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Monoclonal Antibodies Reactive with Chicken Peritoneal Macrophages: Identification of Macrophage Heterogeneity (42382)

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Abstract. Mouse monoclonal antibodies (MCAs) were prepared against chicken inflammatory macrophages for the purpose of analyzing macrophage heterogeneity. Macrophage-rich adherent peritoneal exudate cells harvested from Cornell K-strain chickens 42 hr after stimulation with Sephadex were used as immunogens in the production of the monoclonal antibodies. Eight hybridoma clones producing antibodies reactive with chicken peritoneal macrophages were subjected to characterization. While six of the monoclonal antibodies cross-reacted with various hematopoietic cell types, two MCAs (CMTD-1 and -2) were restricted in reactivity to macrophages. CMTD-1 was found to react with activated peritoneal macrophages generated by several irritants. In contrast, CMTD-2 identified a subpopulation of macrophages elicited by specific carbohydrate-based stimulants. This presumably was based on the cross-reaction of this MCA with specific carbohydrate linkages. Analysis using flow cytometry revealed the time-dependent appearance of CMTD-2-positive peritoneal macrophages between 24 and 52 hr after ip Sephadex injection. This subpopulation of peritoneal macrophages was found to be heterogeneous for the ability to undergo *in vitro* phagocytosis of sheep erythrocytes. CMTD-2-positive cells were also detected in the thyroids of 2-week-old Obese strain chickens with spontaneous autoimmune thyroiditis and at a low incidence in the spleens of normal chickens.

Characterization of mammalian macrophage differentiation and activation has been greatly facilitated by the application of hybridoma technology. Murine macrophages have been investigated using monoclonal antibodies against the Fc receptor (1), Ia antigens (2), Mac-1 (3), Mac-2 (4), and several activation-dependent markers (5–8). Similarly, human monocytes and macrophages have been characterized with the use of monoclonal antibodies (9–11). These antibodies have been particularly useful in delineating surface phenotypic changes associated with either the differentiation of the monocyte–macrophage series or the conversion of unstimulated macrophages into inflammatory, primed, or tumoricidal activated macrophages.

In contrast with recent mammalian macrophage research, the avian macrophage has received little attention. Glick *et al.* (12) demonstrated that unlike the rodent models, chickens contain few, if any, resident peritoneal macrophages. As a result, most avian RES investigations have been performed on blood monocytes. However, the ability to elicit chicken peritoneal macrophages by introduction of a stimulant (13, 14) provides a ready source of macrophages for subsequent analysis (15, 16).

The purpose of this study was to prepare and characterize mouse monoclonal antibodies generated against inflammatory peritoneal macrophages of the chicken. Analysis of the monoclonal antibody reactivity patterns included age and genetic factors and the differentiation and activation status of the monocyte–macrophage lineage. The results are discussed in light of previous studies on chicken macrophage function.

Materials and Methods. *Biological materials.* Avian leukocytes were examined from 2-week- to 18-month-old female White Leghorn chickens of the following lines: Cornell K-strain ($B^{15}B^{15}$) (17, 18), Special C ($B^{13}B^{13}$) (19), Hyline SC ($B^{2}B^{2}$) (Johnson, Iowa), and Obese ($B^{13}B^{13}$) (20). Six-month-old female Balb/c mice (Jackson Laboratories) were used as recipients for immunizations. The PAI myeloma cell line was developed by Dr. T. Stahelin (Basel) and provided by Dr. T. Stahelin and Dr. E. Racker (Cornell). Hybridoma production using this line has been previously de-

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scribed (21). The LSCC-CU10 avian lymphoblastoid tumor cell line was used for analysis of the monoclonal antibodies and has been previously described (22).

Leukocyte isolation. Bursal, thymic, Harderian gland, splenic, and blood leukocytes were obtained from chickens that had not received exposure to a stimulant. The organs were removed and mechanically minced in cold RPMI 1640 growth media (GIBCO) with antibiotics. After filtration of the suspension through a nylon mesh, lymphocytes were isolated on Ficoll–Hypaque (Pharmacia) gradients and rinsed in cold RPMI 1640. In certain isolations, splenic lymphocytes were further separated on a nylon wool column as previously described (23). Peripheral blood leukocytes were obtained after Ficoll separation of whole blood collected by venipuncture in sodium EDTA. Peripheral blood monocytes were isolated from this preparation by plating the blood leukocytes in RPMI 1640 growth media into microtest plates (50 μl/well of 10^7 cells/ml), incubating at 37°C, 5% CO_2 for 30 min, and removing nonadherent cells by rinsing with sterile 0.75% saline (37°C). Adherent peritoneal exudate cells (PECs) were obtained as previously described (16). Briefly, the peritoneal cavity was flushed with cold sterile Heparin–saline using macrophage harvesters (13) and the exudate placed into siliconized tubes. Following collection of the cells by centrifugation and washing in cold RPMI 1640 media, adherent cells were collected onto glass coverslips or plastic microtest plates by incubating at 37°C, 5% CO_2, for 1 hr followed by a rinse with 0.75% sterile saline.

Bone marrow cell isolation. The femur and humerus from each of four 5-week-old female K-strain chickens were removed. After removal of the epiphyses, the shafts were flushed with RPMI 1640 with antibiotics and the cells collected into siliconized tubes. Cells were rinsed in cold PBS and collected on glass slides following centrifugation using a cytospin (Shandon). Air-dryed preparations were methanol fixed and subjected to either May–Grünwald–Giemsa staining or indirect immunofluorescence as described later. Duplicate slides from each chicken were analyzed.

Hybridoma production. Pooled PECs were obtained from seven 5-week-old female K-strain chickens 42 hr after stimulation by injection with 3% Sephadex G-40 superfine (20–50 μ diameter) (Sigma) in 0.75% saline. The dosage of Sephadex was 1 ml/100 g body weight (14, 16). After collection of adherent cells onto plastic petri dishes, the cells were removed with a rubber scraper and placed in 0.15 *M* phosphate-buffered saline (PBS; pH 7.2). Five Balb/c mice were given three biweekly ip injections of 0.2 ml of 1.3×10^7 adherent PECs in PBS. A fourth injection was administered iv 3 days prior to fusion. Fusion of the splenic leukocytes with the PAI myeloma line and subsequent HAT selection of hybrid cells was performed as previously described (21). Initial screening was performed approximately 10–14 days post fusion using the avidin–biotin immunoperoxidase (ABC) technique (24) (Vector Laboratories, Burlingame, Calif.). Both Sephadex-stimulated adherent PECs and bursal lymphocytes were used in initial screenings. The latter cells were attached to the microtest wells by first incubating the wells with 0.1% poly-L-lysine in 0.15 *M* PBS (Sigma) for 90 min at 37°C followed by a PBS rinse. Fifty microliters per milliliter of 2×10^6 lymphocytes in PBS was then plated into each well and incubated for 1 hr at 4°C. For hybridoma wells containing supernatants positive for peritoneal macrophages, the cells were subjected to two single cell clonings by limited dilution. ABC immunoperoxidase screening was performed at each step. Clones producing macrophage-positive supernatants were subsequently subjected to further analysis. Classification of monoclonal antibodies by isotype was performed using rabbit anti-mouse Ig reagents specific for μ, α, γ_1, γ_{2a}, γ_{2b}, γ_3 heavy chains and for κ and λ light chains (Bethyl Laboratories, Montgomery, Tex.). Immunodiffusion was employed for isotype characterization as previously described (21).

Sources of adherent peritoneal macrophages. In addition to the adherent PECs collected 42 hr after Sephadex stimulation, macrophages were also harvested 6, 24, and 52 hr after Sephadex injection. Unstimulated chickens also served as a source of macrophages although the harvestible resident peritoneal macrophage population was extremely low, as expected (12). To determine the effect of the stimulant, peritoneal macrophages were harvested 42 hr after injections of 3% starch

(amylopectin) (A. E. Staley, Decatur, Ill.) in 0.75% sterile saline, 3% sucrose in saline, 3% dextran (Pharmacia) in saline, 5 ml/chicken of 20% *Enterobacter cloacae* supernatant in RPMI 1640 media, and 5 ml/chicken of 20% nutrient broth (Difco) in RPMI 1640 media. Starch-elicited macrophages were also analyzed both 6 hr after a single injection and 42 hr after the last of three daily injections of a 3% solution in 0.75% saline. Peritoneal macrophages were also obtained 5 days after injection of killed *Corynebacterium parvum* bacteria (Burroughs–Wellcome, Inc.) and after ferritin stimulation consisting of a sensitizing s.c. innoculum of 0.2 mg ferritin in Freund's complete adjuvant followed 1 week later by an ip injection of 3.2 mg of ferritin/ml/100 g body weight. Peritoneal macrophages were harvested 42 hr later.

Cultured macrophages. Following 42 hr of Sephadex stimulation, adherent PECs were collected from 5-week-old K-strain chickens and plated onto coverslips at a concentration of 10^6 cells/ml in RPMI 1640 media with 10% fetal calf serum and 1% antibiotics. After attachment for 1 hr at 37°C, 5% CO_2, the coverslips were washed with sterile 0.75% saline and fresh media applied. Medium was replaced every 48 hr during a 1-week incubation of 37°C, 5% CO_2. Fresh 42-hr Sephadex-stimulated macrophages were collected by adherence for comparison with the culture cells.

Thyroid sections. Frozen sections of Obese strain thyroids from 2- and 6-week-old chickens were generously provided by Dr. W. Gause and Dr. J. Marsh, Cornell University. The Obese strain chicken develops a spontaneous autoimmune thyroiditis (SAT) that is characterized by extensive leukocyte infiltration of the thyroid gland (25). Thyroid sections from six individual 2-week-old chickens and four 6-week-old chickens were examined using MCAs 1C9 and CMTD-2, and the PAI control.

Monoclonal antibody reactivity. Reactivity of the hybridoma supernatants with macrophage and lymphocyte isolates was determined using both the ABC immunoperoxidase (24) and indirect immunofluorescence techniques. For the immunoperoxidase assay, cells were attached to the plates either by adherence for macrophages and blood monocytes or by the attachment of nonadherent lymphocytes to poly-L-lysine (Sigma)-treated plates. A 1.5-hr incubation at 37°C of 0.1 poly-L-lysine in 0.15 *M* PBS was performed prior to rinsing the plates in PBS. Lymphocytes were incubated with the treated plates for 1 hr at 4°C. After cell attachment, plates were immersed in methanol with 0.3% H_2O_2 (Sigma) for 30 min. After a 30-min wash in 0.5 *M* PBS, diluted normal serum (horse serum for IgG; goat serum for IgM) was added to the plates and incubated for 20 min. Excess serum was removed from the plates, hybridoma supernatant was added to each well, and the plates were incubated for 30 min at room temperature. Both PAI supernatant and PBS were used as controls. Following a 15-min wash in PBS, biotinylated horse anti-mouse IgG or biotinylated goat anti-mouse IgM (Vector Laboratories) was added to each well and incubated 30 min at room temperature. This was followed by a 15-min PBS wash. Finally, the Vectastain ABC reagent was added to each well and incubated for 45 min, followed by a 10-min wash in PBS. To develop the colorimetric reaction, peroxidase substrate solution consisting of equal volumes of 0.02% H_2O_2 in distilled water and 0.1% DAB in 0.1 *M* Tris buffer, pH 7.2, with 10 m*M* imidazole (Sigma) was added to each well. Five minutes later the plates were rinsed with tap water and scored with an Olympus inverted microscope to determine cellular staining patterns.

For indirect immunofluorescence, the PEC cell suspension was prepared as previously described, with 45 ml of suspension being prepared at a concentration of approximately 10^6 cells/ml. Five milliliters of cell suspension was plated onto each of nine plastic petri dishes (100 mm) and incubated for 20 min at 37°C, 5% CO_2. The plates were decanted and gently washed with sterile saline solution. Approximately 1.5 ml of ice-cold PBS with 0.1% EDTA (ethylenediaminetetraacetate) was added to each plate and the cells were removed using a rubber scraper. These cells were transferred to siliconized glass tubes and washed twice in ice-cold PBS. Aliquots (1 ml) containing 10^6 cells/ml were added to individual siliconized glass tubes.

At this time, all tubes containing aliquots of lymphoid cells, adherent cells, or swollen Sephadex G-40 particles were centrifuged and gently resuspended in 1 ml of methanol and

aspirated to disrupt clumps. Fixation was continued for 20 min. Cells were washed three times in PBS and the pellets resuspended in 1 ml of PBS. To this, 0.5 ml of hybridoma supernatant or control supernatant was added. Incubation was performed at 4°C for 30 min. Cells were washed two times with cold PBS and pelleted. To the pellets was added 200 μl of 1/16 dilution of affinity-purified FITC-conjugated goat anti-mouse IgG and IgM (H + L) (Jackson Laboratories, Lot 2907). Suspensions were incubated at 4°C for 30 min in the dark and rinsed twice with cold PBS. Pellets were resuspended in 1 ml PBS and 20 μl was mixed with an equal volume of 9:1 glycerol:PBS buffer (pH 8.0) for analysis on an Olympus Vanox fluorescence microscope.

For analysis of Obese strain thyroid sections, the frozen sections were fixed on slides with methanol for 20 min, washed with PBS, reacted with normal goat serum (GIBCO), and then reacted with the hybridoma supernatants or the PAI control supernatant for 30 min at room temperature (RT) prior to washing with cold PBS. The sections were then reacted with a 1/8 dilution of the FITC-conjugated secondary antibody in PBS at RT followed by washing with cold PBS. Fluorescent staining was viewed as previously described.

Flow cytometry analysis was also performed on methanol-fixed preparations using a Becton–Dickinson II fluorescence-activated cell sorter. Relative cell number versus fluorescent intensity was determined.

To determine the functional activity of CMTD-2-positive macrophages, macrophage-rich monolayers of 42-hr Sephadex-stimulated adherent PECs were collected on glass coverslips and fed sheep erythrocytes (1% in RPMI 1640 media) for 45 min at 37°C. Free erythrocytes were removed by rinsing and the monolayers fixed in methanol. The coverslip preparations were then treated for immunofluorescence as previously described. The results of two separate experiments were obtained.

Results. Six hundred wells with growing colonies 1–1.5 weeks post fusion were initially screened and 55 wells produced supernatants reactive with Sephadex-stimulated peritoneal macrophages from K-strain chickens. Both the IgM and IgG kits for ABC immunoperoxidase gave identical results due to light chain cross-reactivity. Seven clones producing supernatants with different patterns of leukocyte reactivity were selected for subsequent analysis (Table I). Clone 8E2 antibody reacted with all leukocytes examined but did not cross-react with erythrocytes. In contrast, monoclonal antibody (MCA) 4D3 reacted with all sources of leukocytes and was strongly cross-reactive with erythrocytes from 1-week-, 5-week-, and 18-month-old K-strain chickens. MCAs 1C9 and 1B5 reacted with subpopulations of leukocytes from most sources while MCA 9F8 was unreactive with bursal and Harderian gland lymphocytes; these MCAs reacted predominantly with nylon wool adherent leukocytes from the spleen (data not shown). Two clones produced supernatants that were restricted in reactivity to tissue macrophages. MCA CMTD-1 lightly stained all Sephadex-stimulated peritoneal macrophages, reacted with a small population of splenic leukocytes, but failed to react with blood monocytes, bone marrow cells, or lymphocytes. MCA CMTD-2 exhibited a similar reactivity pattern except that approximately 10% of the stimulated peritoneal macrophages were reactive. This subpopulation was intensely stained. Figure 1 illustrates the immunoperoxidase staining of selected MCAs with 42-hr Sephadex-elicited peritoneal macrophages.

Table II illustrates the influence of the stimulant and the kinetics of macrophage recruitment in eliciting peritoneal macrophages reactive with MCAs CMTD-1 and CMTD-2. The former monoclonal antibody was relatively unreactive with unstimulated macrophages and did not react with either 6-hr Sephadex-elicited macrophages or those stimulated with starch or nutrient broth. However, the majority of 42-hr Sephadex-stimulated macrophages and a subpopulation of cells recruited with *E. cloacae* culture supernatant were found to react with MCA CMTD-1. MCA CMTD-2 was even more restricted in reactivity, staining only subpopulations of cells collected 42 hr after injection with Sephadex, dextran, or sucrose.

MCA 9F8 was less reactive with macrophages from 6-hr Sephadex treatment and macrophages stimulated by *E. cloacae* and nutrient broth supernatants than with other macrophages obtained using stimulation protocols. MCAs 1B5 and 4D3 were found to

TABLE I. REACTIVITY OF HYBRIDOMA SUPERNATANTS WITH DIFFERENT CELL TYPES

		Reactivity[a]									
				Lymphocytes							
Hybridoma clone	Isotype	Peritoneal Mϕs[b]	Splenic leukocytes	Bursal	Thymic	Harderian	PBLs[c]	PBMs[d]	BMCs[e]	CU-10[f]	RBCs[g]
CMTD-1	IgM(κ)	+	+ (5)[h]	−	−	−	−	−	−	−	−
CMTD-2	IgM(κ)	+ (10)	+ (5)	−	−	−	−	−	−	−	−
9F8	IgM(κ)	+	+	−	+	−	+	−	NT[i]	+	−
1C9	IgM(λ)	+	+ (20)	−	± (10)	+	+	±	+	+	−
1B5	IgM(κ)	+	+ (90)	+	+	+	+	+	NT	+	−
4D3	IgM(κ)	+	+ (60)	+	+	±	+	+	NT	+	−
8E2	IgM(κ)	+	+	+	+	+	+	+	NT	+	+
PAI[j]	−	−	−	−	−	−	−	−	−	−	−

[a] Determined microscopically by the ABC immunoperoxidase technique using the anti-IgG reagent (Vector Laboratories) except for erythrocyte reactivity which was determined by hemagglutination and bone marrow cell reactivity which employed indirect immunofluorescence.
[b] Elicited by Sephadex stimulation with cells harvested 42 hr after injection.
[c] PBLs, peripheral blood lymphocytes.
[d] PBMs, peripheral blood monocytes.
[e] BMCs, bone marrow cells.
[f] CU-10 is a lymphoblastoid tumor cell line associated with avian leukosis virus.
[g] RBCs, erythrocytes.
[h] Parentheses enclose the percentage of positive cells where reactivity with subpopulations was evident.
[i] NT, not tested.
[j] PAI is the myeloma cell line.

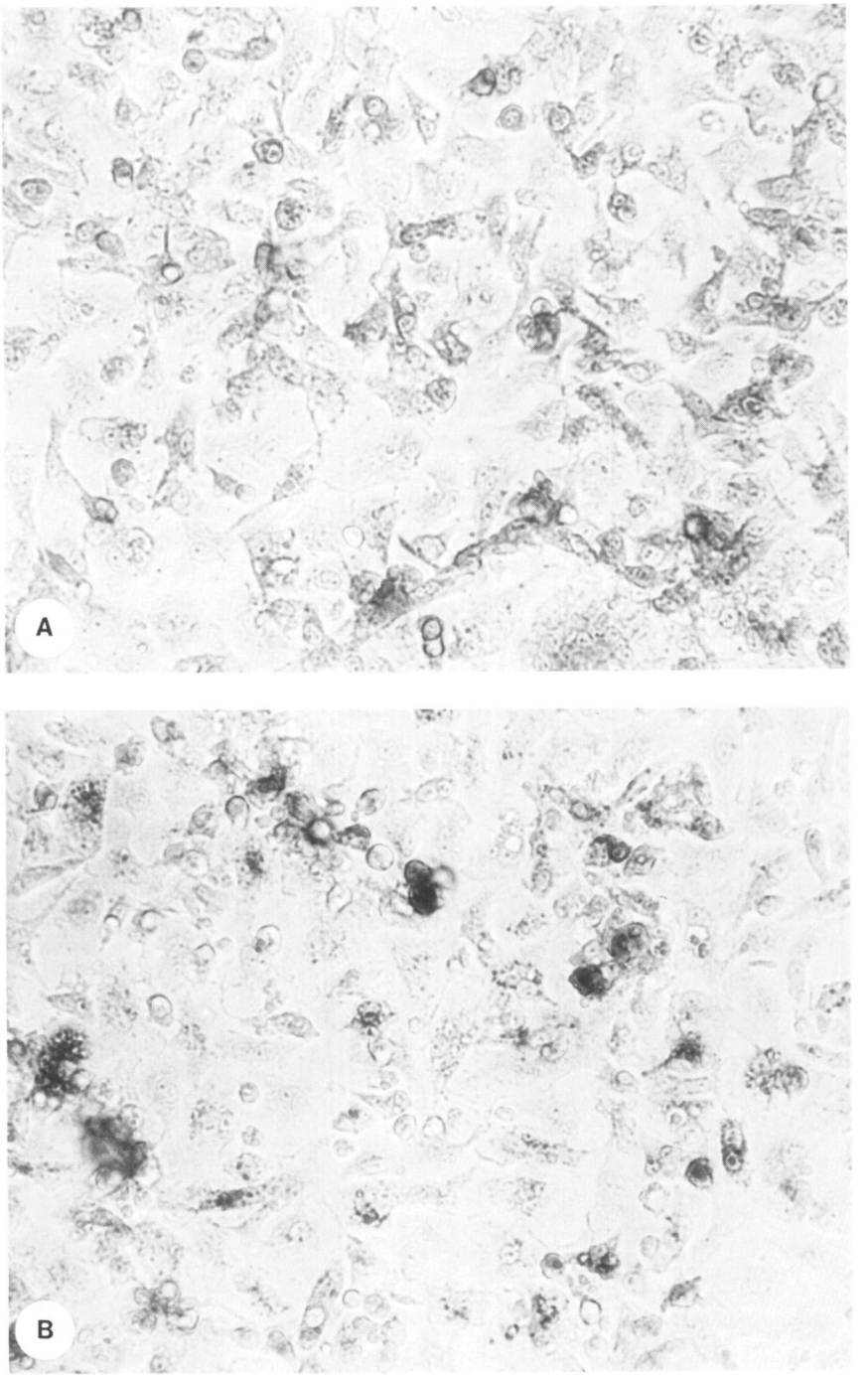

FIG. 1. Immunoperoxidase (ABC) staining of selected hybridoma supernatants and the PAI myeloma cell line supernatant with adherent peritoneal exudate cells collected 42 hr after Sephadex stimulation is shown. (A) CMTD-1, (B) CMTD-2, (C) 8E2, (D) PAI control. The magnification is 400×.

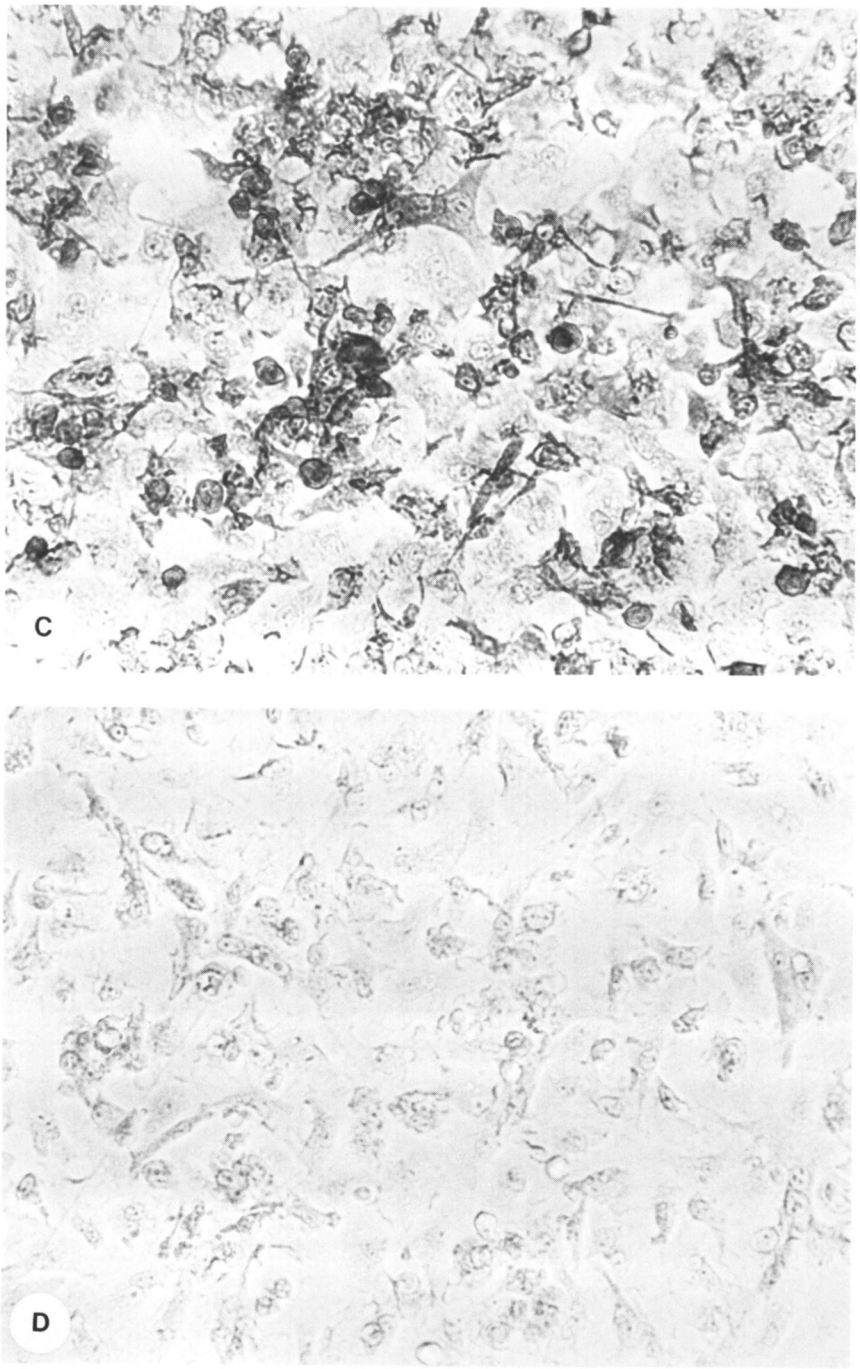

FIG. 1—*Continued.*

react with only subpopulations of macrophages following 6-hr Sephadex stimulation. Differences in stimulation protocol appeared to have little influence on the reactivity of MCAs 1C9 and 8E2 with adherent PECs (Table II).

TABLE II. EFFECT OF STIMULANT ON HYBRIDOMA REACTIVITY

Hybridoma clone	Adherent peritoneal exudate cell source[a]										
	Unstimulated[b]	Sephadex		Starch		*E. cloacae* supernatant, 42 hr[b]	Nutrient broth, 42 hr[b]	Ferritin 42 hr[c]	Sucrose 42 hr[c]	*C. parvum* 120 hr[c]	Dextran 42 hr[c]
		42 hr[b,c]	6 hr[b]	42 hr[c]	6 hr[b]						
CMTD-1	±	+	−	ND[d]	−	+ (25)[e]	−	ND	ND	ND	ND
CMTD-2	−	+	−	−	−	−	−	−	+	−	+
9F8	±	+	±	ND	+	±	±	ND	ND	ND	ND
1C9	+	+	+	+	+	+	+	+	+	+	+
1B5	+	+	+ (5)	+	+ (1)	+ (50)	−	ND	ND	ND	ND
4D3	+	+	+ (5)	+	+	+	+	ND	ND	ND	ND
8E2	+	+	+	+	+	+	+	ND	ND	ND	ND
PAI[f]	−	−	−	−	−	−	−	−	−	−	−

[a] Pooled 5-week-old female Cornell K-strain chickens were used in each assay.
[b] Results obtained by ABC immunoperoxidase staining.
[c] Results obtained by indirect immunofluorescence.
[d] ND, not done.
[e] Parentheses enclose the percentage of positive cells.
[f] PAI is the myeloma cell line.

Sephadex-stimulated (42-hr) macrophages from 2-week-, 5-week-, and 18-month-old (adult) K-strain chickens were compared for reactivity with the MCAs using the ABC immunoperoxidase procedure. No age-related differences in staining patterns were observed using MCAs 4D3, 8E2, and 1C9. MCA CMTD-1 appeared to decrease in staining intensity with adult chicken macrophages while MCA CMTD-2 reacted with a slightly higher subpopulation of peritoneal macrophages (30%) from the adult than were observed at 2 weeks (15%) or 5 weeks (10%). MCA 9F8 increased in macrophage-staining intensity with increasing age of the chicken and while MCA 1B5 provided a rather uniform macrophage staining in young chickens, adult peritoneal macrophages were heterogeneous for the intensity of staining.

Genetic variation in macrophage reactivity with the monoclonal antibodies was examined using 42-hr Sephadex-stimulated macrophages. All MCAs exhibited a similar reactivity with macrophages isolated from the Cornell K strain ($B^{15}B^{15}$) and Special C strain ($B^{13}B^{13}$). In addition, no intrastrain variation was observed using peritoneal macrophages isolated from four individual K-strain and Special C-strain females. Using Hyline SC (B^2B^2) strain macrophages, MCA 1B5 exhibited little reactivity and MCA 4D3 was apparently unreactive. All other MCAs produced the same staining patterns as were obtained with K-strain and Special C-strain macrophages.

The leukocyte staining patterns of several MCAs observed by the ABC immunoperoxidase technique were examined by indirect immunofluorescence. While MCAs CMTD-1 and 1C9 produced staining patterns consistent with cell surface epitopes, MCA CMTD-2 clearly detected an intracellular component. This was consistent with the inability of the latter antibody to stain unfixed macrophages.

The effect of prolonged *in vitro* culture on the retention of epitopes was examined (data not shown). Following a 1-week culture of 42-hr Sephadex-stimulated K-strain macrophages, a preponderance of giant cells was observed. While these giant cells possessed little phagocytic activity, the mononuclear cells remaining in the cultures were still phagocytic for opsonized sheep erythrocytes. MCA CMTD-1 stained the 1-week cultures well if

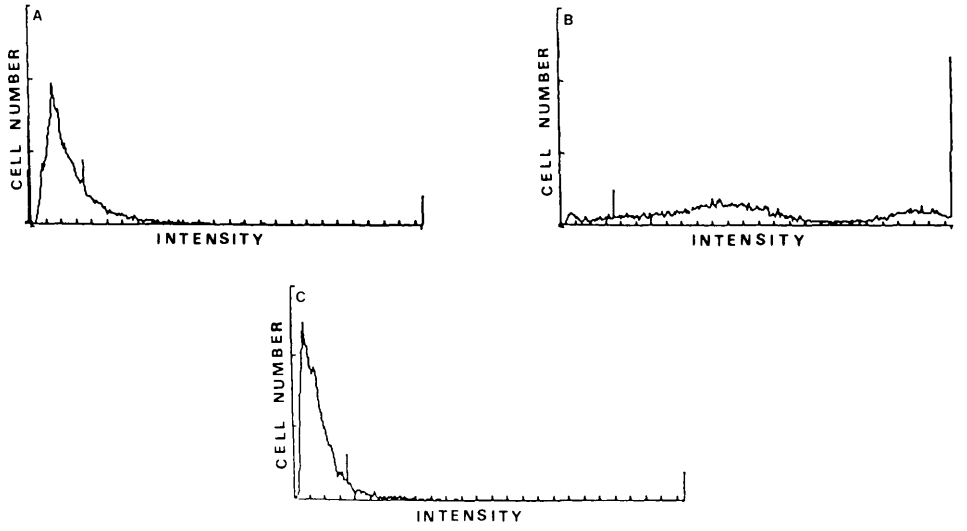

FIG. 2. Flow cytometry analysis of Sephadex-stimulated adherent peritoneal exudate cells reacted with supernatant from hybridoma clones (A) CMTD-2 (designated as 8F10), (B) 1C9, and (C) the PAI control. FITC-labeled secondary antibodies were used. Cells for A, B, and C were obtained 42 hr after stimulation. A Becton–Dickinson II fluorescence-activated cell sorter was employed with a cell count of 10,240.

not more intensely than 1-hr isolates. Likewise, MCA 1C9 reacted well with the cultured macrophages. In contrast, MCA CMTD-2 was markedly reduced in reactivity with the 1-week macrophages.

Flow cytometry was employed as a verification of the reactivity of selected MCAs with subpopulations of specific leukocytes. Table III provides a comparison of the reactivities of CMTD-2 and 1C9 antibodies with Sephadex-stimulated macrophages collected at various time points after injection. The significance of kinetics on the production of Sephadex-stimulated macrophages reactive with CMTD-2 antibody is evident. The fluorescent plots generated by flow cytometry analysis are illustrated in Fig. 2.

The specificity of MCA CMTD-2 was examined in greater detail than that of other monoclonal antibodies. CMTD-2 was found to react directly with swollen Sephadex G-40 superfine beads unlike the lack of reactivity of the other antibodies (data not shown). Presumably, this indicates that CMTD-2 antibody binds a carbohydrate epitope on the polymerized dextran. Nevertheless, naturally occurring

TABLE III. PERCENTAGE OF CELLS POSITIVE FOR REACTIVITY WITH HYBRIDOMA SUPERNATANTS AS DETECTED BY FLOW CYTOMETRY[a]

Hybridoma clone	Cell type[b]				
	% FITC-positive splenic leukocytes	% FITC-positive Sephadex-elicited adherent PECs: Time of harvest (hr)			
		6	24	42	52
CMTD-2	2.7	1.7	2.0	13.4	29.4
1C9	77.0	75.8	84.6	82.3	81.1
PAI control[c]	0.0	0.0	0.0	0.0	0.0

[a] Percentage expressed relative to PAI control values.
[b] The source for all cell types was 5-week-old female Cornell K-strain chickens.
[c] The myeloma cell line.

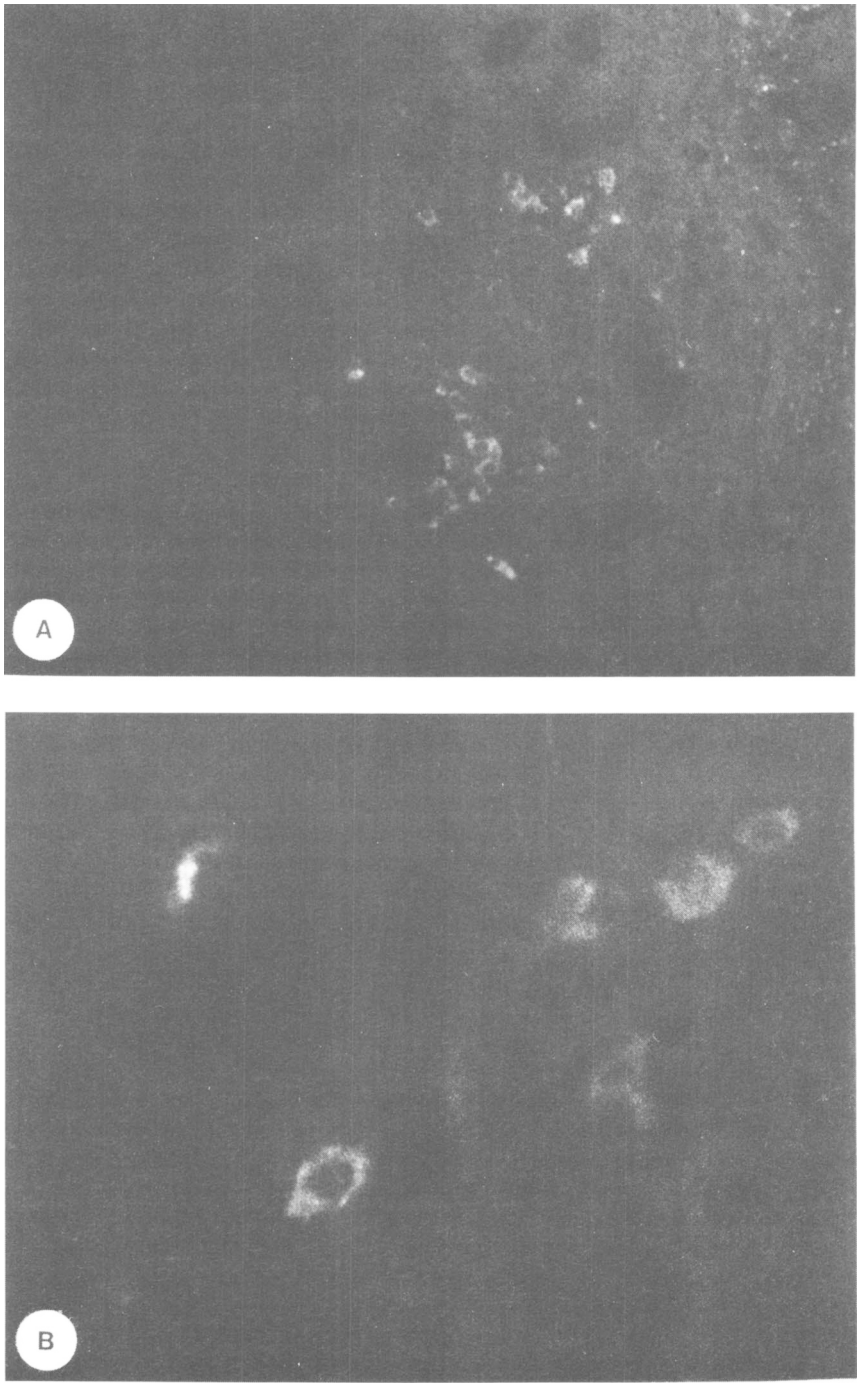

FIG. 3. The immunofluorescent staining of 2-week-old Obese strain chicken thyroid sections (4 μ) using the CMTD-2 hybridoma supernatant is illustrated. (A) 200× magnification; (B) 800× magnification. Affinity-purified FITC-labeled goat anti-mouse Ig was employed as a secondary antibody. (C) The reactivity of CMTD-2 antibody with a low incidence of K strain splenic leukocytes is illustrated. The ABC immuno-peroxidase technique was employed. The magnification is 400×.

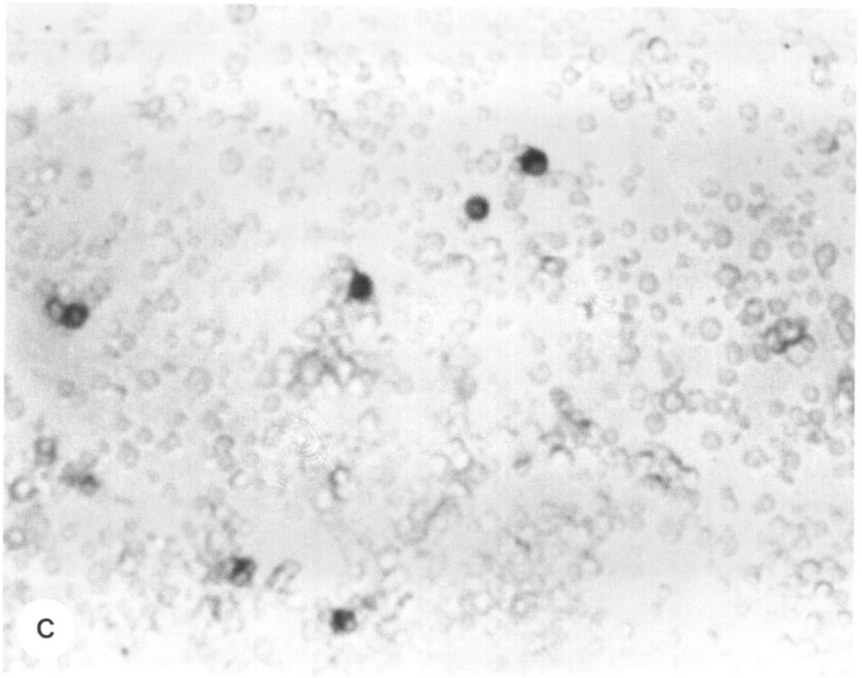

FIG. 3—*Continued.*

cell populations reactive with this MCA were detected in the chicken. A low incidence of macrophage-like cells positive for CMTD-2 was detected in the spleen (Table I, Fig. 3). In addition, the antibody reacted with infiltrating leukocytes during the autoimmune process within the Obese strain thyroid (Fig. 3). CMTD-2-positive leukocytes were found in all thyroid sections examined from six different 2-week-old Obese chickens. However, reactive cells were generally not observed in comparable 6-week-old thyroid sections.

The functional phenotype of CMTD-2-positive peritoneal macrophages was examined using the phagocytosis of sheep erythrocytes as a functional parameter. Figure 4 illustrates the reactivity of a macrophage-rich cell population with CMTD-2 following the opportunity to phagocytize sheep erythrocytes. In the results of two separate experiments, the percentage of CMTD-2-positive macrophages was found to be 24.4 ± 4.2%. Within these cells, 52.5 ± 0.1% were capable of sheep erythrocyte phagocytosis. Overall phagocytosis determined in the first experiment was 39.4%.

Discussion. Production of monoclonal antibodies reactive with mammalian macrophages has proven to be a useful approach for the dissection of macrophage heterogeneity (3, 26). Of the eight monoclonal antibodies generated in this study that react with chicken peritoneal macrophages, CMTD-1 and -2 appear to be most promising for the elucidation of chicken macrophage inflammatory response and subsequent activation. The former antibody failed to react with either peripheral blood monocytes or bone marrow cells; it was found to react primarily with inflammatory macrophages generally independent of the stimulant employed. Macrophages elicited in response to control injections (e.g., nutrient broth) failed to react with the CMTD-1 antibody. In addition, a definite time-dependent effect for reactivity was observed during the inflammatory macrophage peritoneal response. This suggests that cellular reactivity with this antibody is dependent upon activation rather than recruitment processes. It is anticipated that this monoclonal antibody will be useful in analyzing the conversion of blood monocytes into macrophages. The epitope detected by CMTD-1 appears to constitute a cell surface component.

In contrast, the production of a subpopulation of peritoneal macrophages reactive with CMTD-2 was both time dependent and stim-

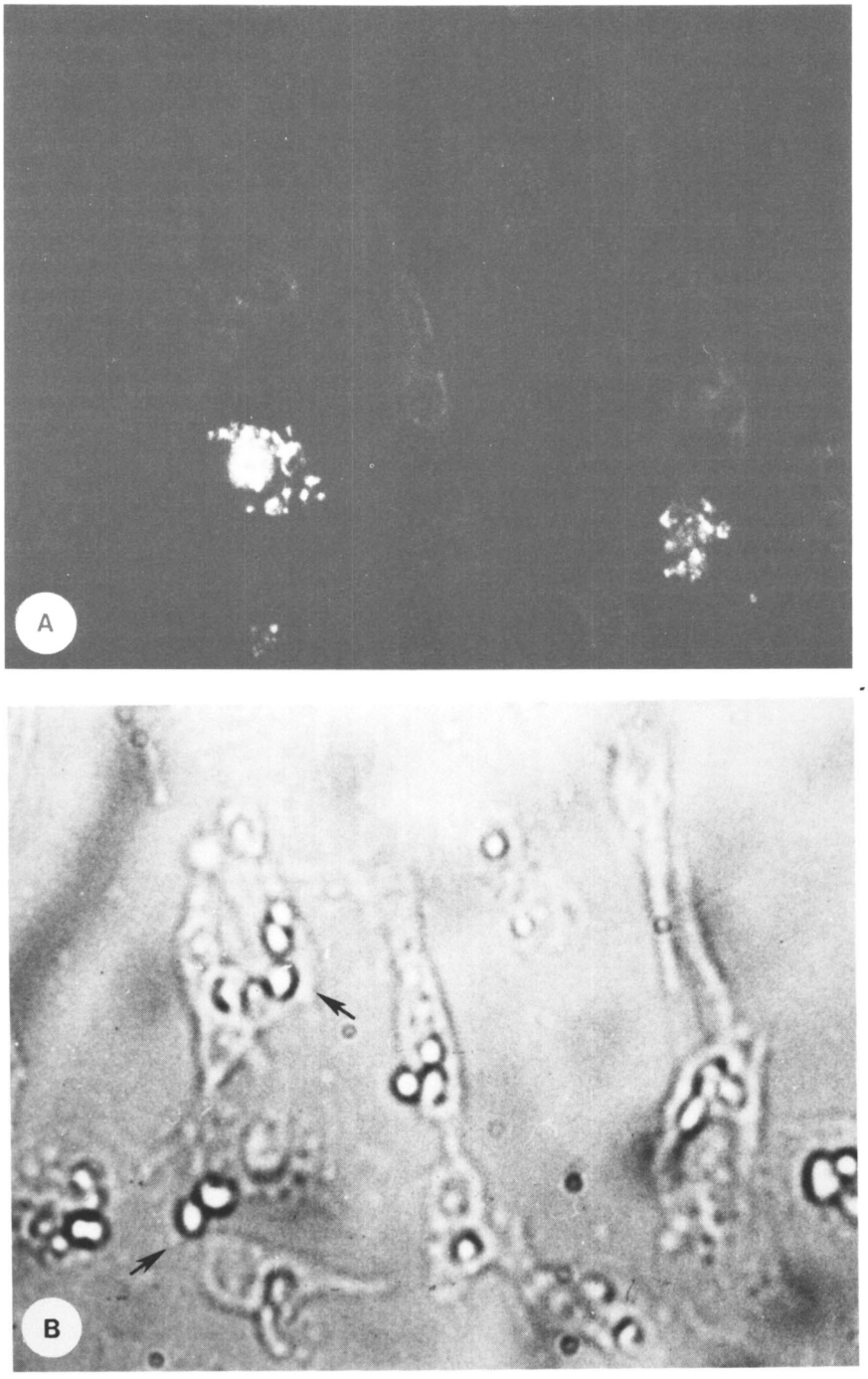

FIG. 4. The immunofluorescent staining of macrophage-rich monolayers from Sephadex-stimulated chickens is shown. Cultures were fed sheep erythrocytes prior to fixation and staining with CMTD-2 supernatant and FITC-labeled secondary antibody. Selected cells with engulfed sheep erythrocytes are denoted with arrows. (A) Dark field FITC immunofluorescence; (B) bright field. The magnification is 800×.

ulant dependent. CMTD-2-positive cells were found to increase between 24 and 52 hr after Sephadex ip injection. CMTD-2 antibody was found to react with cytoplasmic epitopes in these cells based on both the staining pattern and the requirement for prior methanol fixation. Since this monoclonal antibody was capable of reacting directly with Sephadex beads, this suggests that cells positive for CMTD-2 possess internalized dextran-based epitopes. However, the kinetics of appearance of this cell population did not correspond with the incidence of macrophages that retained phagocytic capabilities. At least one explanation for this observation is that only a subpopulation of the peritoneal macrophages capable of engulfing sheep erythrocytes may be able to ingest Sephadex beads or their breakdown products. The observation that about half of the CMTD-2-positive macrophages retained phagocytic activity for sheep erythrocytes has at least two possible explanations. Macrophages saturated with Sephadex may be incapable of further ingestion of any target, or only a subpopulation of these cells may be capable of sheep erythrocyte phagocytosis. Additional functional analyses are required to distinguish between these possibilities.

Although the reactivity of CMTD-2 with chicken macrophages would appear to be a secondary effect based on carbohydrate ingestion, the usefulness of this MCA is not restricted to experimentally induced inflammation. CMTD-2-positive leukocytes were detected at a low incidence in normal K-strain chicken spleens. Presumably, this cell population represents a phagocytic cell possessing the appropriate carbohydrate epitope. Similarly, a CMTD-2-positive leukocyte population was found to occur in the thyroids of OS-strain chickens. This strain possesses a genetically determined spontaneous autoimmune thyroiditis (SAT) (25). Cells positive for CMTD-2 were detected in all thyroid sections from four separate 2-week-old OS-strain chickens. In contrast, thyroid sections from 6-week-old chickens were essentially negative for these cells. Based on these findings, we would hypothesize that early in the development of the SAT, macrophages infiltrate the thyroid and internalize compounds bearing the appropriate epitope. As the disease progresses, the cells either leave the thyroid or simply lose reactivity with CMTD-2 as a result of enzymatic destruction of the epitope.

Little is known about the specificities of the six remaining monoclonal antibodies reactive with chicken peritoneal macrophages. MCA 9F8 exhibits an interesting reactivity pattern; while this antibody cross-reacted with certain lymphoid populations, it did not react with blood monocytes. As a result, it should also prove useful in analysis of monocyte–macrophage alterations. MCA 1C9 was found to provide a consistent level of reactivity with adherent PECs during the course of the Sephadex response. This could reflect the rather stable population of macrophages (80–85%) that is known to be present among adherent PECs over a broad time course of Sephadex stimulation (Y. Chu and R. R. Dietert, manuscript in preparation).

With this battery of monoclonal antibodies reactive with chicken macrophages, it may now be possible to examine the relationship between and potential overlaps of macrophage populations for such functions as antigen presentation (27), suppression (28–30) and bactericidal abilities (31). In addition, the recruitment of blood monocytes into tissues and subsequent activation may be examined using selected monoclonal antibodies as probes.

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