the presumptive Fab fragments with only two bands (Fig. 1). The intact antibodies and digests reacted with subclass-specific antisera, but the flow-through Fab fractions did not; all reacted with rabbit anti-mouse IgG (heavy and light chains).

Antibodies were labeled with Na-^{125}I (carrier-free; New England Nuclear, Boston, MA) using Iodo-Gen (Pierce Chemical Co., Rockford, IL) as the oxidizing agent (27) as described (2). The iodinated and noniodinated antibodies competed for binding to monocytes with equal efficiency (see Results).

Binding of Monoclonal Antibodies to Cells In Vitro. Indirect immunofluorescence was done using a sensitive biotin-avidin system as described (14). Briefly, cells were exposed

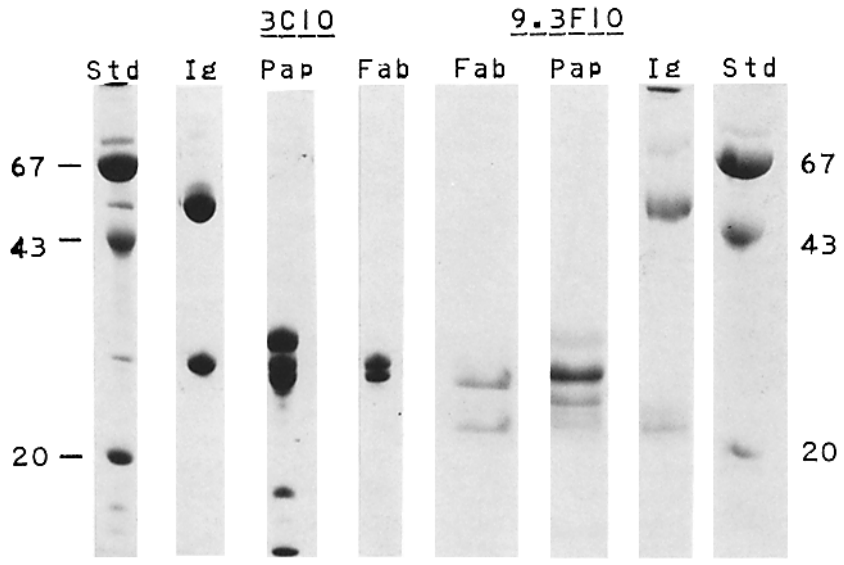

FIGURE 1. Analysis of 3C10 and 9.3F10 Ig and Fab fragments. One-dimensional 11% SDS-PAGE was run under reducing conditions and stained with Coomassie brilliant blue. 3C10 is antimonocyte and 9.3F10 is an anti-HLA class II. For each antibody, samples were analyzed from the purified Ig fraction (ascites passed over diethylaminoethylcellulose), the papain (Pap) digests, and the Fab fragments purified from the digests. Std, standard.

successively to: (*a*) mouse anti-human monoclonal antibody, (*b*) biotinylated horse anti-mouse Ig (Vector Laboratories Inc., Burlingame, CA), and (*c*) fluorescein-avidin (Vector Laboratories Inc.). Biotinylated horse anti-human Ig (Vector Laboratories Inc.) was used at 20 μg/ml to stain B cells.

Quantitative binding studies with monoclonal antibodies were performed at 4°C with cells attached to 16-mm poly-L-lysine-coated macrowells (3524; Costar) as described above for the screening radioimmunoassay, except that the volume of the well was always maintained at 0.25 ml; 1–4 × 10^5 cells were used per well; and the bound radioactivity was determined by wiping the cells with cotton swabs and counting these in a Packard gamma counter (Packard Instrument Co., Downers Grove, IL). Additional details are provided in the legends to Tables IV–V.

Quantitative binding studies were used to monitor the effect of protease treatment on monocyte-specific and class II antigens. Treatment with proteases was done in suspension at 5 × 10^6 cells/ml in PBS with 1 mg/ml of either TPCK-trypsin (T8642), chymotrypsin (C4129; Sigma Chemical Co.), or pronase (53702; Calbiochem-Behring Corp., La Jolla, CA) for 15 min at 37°C; or by 0.1 IU/ml neuraminidase (vibrio cholerae; 480717; Calbiochem-Behring Corp.) for 45 min at 37°C. Viability was >90% by trypan blue exclusion after each treatment. Cells were then centrifuged onto poly-L-lysine-coated 16-mm wells at 4 × 10^5 cells per well, and the amount of antigen was quantified as above.

Identification of the Polypeptide Carrying the 3C10/1D9 Monocyte Antigen. The "western blot" procedure was modified slightly from those described by Towbin et al. (28) and Johnson et al. (29). In brief, 5 × 10^6 adherent cells were solubilized in lysis buffer, i.e., PBS with 0.5% NP40 detergent, 0.2 trypsin inhibitory units trasylol (aprotinin; A-6012) and 2 mM phenylmethylsulfonylfluoride (P-7626; Sigma Chemical Co.). The lysate was cleared by centrifugation at 600 *g* for 10 min, and then 20,000 *g* for 20 min. The lysate was mixed 1:1 with sample buffer (4% SDS, 24% sucrose, 0.05% bromphenol blue, 100 mM Na_2CO_3, pH 8.5), boiled for 2 min, and electrophoresed 10 cm on a 1-mm, 4–11% gradient SDS-PAGE. The proteins were transferred from the gel lane onto nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, NH) by electrophoresis. The nitrocellulose filter was incubated for 16 h with PBS-Tween (10% horse serum, 0.1% gelatin, 0.1% NaN_3, 0.05% Tween 20 in PBS), which served as both wash and carrier buffer for all incubations. The filter was exposed at 4°C for 20 h to either directly ^{125}I-labeled monoclonal antibody or unlabeled monoclonal antibody (sandwich assay), washed four times over 45 min at room temperature and, for the sandwich assay, exposed to ^{125}I-F(ab′)$_2$ rabbit anti-mouse Ig at 4°C for 20 h. The blot was then washed extensively and autoradiographed for 16–48 h as above.

The immunoprecipitation procedure was carried out on overnight-cultured, adherent mononuclear cells that were surface labeled with ^{125}I and lactoperoxidase/glucose oxidase (30). The cells were solubilized and cleared by centrifugation as described above. Then SDS was added to a final concentration of 0.2%, and the lysate was preabsorbed for 1 h at 4°C with 100 μgm crude mouse immunoglobulin (45% $(NH_4)_2SO_4$ precipitate of nonimmune mouse serum) coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), per 10^6 cell equivalents. The supernatant representing 10^6 cell equivalents was then absorbed with 100 μgm of antibody coupled to Sepharose 4B for 1 h at 4°C. The beads were washed three times in lysis buffer with 2% FCS and two times in lysis buffer alone. The antigen was released by boiling with sample buffer and 50 mM dithiothreitol and electrophoresed for 20 cm. The gel was dried and autoradiographed for 7 d.

Complement-mediated Cytotoxicity. Killing with antibody and rabbit complement was accomplished by exposing cells (1.7–2.5 × 10^6 cells/ml) to antibody (3C10 antimonocyte at 10 μg/ml; 9.3F10 anti-Ia at 3 μg/ml; Leu-1 anti-T cell at 3 μg/ml; BA-1 anti-B cell at 3 μg/ml) and rabbit serum (selected for low background cytotoxicity and stored at −70°C after lyophilization; 1:9 final dilution) for 1 h at 37°C. All reagents were diluted in 0.3% bovine serum albumin, 25 mM Hepes, RPMI 1640 pH 7.4, and deoxyribonuclease (DNAase I; Sigma Chemical Co.) was present at 10 μg/ml. The percentage killed was determined by the reduction of viable cells (trypan blue exclusion test) as compared with the mean of the control incubations; i.e., no treatment, heated (56°C for 30 min)

complement alone, active complement alone, and heated complement plus antibody. The standard error of the averaged controls was <10%. The percentage of monocytes was monitored by staining cytocentrifuge preparations with α-naphthylbutyrate for nonspecific esterase according to the method of Li et al. (31). Cells with diffuse red cytoplasmic staining were scored as monocytes. Staining for myeloperoxidase, with diaminobenzidine tetrahydrochloride and hydrogen peroxide (32), was also used to count monocytes with similar results. However the percentage of monocytes that were peroxidase positive diminished to ~50% after overnight culture.

Dendritic Cell Purification with Cytotoxic Cell-specific Antibodies. To enrich for dendritic cells using cell-specific antibodies, we began by treating 0.5–5 × 10^8 blood mononuclear cells with 3C10 antimonocyte antibody and complement, or complement alone as outlined above. The cells were washed and adhered in 100-mm plastic culture dishes for 1 h at 37°C in 5% FCS medium. The nonadherent cells were removed with five rinses and the adherent monolayers were cultured for 18 h. Most of the adherent cells had released from the plastic, and these were retreated with 3C10 as well as BA-1 and Leu-1 (anti-B and -T cell, respectively) antibodies and complement, or just complement for the control cells. Almost all of the viable cells (~80%) were retrieved by flotation in columns of dense bovine plasma albumin (P = 1.082) as described (14). This fraction was highly enriched for dendritic cells (see Results). An alternative approach to eliminate monocytes was to treat mononuclear cells with 3C10 antibody and complement after, rather than before, adherence to plastic. The former requires less complement, but the latter approach was used in this paper to establish that normal numbers of dendritic cells adhere to plastic after elimination of most monocytes.

Staining Tissue Sections with Monoclonal Antibodies. Skin biopsies were obtained by Dr. W. R. Levis (The Rockefeller University) from patients with leprosy, but normal skin samples were also stained. Spleen and lymph node were provided by the Tumor Procurement Center of Memorial Sloan-Kettering Cancer Center, New York. The tissues, which did not contain tumor cells, were frozen in liquid nitrogen, embedded in OCT embedding medium (Lab-Tek Div., Miles Laboratories Inc., Naperville, IL), sectioned at 6–8 μm with a cryostat, and stored at −20°C. The sections were fixed before staining for 30 s with ice-cold acetone. Immunoperoxidase staining was done as previously described for immunofluorescence of tissue sections (33), except that after treatment of tissue sections with monoclonal antibody and biotinylated horse-anti-mouse IgG, the sections were treated with 20 μg/ml avidin-horseradish peroxidase (Vector Laboratories Inc.), and developed with 0.4 mg/ml 3,3′ diaminobenzidine tetrahydrochloride (Polysciences Inc., Warrington, PA) in 0.05 M Tris buffer, pH 7.6, with 0.01% H_2O_2 for 10 min at room temperature. Sections were stained for 3 min with Gill's hemotoxylin (formulation 1; Fisher Scientific Co., Fairlawn, NJ).

Results

The purpose of this study was to develop monoclonal antibodies that reacted only with mononuclear phagocytes in blood and in tissues, and not with dendritic cells. The monoclonals could then be used to help purify dendritic cells from blood and to show that macrophages and dendritic cells occupy distinct anatomic compartments in spleen, lymph node, and skin.

Cell Specificity of 3C10, 1D9, and 9.3F10 Monoclonal Antibodies. Initial screening with a microradioimmunoassay (see Materials and Methods) indicated that 3C10 and 1D9 reacted with monocytes, whereas 9.3F10 reacted with many cell types and lines known to express class II HLA products. After cloning, we evaluated culture supernatants, purified Ig, and Fab fragments, for reactivity with a large panel of cell types using immunofluorescence and a sensitive biotin-avidin technique. 3C10 and 1D9 stained monocytes strongly, but did not react with a large

panel of other cell types (Table II). A small percentage of cells were stained in the B cell- and dendritic cell-enriched populations, but this percentage corresponded to the number of contaminating monocytes estimated by cytologic criteria or by nonspecific esterase staining. 9.3F10 stained most monocytes, B cells, and dendritic cells, but not T cells, platelets, and granulocytes (Table II). 9.3F10 stained similarly to OKIa (22), further suggesting that it recognized class II HLA antigens. The Fab fragments of 3C10 and 9.3F10 stained a similar panel of cell types to the corresponding intact Ig (not shown). Leu-1 and anti-Ig reacted with most cells in the T and B cell-enriched populations, respectively, but not with monocytes, dendritic cells, and other cell types (Table II).

By indirect immunofluorescence, the 3C10 antigen was stable in culture. Thus 3C10 was expressed on 95% of monocytes that had been cultured for 45 d in teflon beakers to retard adherence (Table II). Glass-adherent monocytes were $3C10^+$ for 21 d, but at 28 d about 25% became $3C10^-$. The latter frequently were multinucleated giant cells. Alveolar macrophages, obtained by bronchial lavage, were all stained with 3C10 and 3C10 Fab fragment, but not with the T lymphocyte specific marker, Leu-1 (Table II).

In the presence of rabbit complement, 3C10 killed >95% of purified monocytes but did not kill populations enriched in other cell types (not shown). 9.3F10 killed 30–70% of monocytes, as well as most B cells and dendritic cells. 3C10 efficiently and selectively depleted monocytes from heterogeneous mixtures of unfractionated mononuclear cells or plastic-adherent cells (Table III). Total cell killing precisely paralleled the loss of cells that stained diffusely for nonspecific esterase, an independent monocyte marker (Table III). >95% of the esterase-

TABLE II

Reactivity of Monoclonal Antibodies with Different Cell Types

Cells	Percentage of cells positive after staining with:						
	3C10	9.3F10	OKIa	Leu-1	Anti-Human Ig	Anti-HLA class I	Percent monocytes
PBMC* 1	30	28	26	60	7	100	31
PBMC 2	25	23	24	53	14	100	25
Monocytes	95	78	78	1	2	100	96
Granulocytes	<1	<1	<1	ND	<1	100	<1
Dendritic cells	10	100	100	6	44	100	10
T Cells (E_n+)	1	5	5	95	2	100	1‡
T lymphoblasts	1	7	7	85	1	100	1
Non-T (E_n−)	4	37	33	8	24	100	4‡
B cells	2	84	81	19	78	100	2
B lymphoblasts	1	84	82	21	68	100	1
Erythrocytes	0	0	0	0	0	ND	0
Platelets	0	0	0	0	0	100	0
45-d cultured monocytes	100	100	ND	0	ND	ND	100
Alveolar macrophages	98	99	ND	0	ND	ND	98

Enriched populations of various cell types were prepared as described in Materials and Methods. The dendritic cell-enriched fraction was 29% dendritic cells, 55% lymphocytes, and 10% monocytes by morphology. The percent monocytes was generally determined by morphology, i.e., circumferential spreading, ruffles, large-phase dense lysosomal granules. ND, not done.

* Peripheral blood mononuclear cells.

‡ Percent monocytes determined by diffuse nonspecific esterase staining.

TABLE III

Summary of Cytotoxicity Experiments with Antimonocyte and Anti-Ia Antibodies and Rabbit Complement

Population	Antibody	*n*	Untreated cells	Antibody- and complement-treated cells	
			Percent esterase$^+$	Percent killed	Percent esterase$^+$
Peripheral blood mononuclear cells	3C10	11	31 ± 8	27 ± 9	2 ± 1
	9.3F10	4	31 ± 9	20 ± 7	14 ± 13
Adherent mononuclear cells	3C10	5	54 ± 9	52 ± 6	2 ± 2
	9.3F10	1	54	65	35

Unfractionated mononuclear cells or plastic-adherent cells (released after overnight culture) were treated with antimonocyte (3C10) or anti-Ia (9.3F10) antibodies and rabbit complement. We then counted the percent (mean ± standard error) of cells that were stained diffusely for nonspecific esterase in aliquots of untreated and antibody plus complement-treated cells. The percent killed was determined by trypan blue staining.

TABLE IV

Number of 3C10, 1D9 (Antimonocyte), and 9.3F10 (Anti-Ia) Binding Sites per Monocyte

^{125}I-labeled antibody	ng of antibody bound per 4×10^5 monocytes (molecules of antibody bound per monocyte)		
	Experiment A	Experiment B	Experiment C
3C10 (intact Ig)	3.6 (38,000)	4.9 (47,000)	–
3C10 (Fab fragment)	1.3 (38,000)	1.5 (46,000)	1.0 (37,000)
1D9 (intact Ig)	3.4 (34,000)	–	–
9.3F10 (intact Ig)	23 (230,000)	–	–
9.3F10 (Fab fragment)	–	–	7.5 (225,000)

In experiment A, saturating doses of ^{125}I-antibody (3–12 μg/ml) were added, while in experiment B, a constant dose of ^{125}I-antibody (0.2 μg/ml for intact and 1.0 μg/ml for Fab) was mixed with varying doses of unlabeled antibody (0–12 μg/ml for intact, 0–32 μg/ml for Fab). The values given are means of at least three saturating doses, the standard deviation being <10%. Nanograms of antibody bound was determined by: [(cpm bound − no-cell control)/specific activity of the antibody]. The no-cell control was <2% of the total bound. 1 ng of intact Ig (~150 kD) was assumed to be 4.0×10^9 molecules and 1 ng Fab (~50 kD) 1.2×10^{10} molecules. The monocytes were studied after 1 d of culture.

positive cells were killed and the preparations remained monocyte depleted for >1 wk in culture. In contrast, the cytolytic anti-class II reagent 9.3F10 did not kill all the monocytes judged to be 9.3F10 reactive by immunofluorescence (Table III). We conclude that 3C10 and 1D9 recognize an antigen that is mononuclear phagocyte specific and is present on most if not all fresh and cultured monocytes and alveolar macrophages.

Quantitative Binding Studies. Saturable binding of the antimonocyte and anti-class II monoclonal antibodies occurred at 0.3–1.0 μg/ml for $1–4 \times 10^5$ cells. Monocytes expressed $3.7–4.7 \times 10^4$ binding sites for 3C10, 3C10 Fab, and 1D9, and $1.3–2.3 \times 10^5$ binding sites for the anti-class II reagent, 9.3F10, and its Fab fragment (see Table IV). When monocytes were cultured for 6 d, the number of 3C10 binding sites rose to 1.8×10^5 per cell (not shown).

Quantitative binding studies (not shown) established that the molecule carrying the 3C10 antigen was sensitive to trypsin, pronase, and chymotrypsin but not to neuraminidase. The 9.3F10 Ia-like determinant was not protease sensitive.

Binding of ^{125}I-3C10 was blocked by cold 1D9 or cold 3C10 Fab (Table V). However, all the other antibodies in our panel (Table 1), which included several antibodies to HLA-class I and II antigens did not inhibit 3C10 binding (see Table V). We conclude that 3C10 and 1D9 bind to a similar, non-HLA determinant, and that binding is not Fc mediated.

Identification of Polypeptides Bearing the 3C10, 1D9, and 9.3F10 Antigens. 3C10 precipitated a major 55 kD polypeptide from monocytes that had been surface radiolabeled with Na-^{125}I and lactoperoxidase-glucose oxidase (Fig. 2, *left*). The same polypeptide was identified with 3C10 Fab. As anticipated, 9.3F10 reacted with a 33-29 kD doublet typical of the alpha and beta chains of HLA-class II products (Fig. 2).

By Western blotting, 3C10 (Fig. 2, *right*) and 1D9 (not shown) again reacted with a 55 kD polypeptide. In some blots, a minor 53 kD band reacted with 3C10. This might have represented an intracellular precursor for the 55 kD 3C10 surface polypeptide (Fig. 2, *right*).

Use of Antimonocyte Antibodies in the Purification of Dendritic Cells. Although blood dendritic cells adhere to plastic, they are far outnumbered by monocytes (~20-fold) and lymphocytes. We first noted that 3C10 (antimonocyte), BA-1 (anti-B cell), and Leu-1 (anti-T cell) efficiently killed populations enriched in monocytes, B cells, and T cells, respectively, but not dendritic cells (not shown). We therefore devised a procedure (see Materials and Methods) in which monocyte-depleted adherent cells were prepared, cultured overnight, and reexposed to complement and a battery of monoclonals: 3C10, BA-1, and Leu-1. 80% of the cells that survived cytolysis could be floated free from the dead cells on dense albumin columns. The recovery was 0.1–0.2% of the total starting mononuclear cells. By cytology (Fig. 3, *a–c*), ~70% of the final low density population were

TABLE V

Competition for ^{125}I-3C10 Binding by Unlabeled Antibodies

Unlabeled antibody	Mean cpm minus background	Percent binding compared to no unlabeled antibody
No unlabeled antibody	11,258	–
9.3F10 (anti-Ia)	10,715	95
1E8 (antigranulocyte monocyte)	10,737	95
T29/33 (antileukocyte)	11,312	100
1B4 (anti-C_{3bi} receptor)	15,510	138
1D9 (antimonocyte)	956	8
3C10 (antimonocyte)	135	1
3G8 Fab (anti-Fc γ receptor)	10,751	95
3C10 Fab (antimonocyte)	1,085	10

^{125}I-3C10 (1 μg/ml) was added to 4×10^5 monocytes that previously had been exposed for 60 min to either no other antibody, or to saturating levels (50 μg/ml for all except T29/33, which was 1:100 dilution of ascites) of several noniodinated monoclonal antibodies.

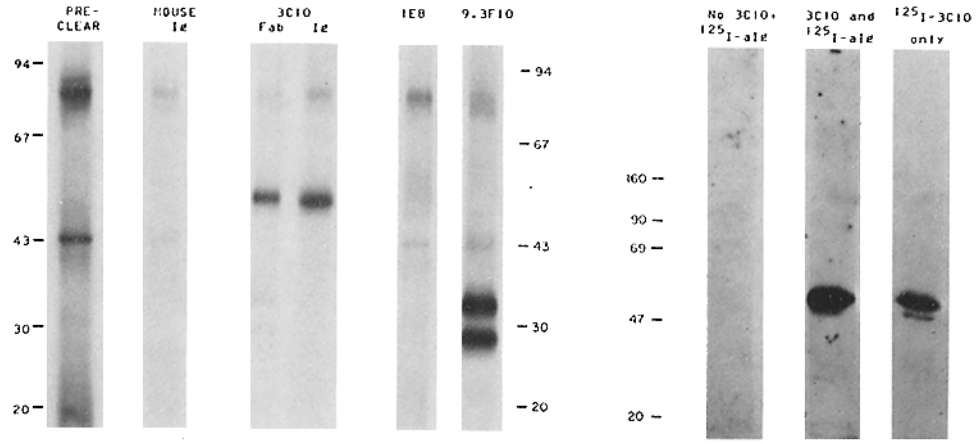

FIGURE 2. Immunoprecipitation (*left*) and immunoblotting (*right*) analysis of the polypeptides carrying the 3C10 and 9.3F10 antigens. In the immunoprecipitation experiment, a radioiodinated monocyte lysate was precleared on Sepharose coupled to mouse Ig and then divided into aliquots for precipitation with Sepharose coupled to 3C10 (antimonocyte), 1E8 (antimonocyte, granulocyte), 9.3F10 (anti-class II), or mouse Ig. The blotting experiment used directly labeled ^{125}I-3C10 Ig at 0.08 μg/ml, 7×10^6 cpm/μg; or an indirect approach (3C10 at 1 μg/ml followed by ^{125}I-F(ab')$_2$ rabbit anti-mouse Ig at 0.05 μg/ml, 6×10^6 cpm/μg).

dendritic cells, and total yield was comparable to controls that had been exposed to complement only (Table VI). The dendritic cell fraction contained <1–4% monocytes, identified by cytology or nonspecific esterase staining. Likewise B and T cells were depleted to <2%, as monitored by immunofluorescence with anti-Ig and Leu-2a and -3a antibodies. Controls exposed to complement in the absence of antibodies were ~70% monocytes (Table VI, Fig. 3*d*). Therefore, dendritic cells in blood (~0.5% of total mononuclear cells) can be purified by a technique that uses their adherence property and the fact that they lack the differentiation markers of other cell types.

Distribution of Macrophages in Tissue Sections. Since 3C10 reacted with all monocytes and alveolar macrophages, but not with dendritic cells, this antibody was used to outline the distribution of macrophages in frozen sections of spleen, lymph node, and skin. In spleen, both 3C10 and 3C10 Fab stained large numbers of macrophages in the red pulp (Fig. 4). Lymphocytes in white pulp, fibroblasts in connective tissue trabeculae, and endothelial and smooth muscle cells of blood vessel walls were not stained. White pulp nodules, which probably contained most of the dendritic cells (34, 35), had few 3C10$^+$ cells in either T (Figs. 4, 5) or B (not shown) regions. Some 3C10$^+$ cells, presumably macrophages, abutted the central white pulp arteries (Fig. 5). Leu-1 stained collections of T lymphocytes close to the central arteries (Figs. 4, 5) as well as scattered cells in both red pulp (Fig. 4) and B cell follicles (not shown). The anti-Ia antibody, 9.3F10, stained both red and white pulp intensely. Ia$^+$ cells in T-dependent areas probably corresponded to dendritic or interdigitating cells (see Discussion) and far outnumbered 3C10$^+$ macrophages (Fig. 5).

In node (Fig. 6), 3C10$^+$ cells were abundant along all of the lymphatic channels. This included the subcapsular sinus into which afferent lymphatics empty; the

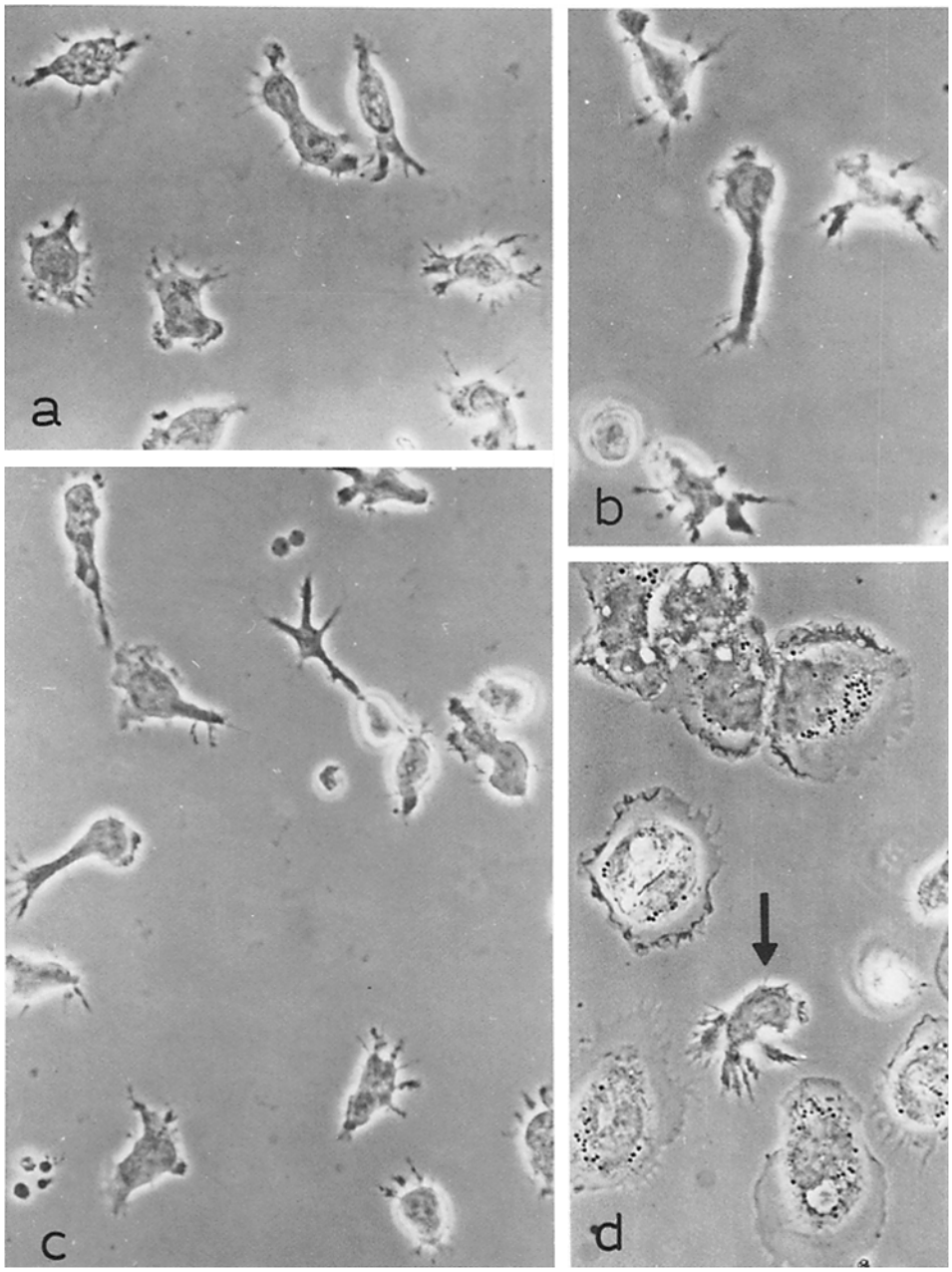

FIGURE 3. Phase-contrast microscopy of low density adherent cells from blood. (*a–c*) Purified dendritic cell fraction, prepared by cytolytic elimination of monocytes and lymphocytes as described in Materials and Methods. Most of the cells exhibit the typical dendritic appearance described previously (14). (*d*) Control low density population exposed to complement only; most of the cells have the characteristic features of monocytes, while ~10% are dendritic cells (arrow). (*a*) × 750, (*b*) × 1100, (*c*) × 750, (*d*) × 700.

TABLE VI

Preparation of Human Dendritic Cells by Eliminating Other Cell Types with Monoclonal Antibodies and Complement

Treatment	Starting number of mononuclear cells	Number of low-density adherent cells	Differential count of low-density fractions		
			DC	Monocytes	Lymphs
				%	
Experiment 1					
Complement only	5×10^7	1.4×10^6	8	80	12
Complement + 3C10	2×10^8	5×10^5	43	1	57
Experiment 2					
Complement only	1.5×10^8	7×10^6	7	62	31
Complement + antibodies (3C10 + BA-1 + Leu-1)	3.5×10^8	6×10^5	76	1	23
Experiment 3					
Complement only	1×10^8	4.6×10^6	12	71	17
Complement + antibodies (3C10 + BA-1 + Leu-1)	5×10^8	5×10^5	80	1	20

Mononuclear cells were obtained from Ficoll-Hypaque columns and processed, as described in Materials and Methods, to deplete the adherent fraction of monocytes. Then the adherent cells that came off the plastic surface after overnight culture were treated again with complement with or without monoclonal antibodies as indicated. The cells resistant to cytolysis were recovered on albumin columns (low-density adherent cells) and analyzed by phase-contrast microscopy. Round cells were classified as "lymphocytes" since they did not look like dendritic cells (DC) or monocytes; however the BA-1- and Leu-1-treated populations lacked typical B and T cells by surface markers (see Results).

intermediate sinuses that penetrate the cortex along connective tissue trabeculae; and the medullary cords that contain the efferent lymphatics. 3C10 reactivity was also noted in the center of secondary follicles (germinal centers; not shown). However, $3C10^+$ cells were scarce in thymus-dependent regions (identified by abundant Leu-1-positive T cells, and the presence of postcapillary venules) and in follicular collections of small B cells (Fig. 6).

In normal skin (not shown), a few $3C10^+$ cells were scattered in the dermis. In epidermis, Langerhans' cells were stained with OKT 6 (25), T29/33 (antileukocyte) (21), and 9.3F10 (anti-Ia) reagents, but not with 3C10. Skin biopsies were also studied from patients with lepromatous leprosy (Fig. 7), since these contain macrophage-rich dermal infiltrates and in many cases, abundant epidermal Langerhans' cells (33). The infiltrates contained many $3C10^+$ cells, but again epidermal Langerhans' cells failed to stain with 3C10 (Fig. 7).

We conclude that 3C10 identifies mononuclear phagocytes that are resident in dermis, lymph node, and spleen, and that migrate into inflammatory foci. Few macrophages are noted in those regions considered to be rich in dendritic cells; that is, the two cell types are segregated anatomically.

Discussion

We have described an antigen that is widely distributed on human mononuclear phagocytes. The antigen is detected by the monoclonal antibodies, 3C10 and 1D9, and is present on most if not all blood monocytes, alveolar macrophages, and macrophages in sections of skin, lymph node, and spleen. 3C10 and 1D9

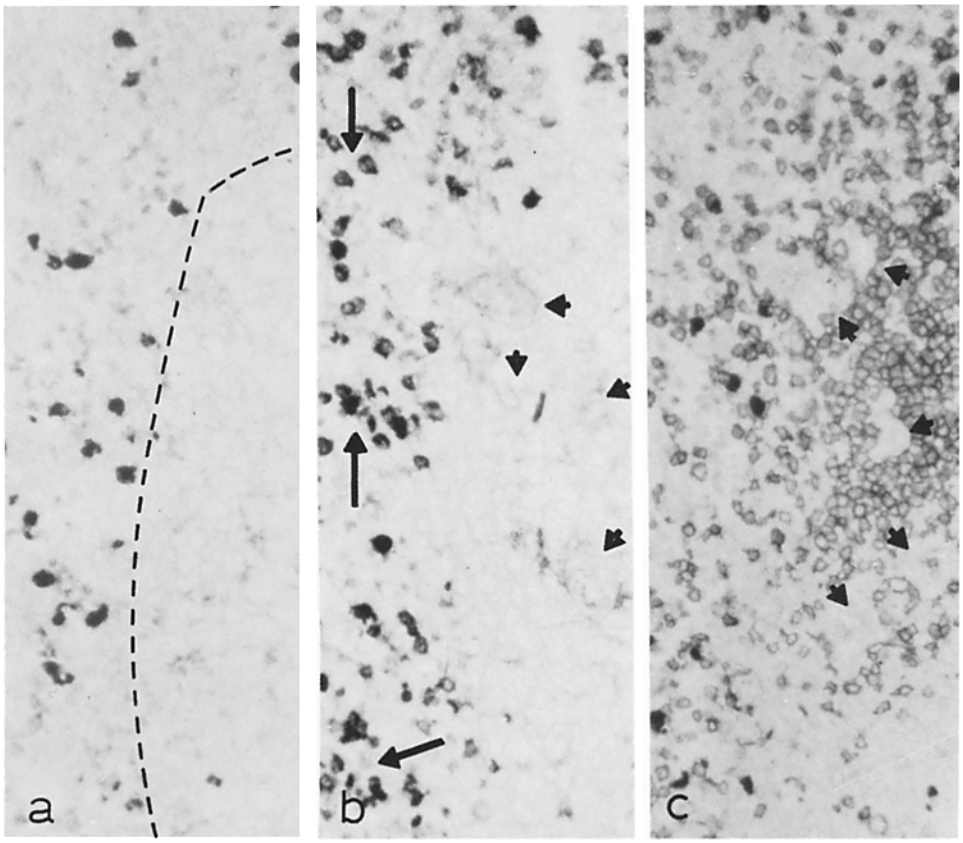

FIGURE 4. Immunocytochemistry of the junction (dotted line) of red and white pulp in spleen. Adjacent sections were stained with no primary antibody (*a*); 3C10 antimacrophage (*b*); and Leu-1 anti-T cell (*c*). The red pulp contains some opaque profiles (*a*) which represent background staining occurring in the absence of primary antibody. The red pulp has many $3C10^+$ macrophages (*b*, arrows) and scattered T cells (*c*). The white pulp nodule, with central arteries (arrowheads), contains an area rich in Leu-1^+ cells (T area) that lacks $3C10^+$ cells. × 200.

probably recognize a related if not identical antigen. Specifically, the reagents compete for binding to monocytes and immunoprecipitate a similar 55 kD surface molecule. In both binding and immunoprecipitation assays, 3C10 Fab behaves identically to intact Ig (Tables IV, V, Fig. 2). This is important since monocytes exhibit Fc receptors, which have a molecular weight of ~55,000 (16, 20). The 3C10-1D9 antigen is clearly distinct from HLA-class II or Ia-like determinants which in this paper were identified primarily with a new monoclonal, 9.3F10 (Tables II–IV, Fig. 2). Of the many antimonocyte antibodies that have been produced, 3C10 and 1D9 most closely resemble the anti-Mo2 reagents described by Todd et al. (7, 10).

Use of Antimonocyte Antibodies in the Purification of Dendritic Cells. The 3C10 and ID9 antibodies do not stain (Table II) or kill dendritic cells enriched as described previously (14). In fact elimination of monocytes with 3C10 antibody and rabbit complement can be used to enrich dendritic cells in considerable

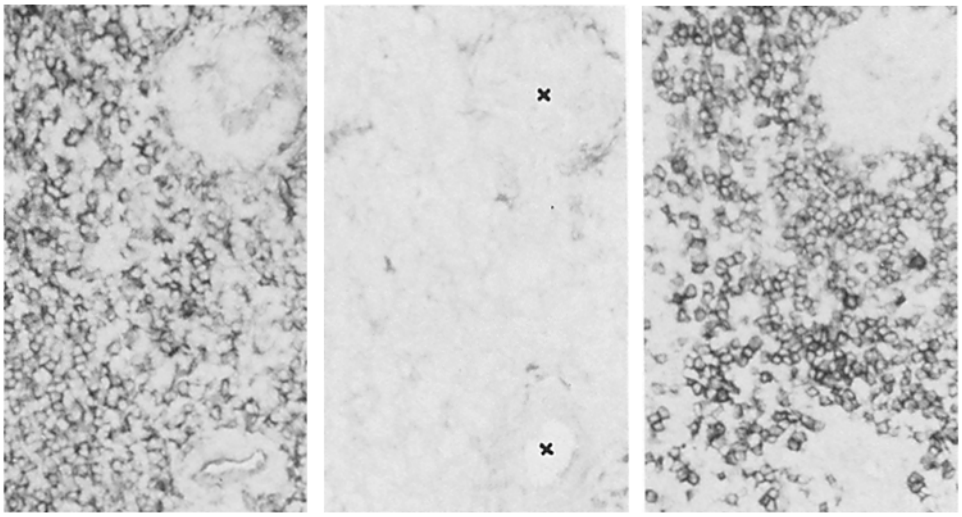

FIGURE 5. Immunocytochemistry of the periarterial region of splenic white pulp. Adjacent sections were stained with 9.3F10 anti-Ia (*left*), 3C10 antimacrophage (*middle*), and Leu-1 anti-T cell (*right*). The sections illustrate that the T area has few macrophages but many Ia^+ cells. The latter probably include interdigitating or dendritic cells (see Discussion). × 200.

purity and yield (Table VI, Fig. 3). The enriched fractions that can be depleted of B and T cells, with BA-1 and Leu-1 antibodies, contain 65–80% dendritic cells. The identity of the remaining 20–35% is unclear but could include dendritic cells that do not spread well at the time of assay. In the accompanying paper (36), we also show that heterogeneous cell mixtures can be separated into $1D9^+$ and $1D9^-$ fractions; the former are highly enriched in monocytes and the latter contain the dendritic cells.

The availability of the 3C10-1D9 antibodies is particularly timely, since the separation of dendritic cells from mononuclear phagocytes in man (14) is more demanding than in mouse (12). In both species, dendritic cells adhere to plastic and account for <1% of total nucleated cells. However, human blood contains large numbers of adherent monocytes (15–30%); mouse lymphoid tissues release <1–2% adherent macrophages. Both monocytes and dendritic cells in man have a low buoyant density; in mouse, most (~2/3) dendritic cells float on dense albumin columns, whereas most macrophages typically pellet. These experimental difficulties can be overcome by using 3C10 and 1D9 to identify and separate the two cell types (Tables II, VI, Fig. 3). The experiments described here represent the first time that antimacrophage antibodies have been used in the purification of dendritic cells.

Distribution of Macrophages and Dendritic Cells in Tissues. Both 3C10 and its Fab fragment are effective for immunocytochemical staining of tissue sections. Endothelial cells, fibroblasts, lymphocytes, and dendritic cells do not stain with 3C10, but many $3C10^+$ cells are found in regions that are known to be rich in macrophages: the red pulp of spleen; the areas surrounding lymphatic vessels in lymph node capsule, cortex, and medulla; and the dermal inflammatory infiltrates of leprosy (Figs. 4–7). Since we have not used two macrophage labels simulta-

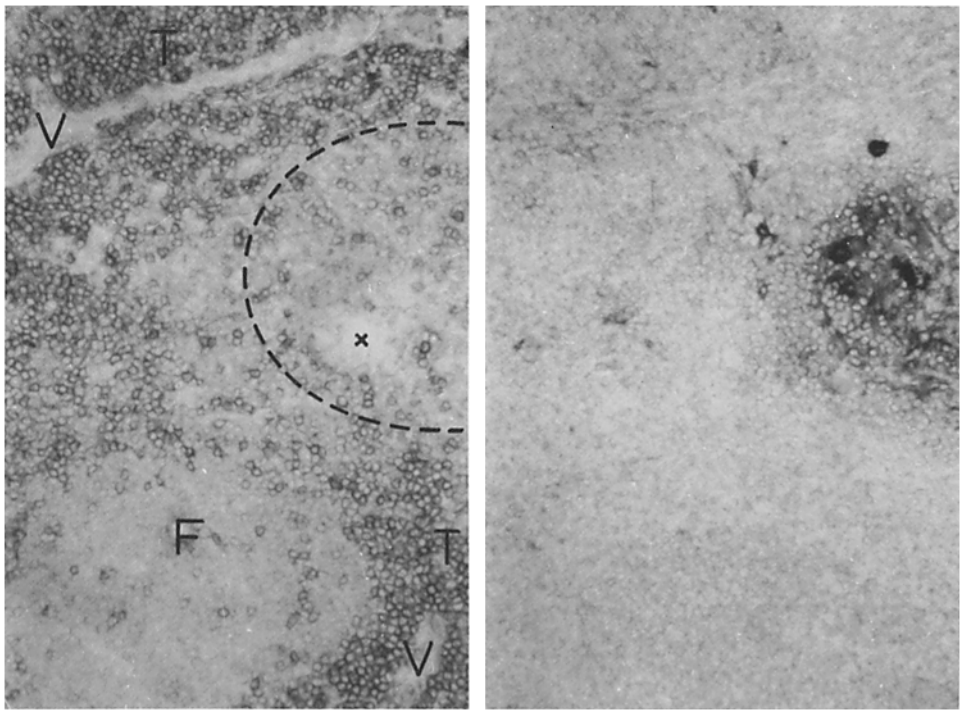

FIGURE 6. Immunocytochemistry of lymph node cortex. Adjacent sections were stained with Leu-1, anti-T cell (*left*) and 3C10, antimacrophage (*right*). Lymph node cortex contains two kinds of lymphatic tissue: one is rich in Leu-1$^+$ T cells (*T*) and contains many postcapillary venules (*V*); the other contains follicles of Leu-1$^-$ B cells (*F*). Penetrating the cortex are connective tissue trabeculae (×) that course from subcapsular cortex to the hilum. The trabeculae are lined with lymphatic channels (not evident in frozen sections) and sheets of 3C10$^+$ macrophages (region with dotted line). × 160.

neously, we cannot be certain that all tissue macrophages stain with 3C10. However, the staining is prominent, and the number of reactive cells is abundant. It seems likely that 3C10 acts as a panmacrophage reagent in the tissues we have studied.

Strikingly, 3C10$^+$ cells are not found in several compartments that are thought to be rich in dendritic cells: white pulp of spleen, cortical lymphatic tissue of node, and epidermis (Figs. 4–7). Staining with 3C10 therefore reveals that most macrophages and dendritic cells are segregated from one another, a distinction that has never been so dramatically evident. In mouse spleen, dendritic cells can be isolated from white but not red pulp (34), and can be visualized by electron microscopy in white pulp (35). The white pulp dendritic cells resemble the "interdigitating cells" (37, 38) first described by Veldman. Interdigitating cells are concentrated in thymus-dependent regions of lymphoid organs in all mammals. Several studies have likened interdigitating cells in tissue sections to dendritic cells isolated in vitro (39, reviewed in 40). In epidermis, Langerhans' cells are stellate, bone marrow-derived, and Ia$^+$ (reviewed in 41). Human Langerhans' cells, identified in this study by their reactivity with anti-Ia, anti-leukocyte, and OKT 6 antibodies, are 3C10$^-$; likewise mouse epidermal Langer-

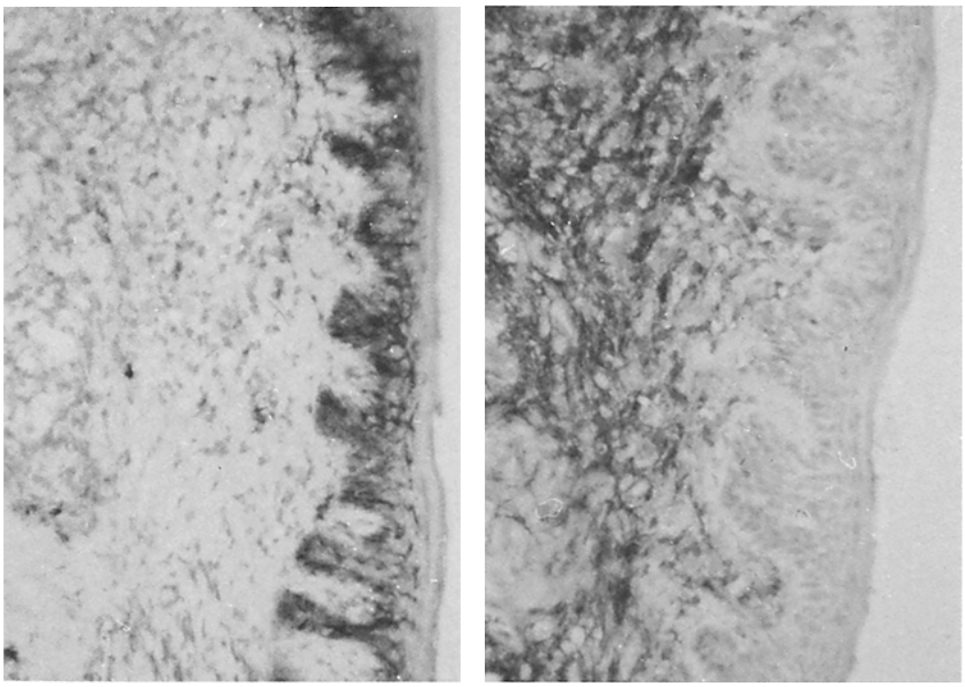

FIGURE 7. Immunocytochemistry of a skin biopsy from a patient with lepromatous leprosy. Adjacent sections were stained with OKT6, anti-Langerhans' cell (*left*) and 3C10, antimacrophage (*right*). A network of Langerhans' cell processes is evident in the epidermis, but this network lacks the 3C10 determinant. In contrast, the dermal infiltrates of leprosy represent a dense network of $3C10^+$ $OKT6^-$ macrophages, and (not shown) OKT 8^+4^- lymphocytes (33). Although Langerhans' cells can increase in number in leprosy epidermis (33), their surface markers ($OKT6^+$, $3C10^-$, Ia^+) are comparable to normal skin. × 160.

hans' cells do not react with the F4/80, mac-1, and 2.4G2 monoclonals (Witmer and Steinman, unpublished results), each of which binds to macrophages but not to dendritic cells (2). Therefore Langerhans' cells, which frequently are likened to macrophages, may be part of the dendritic cell system.

The segregation of macrophages and dendritic cells in spleen and lymph node seems appropriate for the two main functions performed by lymphoid organs—clearance of antigen, and development of immune responses. The macrophage is the "versatile element of inflammation" (42). It is endowed with endocytic and microbicidal capacities, and these functions are enhanced by the Ig and lymphokine products of stimulated lymphocytes. Macrophages are concentrated in those regions where antigen first leaves vascular and lymphatic channels, namely the red pulp of spleen and the lymphatic sinuses of node. Therefore, macrophages are positioned to scavenge and clear antigen, and likely constitute a major first line of host defense. In contrast, dendritic cells are "nature's adjuvant," the principal accessory cell required for the stimulation of many lymphocyte responses (13). Dendritic cells likely are poised near recirculating lymphocytes, and are accessible to antigen that escapes macrophage clearance. Although additional immunocytochemical studies of antigen-stimulated tissues are needed, during immune responses lymphoblasts first develop in regions that are macrophage

poor and dendritic cell rich (see 43–46). These anatomical findings are an important adjunct to in vitro studies that compare the accessory function of human macrophages and dendritic cells. The latter are the subject of the accompanying paper (36).

Summary

3C10 and 1D9 are two related monoclonal antibodies that specifically identify human mononuclear phagocytes in a large number of sites, including blood monocytes, alveolar macrophages, and macrophages in tissue sections of spleen, lymph node, and skin. The antigen persists on monocytes cultured for >4 wk, but it is not found on giant cells. The 3C10-1D9 determinant is carried by a 55 kD polypeptide, is expressed at ~40,000 copies per monocyte, and is protease sensitive. The antigen is clearly different from HLA-class II or Ia-like antigens that have been studied with a new monoclonal 9.3F10. The 9.3F10 antigen is found on B cells, dendritic cells and monocytes; is protease resistant, and occurs on a 33-29 kD doublet typical of class II products.

The 3C10 monoclonal provides a clear distinction between human mononuclear phagocytes and dendritic cells. First, monocytes and lymphocytes can be eliminated from plastic-adherent mononuclear cells using 3C10, complement, and two previously described cytotoxic antibodies, BA-1 (anti-B cell) and Leu-1 (anti-T cell). As a result, the trace dendritic cell component of blood can be enriched to considerable purity (65–75%) and yield. Second, immunocytochemical staining of tissue sections reveals that $3C10^+$ macrophages are anatomically segregated from dendritic cells. Large numbers of $3C10^+$ cells are found in red pulp of spleen and in regions surrounding lymphatic channels of lymph node. However, $3C10^+$ macrophages are scarce in white pulp of spleen and the lymphocyte-rich cortex of node that are the sites where dendritic cells are localized. $3C10^+$ cells in skin are found in the dermis, particularly in leprosy infiltrates, but the Langerhans' cells of epidermis are $3C10^-$. The distinctive localization of macrophages and dendritic cells is consistent with their respective functions as effector and accessory cells in the immune response.

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