ANATOMY OF GERMINAL CENTERS IN MOUSE SPLEEN, WITH SPECIAL REFERENCE TO "FOLLICULAR DENDRITIC CELLS"

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ABSTRACT

Lymphocyte proliferation in germinal centers (GC's) is thought to be triggered by antigen retained extracellularly on the surface of special "dendritic" cells. The anatomy and function of these cells have not been studied directly or in detail. We therefore examined mouse spleen GC's developing in response to sheep erythrocyte stimulation.

We found that distinctive "follicular dendritic cells" (FDC's) were present in both the GC and adjacent mantle region of secondary follicles. The large, irregularly shaped nucleus, containing little heterochromatin, allowed for the light microscope (LM) identification of FDC's. By EM, the cell was stellate in shape sending out long, thin sheets of cytoplasm which could fold and coil into complex arrays. The processes were coated extracellularly by an amorphous electron-dense material of varying thickness, as well as particulates including variable numbers of virions. The FDC cytoplasm lacked organelles of active secretory and endocytic cells, such as well-developed rough endoplasmic reticulum (RER) and lysosomes. These anatomical features readily distinguished FDC's from other cell types, even those that were extended in shape.

To pursue these descriptive findings, we injected three electron-dense tracers i.v. and sacrificed the mice 1 h–10 days thereafter. Colloidal carbon, colloidal thorium dioxide ($cThO_2$), and soluble horseradish peroxidase (HRP) were actively sequestered into the vacuolar system of macrophages but were interiorized only in trace amounts by FDC's. Therefore, FDC's are not macrophages by cytologic and functional criteria. FDC's did display a unique property. Both colloidal carbon and thorium dioxide, which are nonimmunogens, could be visualized extracellularly on the cell surface for several days. The meaning of this is unclear, but the association of colloid with FDC's appeared to slow the movement of particulates through the extracellular space into the GC proper. FDC's were not readily identified in splenic white pulp lacking GC's. They must develop *de novo* then, possibly from novel dendritic cells that we have identified in vitro (Steinman, R. M., and Z. A. Cohn. 1973. *J. Exp. Med.* **137**:1142–1162).

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Cytologists have known for several decades that germinal centers (GC's)[1] develop in lymphoid organs in response to antigenic stimulation. The GC is composed primarily of two cell types: proliferating lymphocytes or lymphoblasts, and unusual large phagocytic cells known as "tingible body macrophages" (TBM's). The lymphoblasts are thought to be B cells which can form antibody (22, 26) and/or develop into small, memory cells for subsequent antibody responses (4, 33). The TBM's are known to actively phagocytose at least some of the lymphoblasts, producing dense phagocytic inclusions or tingible bodies (7). However, very little is known about the induction, control, and function of these two cell types.

10–15 years ago, Nossal et al. (19–21) and White (35) provided a fascinating new element to the GC problem. As a result of studies on the distribution of antigens within lymphoid organs, it was suggested that GC's contain another cell type variously referred to as "dendritic reticular cells" or "dendritic macrophages" (6, 9, 10, 13, 16, 19–21, 26, 32, 35, 36). It is thought that this cell differs somehow from typical phagocytic cells, and that it functions to retain antigens as immune complexes, extracellularly on the surface of many fine cytoplasmic processes or dendrites (11, 13, 19, 21; reviewed in reference 10).

The possible importance of dendritic cells and antigen retention in understanding GC function is obvious, so it is surprising that there has been so little work on the problem at the cellular level. To our mind, three sorts of difficulties exist in the current literature. Much of the experimental work has involved light microscope examination of frozen or paraffin-embedded thick sections in which it is difficult to be certain which cells are retaining antigen and whether it is in fact extracellular, e.g., macrophages and lymphocytes are found in the GC region and they likely can ingest and/or bind immune complexes (reviewed in references 3 and 24). The light microscope (LM) studies are also confusing in other respects, e.g., do dendritic cells exist before or after GC development (e.g. reference 6)?; are nonimmunogenic materials retained as well (e.g. reference 6)?; and in what ways do dendritic cells differ from other cell types that are extended in shape like macrophages and connective tissue cells? A second problem is that the few EM studies in this field were performed before currently useful fixation methods and electron-dense tracers were in active use. We still lack a detailed study of the anatomy of the various cell types and of the extracellular space, in that region of lymphoid organs in which antigens are retained, and it is not clear how the anatomy of the GC differs from that of other regions of the lymphoid organ. Finally, special antigen-retaining dendritic cells have not been isolated in vitro. This became of special concern to us when we identified distinctive dendritic cells from mouse peripheral lymphoid organs (28, 29) which were present in animals lacking GC's (30) and which did not bind antigens or immune complexes (29).

In this paper, we present an anatomical description of GC's that have developed in mouse spleen in response to sheep erythrocyte stimulation. Particular emphasis is placed on the presence of unusual "follicular dendritic cells" (FDC's). FDC's can be recognized by light microscopy, are exclusively associated with GC's, can be distinguished from other cell types that are extended in shape (31), and appear to develop *de novo* in association with GC formation. Electron-dense tracers were administered to show that FDC's do not actively endocytose as do macrophages. To our surprise, two of these tracers—colloidal carbon and thorium dioxide—which are nonimmunogens, were retained in the extracellular space selectively in association with FDC's. This property provides an important marker of the FDC, raises new possibilities on its origin and function, and has important implications for the study of antigen retention.

MATERIALS AND METHODS

Mice

Conventionally reared, outbred mice were obtained from The Rockefeller University colony (NCS strain), and inbred mice from The Jackson Laboratories (Bar Harbor, Me.; DBA/2J strain). Outbred, CD-1, specific pathogen-free mice were obtained from The Trudeau Institute (Saranac Lake, N. Y.) and Charles River Breeding Laboratories (Wilmington, Mass.). Mice of

[1] *Abbreviations used in this paper:* $cThO_2$, colloidal thorium dioxide; DAB, diaminobenzidine; EDM, extracellular dense material; FDC, follicular dendritic cell; GC, germinal center; HRP, horseradish peroxidase; LM, light microscope; PALS, periarterial lymphatic sheaths; RER, rough endoplasmic reticulum; TBM, tingible body macrophage.

both sexes, weighing 20–30 g, and 8 wk–8 mo in age, were used.

Antigen

Sheep erythrocytes (0.2 ml of a 5% suspension, about 2×10^8 cells) were administered i.p. or i.v. to induce the formation of GC's. Large typical GC's appeared in the spleen by day 6 and were found for 1–2 wk thereafter. Mice were studied 6–17 days after sheep erythrocyte administration.

Preparation of Tissue Specimens

Mice were anticoagulated with 150 U of heparin i.v. 30 min before sacrifice. The portal vein was exposed and a no. 10 polyethylene cannula (Intramedic PE 10; Clay Adams, Parsippany, N. J.) inserted retrograde at the entry of the splenic into the portal vein. ½ ml of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 was infused over 2 min such that the spleen assumed an orange-tan color. An additional 2 ml of fixative was then perfused. The spleen was removed and sliced into ~100-μm sections with a tissue chopper. If horseradish (HRP) or endogenous peroxidase cytochemical reactivity had to be visualized, the sections were rinsed overnight in buffer before further processing (see below). Otherwise, the slices were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4 for 1 h on ice, stained en bloc with 0.5% uranyl acetate in saline, pH 5.0 for 1 h, dehydrated in graded ethanols, and flat-embedded in Epon.

This fixation-tissue slice regimen is extremely helpful for the study of spleen anatomy (in addition to its requirement for cytochemistry). The slices are fixed uniformly, readily penetrated with Epon, and provide a huge cross-sectional area from which defined portions of the spleen can be selected for EM study.

Tracers

Three tracers, all of which could be visualized by EM, were administered 1 h–10 days before sacrifice, usually to mice primed with sRBC 6–7 days previously. Soluble HRP (Sigma Chemical Co., St. Louis, Mo., type II) was given i.v. at a dose of 3 mg/mouse in a volume of 0.2 ml. Before injection, the HRP solution was centrifuged at 80,000 *g* for 6 h to remove aggregates. The tracer was visualized by the diaminobenzidine (DAB) technique of Graham and Karnovsky (8). Glutaraldehyde-fixed, chopper sections were rinsed overnight in buffer and then treated with the DAB-H_2O_2 substrate for ½ h at room temperature. The slices were rinsed and processed as described above. Colloidal thorium dioxide ($cThO_2$) (Thorotrast, Fellows Testager Div., Fellows Mfr. Co., Inc., Detroit, Mich. and Thoria Sol, Polysciences Inc., Warrington, Pa.) was administered i.v. The Thorotrast preparation was a 25% solution of thorium dioxide in a dextrin base, and a dose of 50 μl was used. Unfortunately, this material is no longer being manufactured. Thoria, which is a 2% solution, was given i.v. at a dose of 0.2–0.5 ml. Colloidal carbon (Pelikan Special Ink, Günther Wagner, Hanover, Germany, distributed by Koh-I-Noor Radiograph, Inc., Bloomsbury, N. J., no. 9195) was given at a dose of 0.2 ml of a 1:4 dilution in saline. Carbon particles were not particularly electron dense and were best visualized in sections not stained with lead or uranyl salts (though the tissue slices were stained en bloc with uranyl acetate).

These tracers did not appear to be immunogens. For soluble HRP, we could not detect anti-HRP antibodies with the hemagglutination technique of Avrameas et al. (2) either during a primary or secondary exposure to soluble enzyme. For colloidal carbon and thorium, we could not find any morphologic evidence of an immune response in pathogen-free mice, i.e., blast transformation, plasma cell formation, or GC development.

RESULTS

Anatomy of FDC's

LIGHT MICROSCOPY: GC's, i.e. large collections of lymphoblasts and interspersed TBM's, were readily noted in mouse spleen some 6 days after sheep erythrocyte stimulation. GC's formed peripherally in the white pulp nodule and were usually separated from the marginal zone by a so-called "mantle," or "corona" region (Figs. 1 and 11). The latter consisted mainly of small lymphocytes (probably recirculating B cells [12, 18]), as well as scattered lymphoblasts, macrophages, plasma cells, and cells which we termed FDC's. The mantle and GC proper together were referred to as a secondary follicle.

FDC's were recognized by their large and unusually shaped nuclei (Fig. 1). Two large nuclear lobes were often noted in section. Heterochromatin was almost totally absent. The nucleoplasm was more phase dense and/or basophilic than other cells. FDC cytoplasm usually could not be distinguished except when large intercellular spaces were evident, and then the cell was noted to be stellate in shape.

These FDC nuclei were found only rarely in "unstimulated" spleen, i.e. from specific pathogen- or germ-free mice, and usually in the infrequent GC that was noted. After extensive GC development in response to sheep erythrocytes, FDC's were distributed entirely in the mantle region and adjacent GC proper. They were not found on the central artery aspect of the GC. We have not yet determined when FDC's appear during the 5–6 days it takes for GC's to develop in response to sheep erythrocytes.

ELECTRON MICROSCOPY: The irregularly

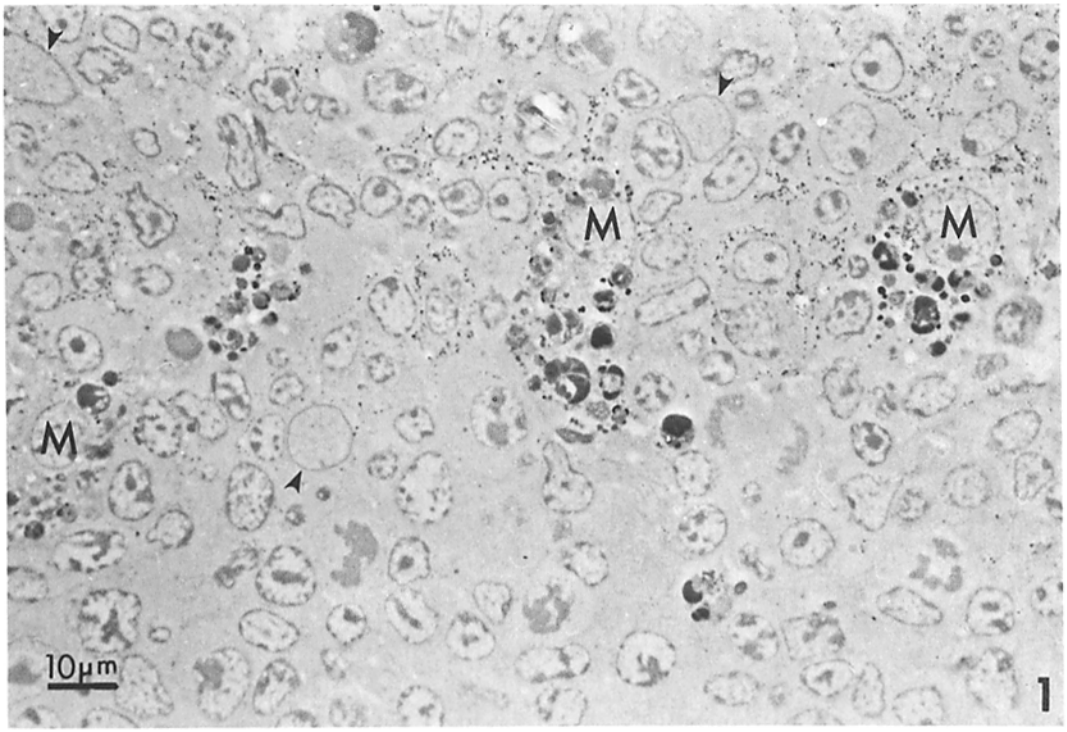


FIGURE 1 Light micrograph of a 2° follicle 7 days after sheep erythrocyte stimulation, and 1 day after colloidal carbon i.v. The top half of the figure is the mantle zone, and the bottom, the periphery of the GC. Most mantle lymphocytes have small nuclei, while GC lymphocytes are larger blasts with many mitotic figures. The distinctive nuclei of three follicular dendritic cells (arrowheads) are recognized by their lack of heterochromation and relatively dense nucleoplasm. The mantle contains macrophages (*M*) filled with granular inclusions. The smaller, very dense (brown) granules contain carbon, and the larger, less dense inclusions are "tingible bodies" of phagocytosed lymphocytes. A fine pepperlike distribution of carbon is noted throughout much of the mantle. This proves to be colloid localized exclusively on FDC processes (Figs. 5 and 7). Little intra- or extracellular carbon is evident in the GC (in contrast to HRP which enters the GC within an hour, see Fig. 9), suggesting the existence of a barrier to the movement of colloid at the mantle-GC interface. × 640.

shaped FDC nucleus, detected by LM, was easy to find in thin sections (Figs. 2–5 and 7–9). It had very little heterochromatin. The nuclear membrane was studded with nuclear pores and lined on its inner aspect by a 300-Å nuclear lamina. It is thought that the lamina subtends the pore complexes in most cells.

The structure and distribution of FDC cytoplasm was a most distinguishing feature. Very little cell body was evident. Instead, the cytoplasm ramified as very thin processes in several directions beginning close to the cell nucleus (Figs. 2–5 and 7–9). Some of the processes were long and straight (Fig. 3), often being continuous for 10–40 μm in a single section. Therefore, the cytoplasm was organized in sheets rather than true dendrites. These thin, flat sheets predominated in FDC's located at the very periphery of the white pulp nodule or more deeply in the GC proper. In other instances (especially at the mantle-GC interface) (Figs. 4, 8, and 10), the processes were folded and coiled, occasionally into unusually complex arrays. But again, the cytoplasm could be followed continuously in a single section for 10 μm or more. Infrequently, desmosomes were noted connecting two processes, presumably between two different FDC's. Other junctional specializations were not evident. We found no morphologic evidence that the FDC was interacting selectively with any one class of cell in the 2° follicle.

The network of FDC intercellular processes was most intricate and well developed in the mantle region and adjacent GC periphery. Macrophages and lymphocytes contributed few processes to the

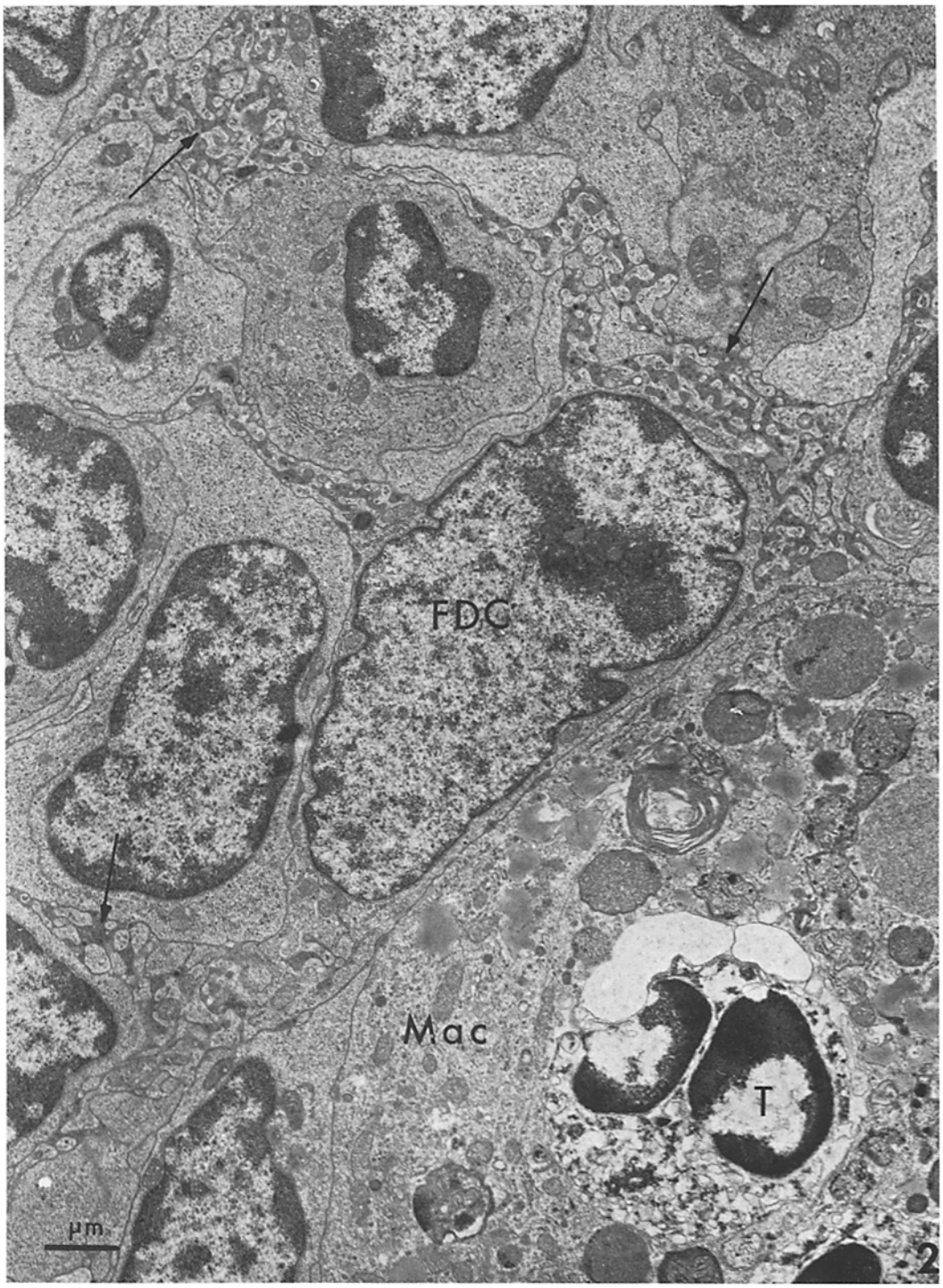


FIGURE 2 An FDC and a portion of a tingible body macrophage (*Mac*) at the junction of mantle and GC regions. The FDC nucleus is characteristically large and irregularly shaped, lacks heterochromatin, and has many nuclear pores. Cytoplasm extends in several directions forming an intricate network of processes adjacent to most lymphocytes in this part of the white pulp. EDM (arrows) outlines many of the FDC processes and, in this particular specimen, is filled with many virus particles (see higher power in Fig. 4). The macrophage contains many membrane-bounded lysosomes including tingible bodies (*T*). The macrophage surface is not elaborated into many fine processes and is not associated with dense material. × 8,300.

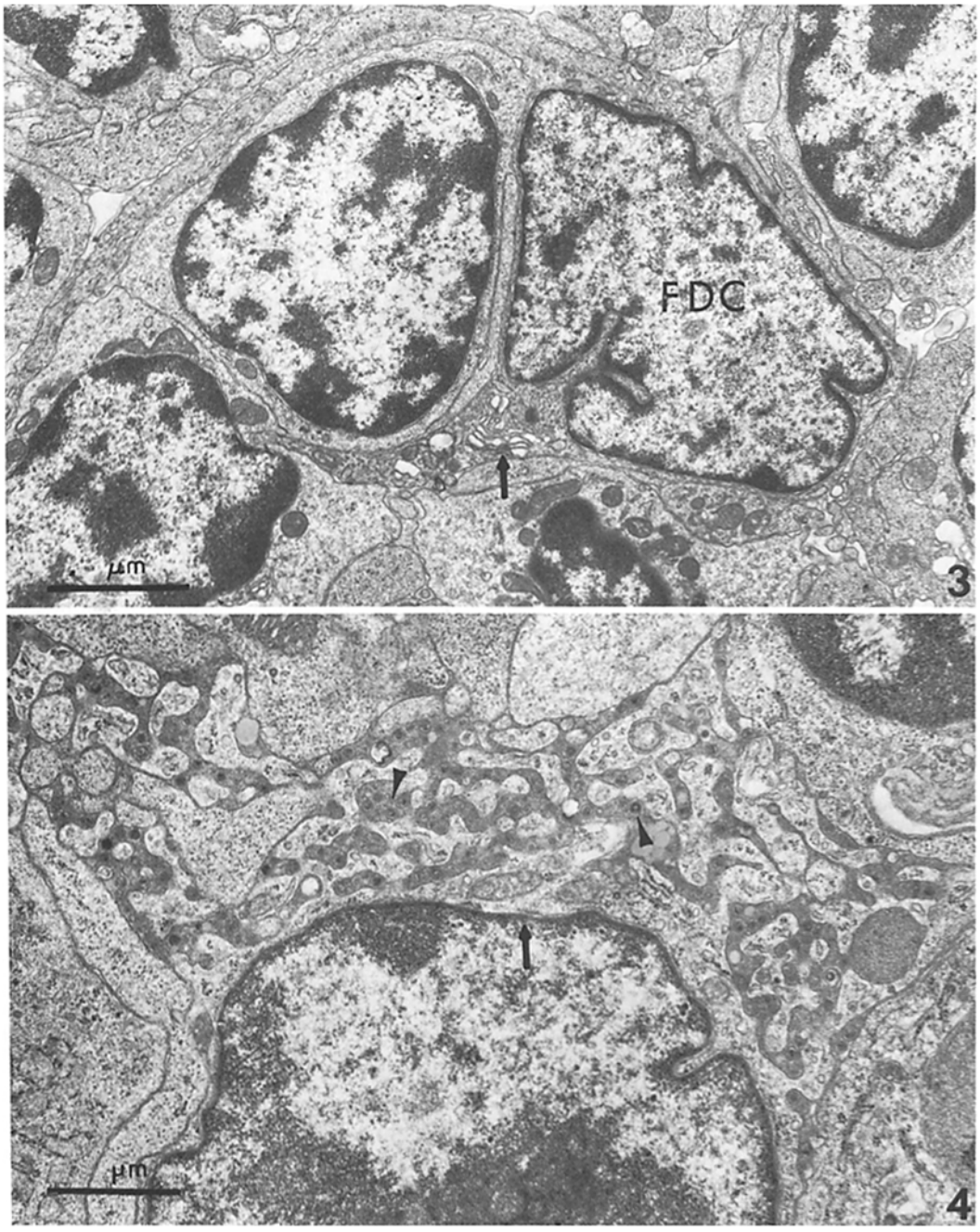


FIGURE 3 An FDC in the mantle region in which the cell shape is as simple as is seen in secondary follicles, i.e., the cytoplasm is arranged in long, thin sheets with little folding or finer ramifications. A Golgi region (arrow) exhibits few associated endocytic or secretory vacuoles, and few organelles occupy the cytoplasm. × 4,850.

FIGURE 4 Higher power of a portion of Fig. 2. The FDC nucleus has a prominent internal lamina (arrow). The cytoplasm can be followed continuously throughout much of its complex organization. Relatively few organelles can be identified, but extracellularly there is abundant dense material and embedded particulates. Some of the latter are clearly virions (e.g., arrowheads). × 19,000.

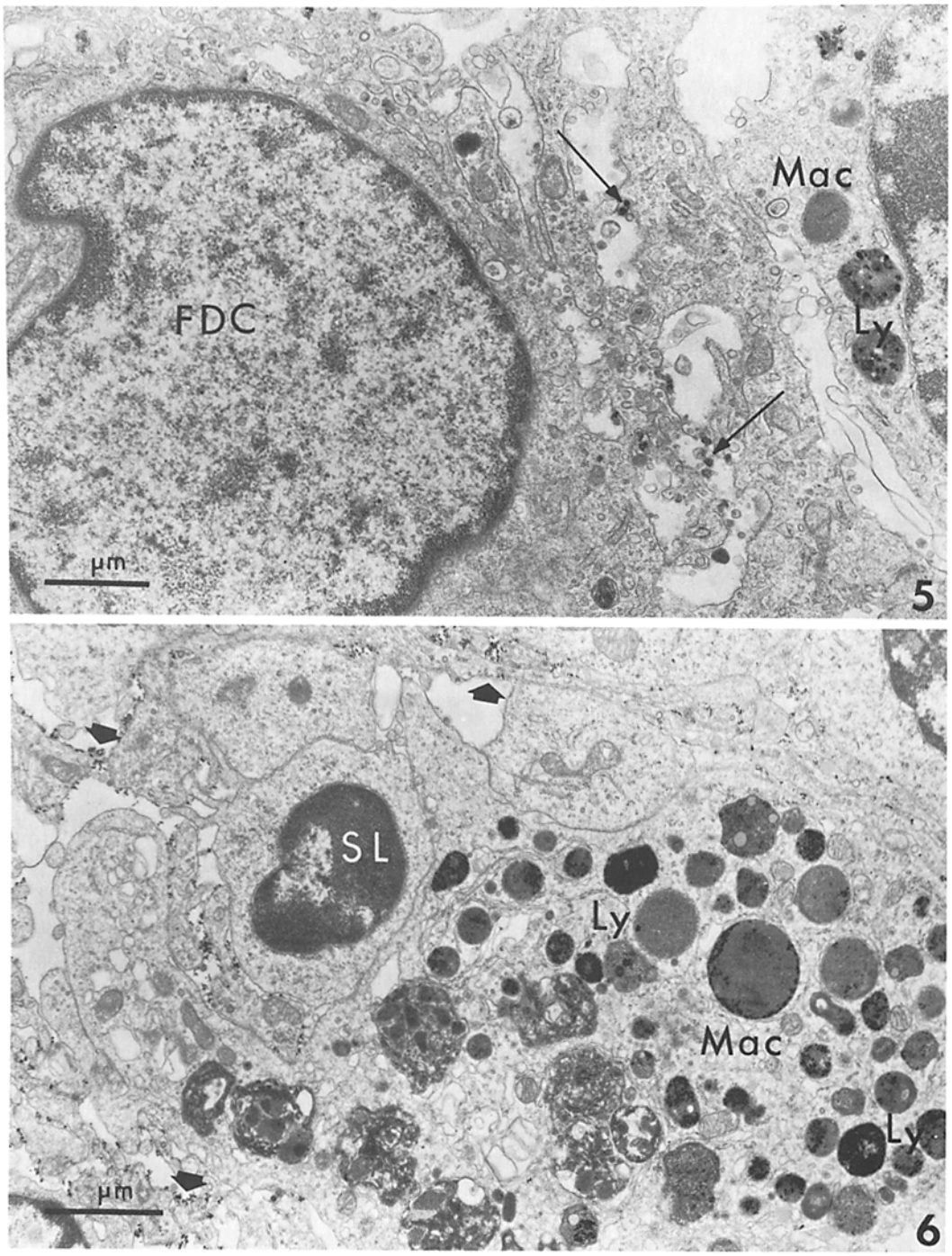


FIGURE 5 Higher power of an FDC and macrophage (*Mac*) 24 h after an injection of colloidal carbon i.v. The more electron-dense particles, representing carbon, are found in two locations: intracellularly in macrophage lysosomes (*Ly*), and extracellularly on the surface of FDC processes (e.g., arrows). × 15,400.

FIGURE 6 Higher power of FDC processes (e.g. arrowheads) and macrophage (*Mac*) cytoplasm to illustrate the distribution of $cThO_2$ 24 h after an injection i.v. Uptake by macrophages renders some of the lysosomes extremely electron dense (*Ly*). The macrophage and lymphocyte (*SL*) surfaces are free of colloid except where the cells are adjacent to the thin FDC processes bearing surface colloid. × 17,500.

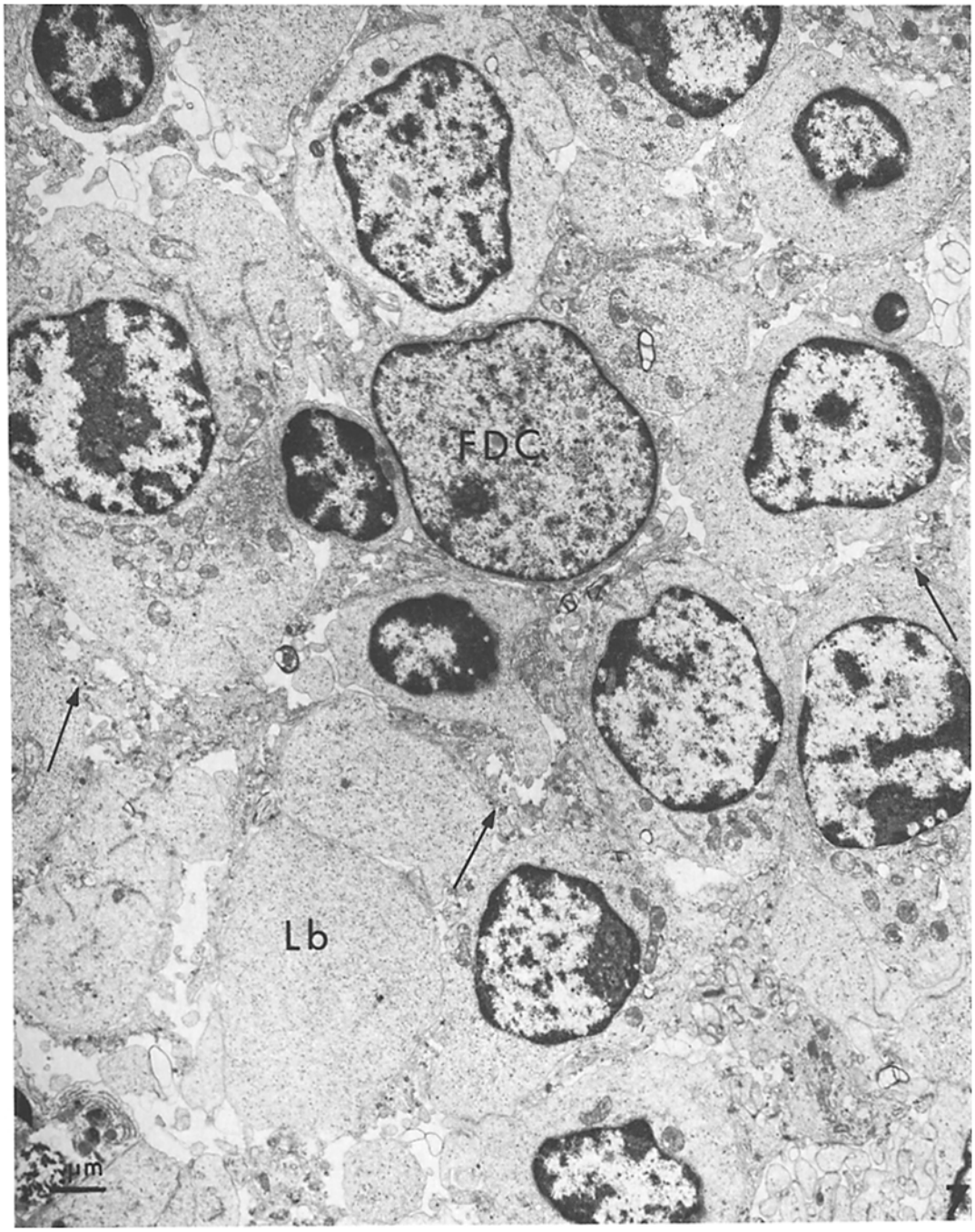


FIGURE 7 Survey micrograph of an FDC at the periphery of a GC 6 h after colloidal carbon i.v. The FDC sends out processes in many directions forming a complex network that is associated with almost every lymphoblast (*Lb*). Electron-dense colloidal carbon particles are evident on the surface of the processes throughout the region (e.g., arrows). × 6,250.

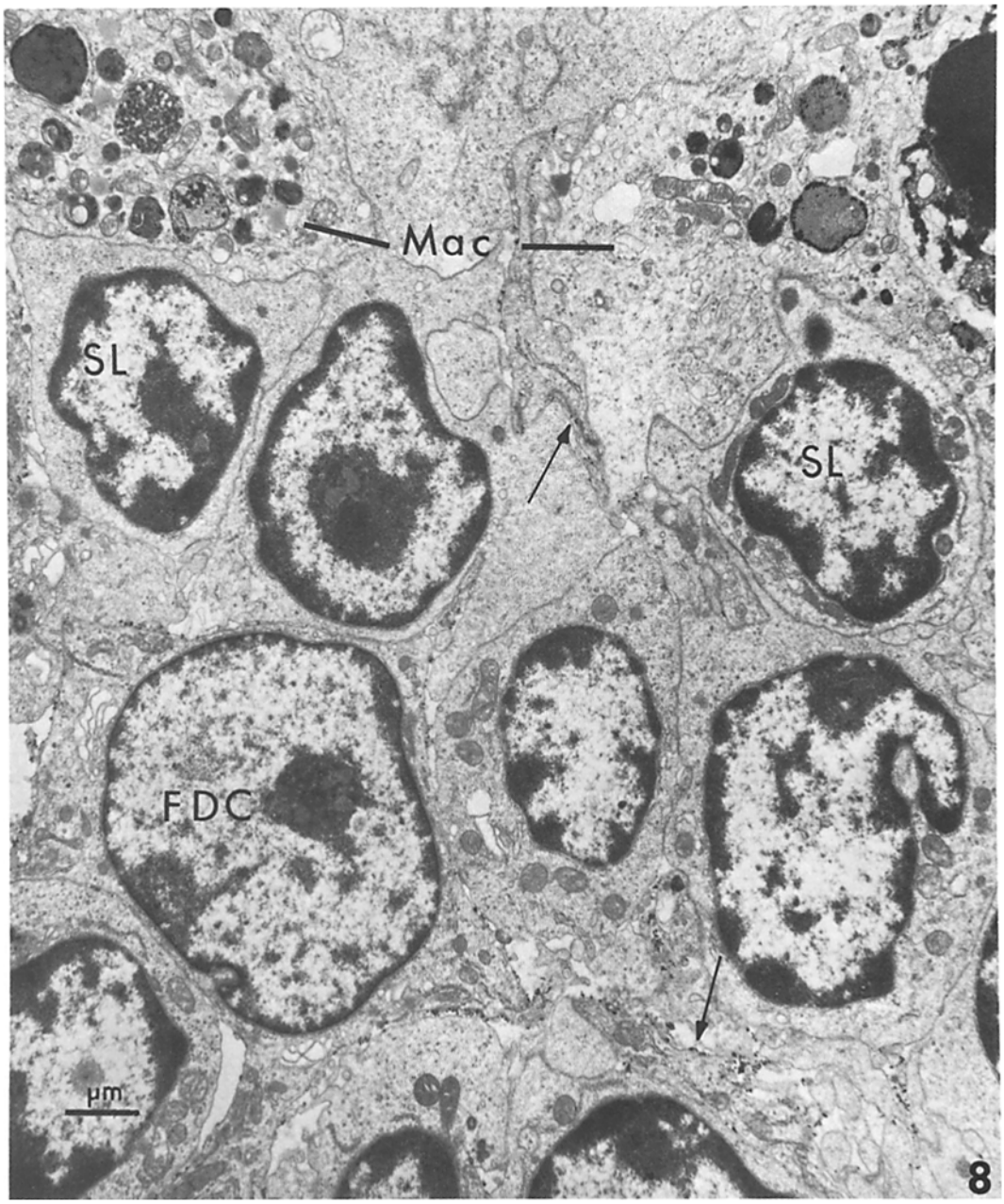


FIGURE 8 Survey micrograph of the mantle region 24 h after $cThO_2$ i.v. In contrast to Fig. 7, the lymphocytes (*SL*) in this region are primarily small cells with few polyribosomes. Colloid has been interiorized into many macrophage (*Mac*) lysosomes and coats many of the FDC processes (e.g., arrows). Lymphocytes do not bind colloid. × 7,500.

network. As one moved through the GC away from the mantle region, the number and complexity of FDC's and their processes diminished, so that processes were almost totally absent on the central artery aspect of the GC. The periarterial lymphatic sheaths (PALS) lacked FDC's as they are defined here, i.e., cells with highly irregular arrays of cytoplasmic processes and nuclei with

scanty heterochromatin. Other cell types with extended shapes were present in the PALS (Fig. 11).

The FDC cytoplasm contained few organelles (Figs. 2–10). There were scattered mitochondria, short slips of rough and smooth endoplasmic reticulum, coated and smooth vesicles, and polyribosomes, but all in small numbers. Microtubules and filament bundles were noted but were infrequent. There appeared to be many Golgi zones, usually at the origin of each set of processes. The lamellar stacks were dilated, but there were few associated, presumptive lysosomes or secretory granules.

The surface of the FDC was almost always coated with electron-dense material (EDM) of widely varying thickness. In most cases, the EDM consisted of tiny hairlike projections, but in others, the EDM filled the entire extracellular space between adjacent processes or between FDC's and other cells (Figs. 2, 4, and 10 b). In section, some of the EDM appeared to lie in membrane-bounded, intracellular channels. However, serial sectioning and tracer studies (see below) established its extracellular nature. The amount and composition of EDM was uniform throughout a particular specimen, rather than varying from cell to cell within the specimen. Extracellular collagen fibrils were not seen, in contrast to the nodular deposits of connective tissue that were found throughout the red and white pulp (17, 23, 31, 34). In fact, typical connective tissue deposits were rare throughout the secondary follicle, except along penetrating blood vessels.

A variety of particulates could be embedded in the EDM, especially in those cases where EDM filled the intercellular spaces (Figs. 2, 4, and 10 b). These included tiny electron-dense particles, electron-transparent vesicles, and typical virions, i.e., membrane-bounded structures with a central electron-dense core and surrounding electron-transparent region. The numbers of virus particles in different experiments varied enormously, but some were always detectable. We found no evidence that virus particles were being produced—either in factories or by budding from the plasma

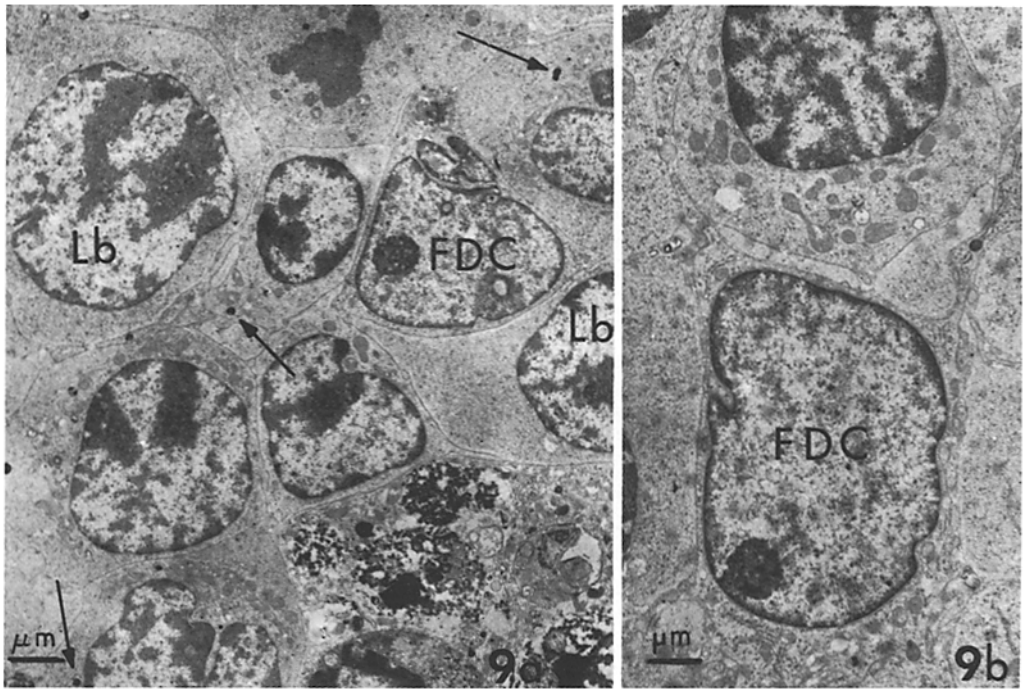


FIGURE 9 These micrographs display several features of the GC proper. FDC's are stellate in shape, but the individual processes do not coil or ramify extensively, as occurs at the mantle-GC junction. As a result, lymphoblasts (*Lb*) are tightly packed, with relatively few intervening processes. 1 h after injection of HRP, reaction product (e.g. arrows) is evident in small numbers of intracellular granules of all cells in the GC. HRP is not associated with the FDC or other cell surfaces. (*a*) × 3,700; (*b*) × 4,600.

membrane – in any of the cell types in this region. When particulates were present in the extracellular space, they were noted only in association with FDC processes and not with the surface of other cells.

In some instances, the GC contained, in addition to typical FDC's, a network of more electron-dense but long and thin cell processes, emanating both from FDC's and typical macrophages. It is possible that these electron-dense variants represent a problem in the fixation of very large GC's, and/or are moribund FDC's and macrophages.

We conclude that 2° lymphoid follicles are "capped" by a network of ramifying FDC's. These cells are located exclusively in that region of lymphoid organs believed to function in antigen retention. Cytologic criteria can be used to identify and distinguish the FDC's from other cell types that are extended in shape (see reference 31 and diagram in Fig. 11). Morphologic distinctions are useful, but functional ones are needed. The distribution of electron-dense markers administered i.v. may be such a functional marker.

Distribution of Markers Injected Intravenously

HRP, colloidal carbon, and $cThO_2$ were injected i.v. and their distribution in spleen was visualized at varying times thereafter (1 h–10 days). The three markers were nonimmunogens (see Materials and Methods), and we were interested in their possible intra- and extracellular distribution. These tracers can be interiorized by bulk uptake during endocytosis, and we wanted to know whether FDC's displayed this critical feature of macrophage function. The possible extracellular retention of these markers was also relevant. Studies on the persistence of antigens and/or immune complexes in 2° follicles (see the introduction) have often failed to include adequate controls for nonimmunogenic materials.

INTRACELLULAR LABELING: Within 3 h of their administration, all three markers could be visualized by LM and EM in intracellular granules (lysosomes) primarily of macrophages (Figs. 5, 6, and 8) and to a lesser extent in connective tissue and endothelial cells. Intracellular uptake was similar in unprimed mice lacking GC's. The only macrophage population that behaved unusually with respect to tracer uptake was the TBM, and this will be discussed below. To our surprise, HRP was also interiorized in small amounts into all lymphocytes of the 2° follicle, producing tiny reactive lysosomes (Fig. 9). The endocytic activity of FDC's was most easily judged using HRP, since colloidal carbon and $cThO_2$ were retained extracellularly on the surface (see below). Only small numbers of HRP-labeled vesicles were present in FDC processes (Fig. 9), and channels filled with EDM did not label. We conclude that FDC's are not active in endocytosis.

Both colloid tracers were evident in abundance in macrophages for the entire period of observation. Cytochemical reaction product of endocytized soluble HRP was lost within a day, as occurs in cells maintained in vitro (27).

EXTRACELLULAR LABELING: Both colloidal carbon and ThO_2 (Figs. 5–10), but not soluble HRP (Fig. 9), produced extracellular labeling, long after their injection (3 h–1 day), but only in the secondary follicle region. This applied to all GC's developing in response to sheep erythrocyte stimulation.

At the LM level, carbon produced a fine, granular, brown stain which was considered to be extracellular since it often outlined the cell borders (Fig. 1). By EM, extracellular carbon particles at 6 h or more after injection were exclusively associated with the surfaces of fine processes, previously shown to arise from FDC's (Figs. 5 and 7). Colloid was not found close to the surface of other cells except when FDC processes were juxtaposed. Because carbon particles were not very electron dense, extracellular retention was more readily followed by LM, once we had established that the carbon was in fact extracellular.

The opposite was true of $cThO_2$. At the LM level, this tracer was only seen in intracellular granules, but with EM an abundant and very dense extracellular staining was evident (Figs. 6, 8, and 10). We presume that the tiny extracellular particles were too small to be resolved by LM. 6–24 h after administration, extracellular $cThO_2$ was entirely associated with FDC processes primarily in the mantle region. Usually the particles were closely adherent to the plasma membrane (Figs. 6, 8, and 10 a), but in cases where abundant EDM was present the colloid appeared to be embedded in the EDM (Fig. 10 b). However, the frequency of colloid per unit of cell surface was similar over the entire FDC, regardless of the number and complexity of cell processes. Extracellular $cThO_2$ was evident for 7–10 days after injection, although the amount decreased steadily after day 1.

FDC'S AND A MANTLE-GC BARRIER: Within an hour of an HRP injection i.v. by LM, small numbers of fine, cytochemically reactive granules

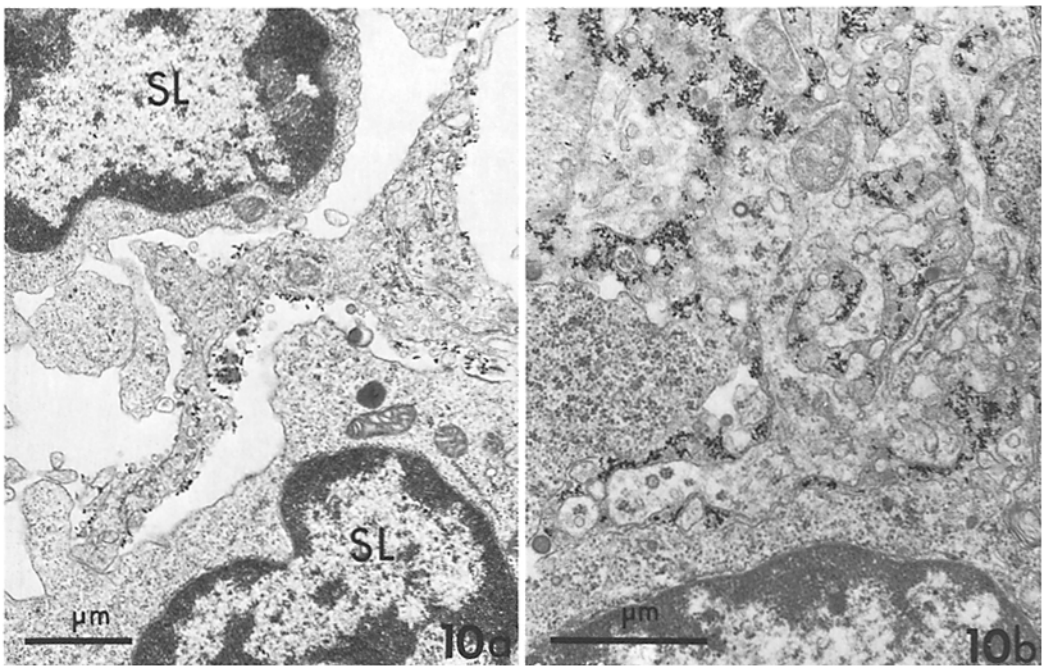


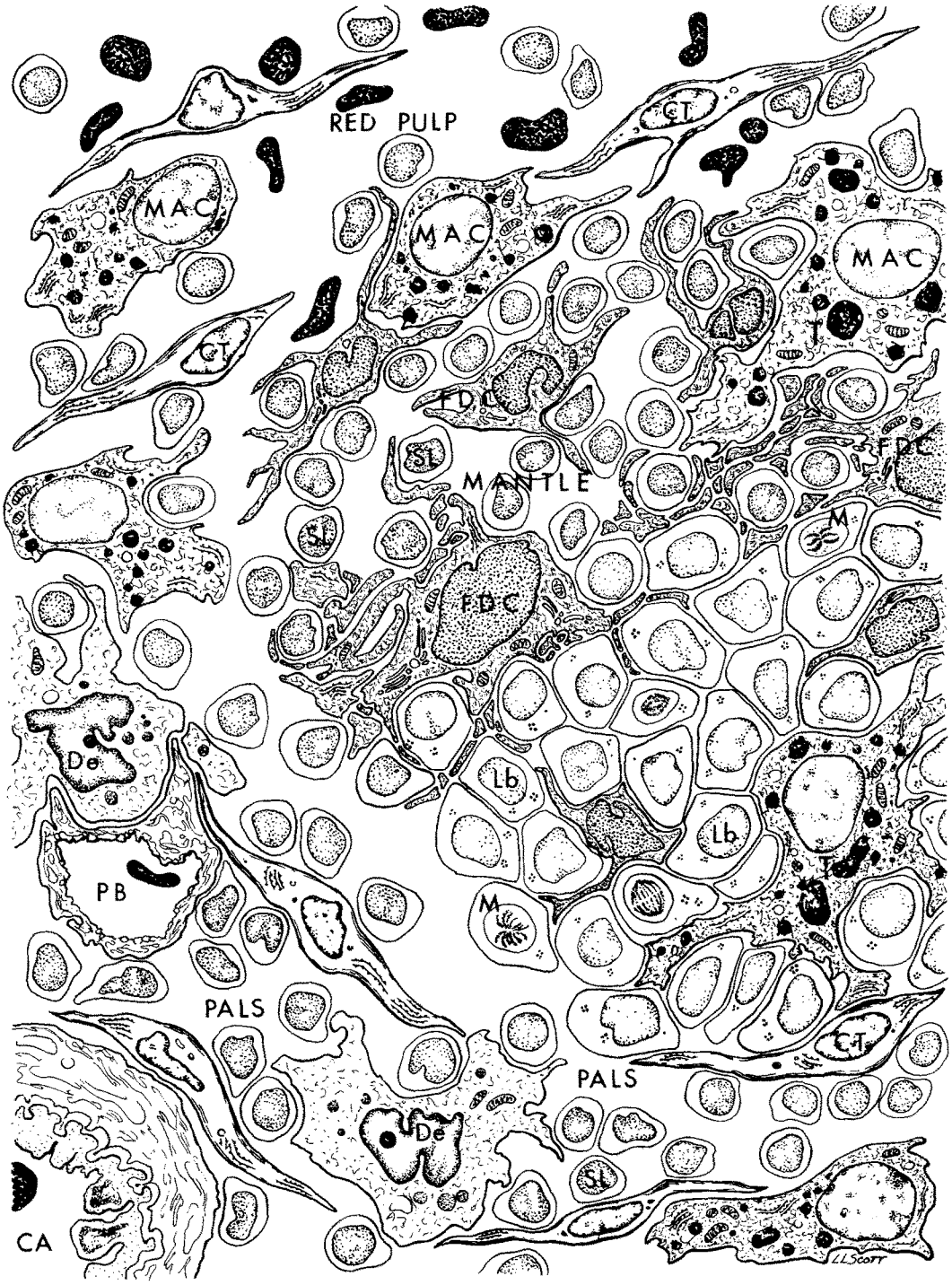
FIGURE 10 Higher power of the distribution of $cThO_2$ in association with the surface of FDC processes. (*a*) Typical selective binding to the surface of FDC processes. Adjacent small lymphocytes (*SL*) are negative. × 14,300. (*b*) A situation in which abundant EDM is associated with the FDC. The colloid appears embedded in the EDM. × 21,700.

were visualized in most cells of the GC, i.e., TBM's, lymphoblasts, and FDC's (Fig. 9). Therefore, the intracellular spaces of the GC must be rapidly accessible to soluble HRP since this becomes available for endocytic uptake. The route presumably involves entry into the marginal zone vascular spaces and then reflux through the white pulp nodule (see Fig. 11).

The kinetics of colloid entry were quite different and were best followed by LM examination of colloidal carbon. 1 h after injection, extracellular carbon was seen in the marginal zone. By 6–24 h, marginal zone extracellular label had disappeared while large amounts of label had built up at the mantle-GC interface (Fig. 1). Mantle and marginal zone macrophages and FDC's had abundant intra- and extracellular staining, respectively (Figs. 5–8). But TBM's and FDC's deeper in the GC were not labeled. Over many days (3–10), carbon was noted deeper in the GC and in fine TBM granules. At these later time points, extracellular carbon particles (and colloidal thorium) were still confined to the surface of FDC's. We think that these changes in the distribution of colloid were most likely due to the slow movement of the colloid and/or FDC's in the intercellular spaces of the GC. We postulate that the association of colloid with FDC's at the mantle-GC interface greatly retards the subsequent movement of colloid relative to soluble HRP, which is not "trapped" in this location.

INJECTION OF PARTICULATES BEFORE GC FORMATION: In our previous study of unstimulated, immersion-fixed spleen (31), cells identical to the FDC were rare. Other cells with extended shapes were noted and are summarized in Fig. 11. When colloids were given to unstimulated mice, primarily endocytic uptake by macrophages was noted and extracellular binding was essentially absent. These findings were repeated in perfusion-fixed spleen. Moreover, when colloid was injected at the time of, or 1 day after, sheep erythrocyte stimulation, colloid retention was not evident on the FDC's that formed during GC development. However, many TBM's were heavily labeled. Also, TBM's had Prussian blue-positive, hemosiderin granules, which means that they had once to be involved in the phagocytosis of endogenous red blood cells.

We conclude that extracellular colloid retention requires the presence of typical FDC's and that these cells develop at some time after sheep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erythrocyte stimulation. TBM's, on the other hand, probably exist as tissue macrophages before GC formation.

DISCUSSION

Anatomy of FDC's

Our study of the anatomy of lymphoid GC's has produced three sets of findings and raises implications for several others. The critical observation was the presence of distinctive cells which we have termed FDC's. The unique features of the FDC were: the large, irregularly shaped, heterochromatin-poor nucleus readily identified by LM; the stellate organization of cytoplasm, predominantly as thin sheets beginning close to the nucleus; the paucity of organelles including those typical of secretory and/or endocytic cells; and the presence of varying amounts of associated EDM and particulates. Micrographs of some profiles similar to those presented here have been published previously. For example, Maximow called attention to distinctive nuclei associated with GC's that seem identical to those of the FDC (15). He called them "primitive reticular cells" and hypothesized that they gave rise to GC lymphoblasts. Hanna and Hunter (10) and Szakal and Hanna (32) also emphasized the existence of unusual labyrinths of cell processes as a site for the extracellular retention of antigens. The existence of specialized dendritic cells has been proposed for some time (e.g. references 13, 16, 19, 20, 35, 36), but it is still difficult to find a thorough description of their anatomy in either the current literature and/or textbooks of histology and cellular immunology.

We realize that use of the adjective "dendritic" presents several problems. It is a descriptive rather than a functional term, and may need to be replaced once more information becomes available. Terms like "macrophage" and "connective tissue cell" have been used to describe cells which have distinctive cytologic features appropriate for their prominent functions of phagocytosis and extracellular matrix formation, respectively (e.g., references 5, 23, 31). Actually, "dendritic" is not anatomically accurate for the FDC. The cytoplasm consists mainly of large, thin sheets of cytoplasm which may fold and coil extensively and/or give rise to finer ramifications. We have also used the term "dendritic" cell in other ways: first, to describe a cell type that was isolated from "unstimulated" lymphoid organs in vitro (28, 30), and second, to describe what we believed was its counterpart *in situ* in spleens of germ-free mice which have few GC's (31). At this time, we think that the latter entities represent part of a common cell lineage with FDC's (see below), so we must clarify these possibilities before changing terminology. Also, current use of the term dendritic relates our work to the previous studies on the

FIGURE 11 A diagram illustrating portions of the regions of spleen after development of GC's, and emphasizing the various cell types that are extended in shape. Diagrams illustrating the nomenclature and relative size of different areas of spleen have been presented previously (e.g., reference 21). This diagram concentrates on white pulp, which is organized around a central artery (*CA*) and penetrating branches (*PB*), and is subdivided into PALS and 2° follicles. Each follicle in turn contains a mantle zone and a GC. GC's contain tightly packed lymphoblasts (*Lb*) exhibiting polyribosomes and mitotic figures (*M*). All macrophages in the GC have large, densely staining, phagocytic inclusions called tingible bodies (*T*). The mantle contains many small lymphocytes (*SL*), probably recirculating B cells (12, 18). Macrophages (*MAC*) are present, though only a minority contain tingible bodies. FDC's are found only in the 2° follicle, and are the most irregularly shaped cell in spleen. The cytoplasm is arranged as long, thin sheets which coil and ramify extensively, especially at the mantle-GC interface. Few intracellular organelles are present. The PALS contain primarily recirculating small T lymphocytes (12, 18). Most lymphocytes are separated from one another by clear spaces (exaggerated in the diagrams), but in contrast to the mantle there are fewer intervening cell processes. Several cell types with extended shapes (31) are present, even in mice lacking GC's, and include another cell referred to as a "dendritic cell" (*De*). Unlike the FDC, the dendritic cell has a distinct peripheral band of nuclear heterochromatin and is less irregular in shape. The red pulp is marked by red blood cells lying in vascular spaces, which in mice lack true sinus linings (25). Many lymphocytes and macrophages are evident. The red pulp of adult mice has islands of marrow or hematopoietic tissue which have been omitted here. Presumptive connective tissue cells (*CT*) are also found. These cells presumably synthesize deposits of extracellular fibrils and matrix (17, 23, 31, 34). They are distributed throughout the red pulp and PALS, and as adventitial cells along blood vessels. They are usually fusiform, very elongate cells with well developed RER and peripheral microfilament bundles.

retention of antigens in GC's, a possibility which also needs further resolution.

FDC's lacked the well-developed vacuolar system of macrophages, and so we presumed that they also lacked the latter's endocytic capacities. To prove this, we administered three nonimmunogenic, electron-dense tracers, all of which could be sequestered by bulk or endocytic uptake. All three markers were actively sequestered by typical macrophages, i.e., cells with many lysosomes and vesicles before injection of tracer. The one exception was the TBM, many of which required days to acquire readily detectable amounts of colloid (see below). Connective tissue and endothelial cells endocytosed to a lesser extent, as was recognized by Aschoff in his early work defining the "reticulo-endothelial" system on the basis of staining with vital dyes (1). FDC's, however, incorporated only very small amounts of tracers. Conceivably, FDC's correspond to the so-called nonphagocytic, stellate "metallophils" described by Marshall using silver impregnation techniques (14). Another specification of the macrophage lineage is the ability to bind and interiorize particles coated with immunoglobulin and/or complement (3, 24). We have been studying the fate of HRP-anti-HRP complexes *in situ* (L. L. Chen and R. M. Steinman, manuscript in preparation), and have again noted that FDC's do not interiorize these markers. So by both cytologic and functional criteria, the FDC is simply not a macrophage. There is no justification that we know of for further use of the term "dendritic macrophage" in spleen or other tissues.

Association of Particulates with FDC's:

The second and surprising set of findings was that colloidal carbon and ThO_2 particles could be retained extracellularly for many days in association with FDC processes, but not at all with the surface of typical macrophages or other cell types. The distinction between the behavior of FDC's and that of macrophages was dramatic, reproducible, and evident even when the two cell types were adjacent to one another. Particle retention may also have been responsible for the exclusive association of virions with FDC processes. We found no morphologic evidence, i.e. virus factories or cell surface budding, that virus was being produced within cells of the 2° follicle, so we presume that circulating virus may be trapped on FDC's much like exogenous colloids.

We cannot propose a mechanism for particle retention, or to be more accurate, particle association with cell processes, since we do not know whether particles remain immobile in any one site. A critical unknown is the nature of the EDM that coats the dendritic cell surface. This EDM was present in widely varying amounts. Possibly, it is produced by the FDC and/or it may represent endogenous materials bound to the plasma membrane, like immune complexes, virus particles, and cell debris. Whatever the mechanism, particle retention serves as a marker for this cell *in situ,* i.e., it represents a functional property that is associated with its distinctive cytologic features. Particle retention may also provide other experimental opportunities. If indeed, circulating endogenous particulates like virus and immune complexes are trapped in association with FDC's, one could experimentally induce GC's to detect and characterize these particulates in disease states.

Although we must extend our studies with the use of antigens and/or immune complexes, it seems likely that the retention of colloids that we have observed bears upon the phenomenon of antigen retention in GC's (see the introduction). The two processes seem to occur in the same region and can bring about retention for days or more (6, 9, 10, 13, 16, 19–21, 32). Our observations point to the need for precise electron microscope techniques to determine what cells are retaining a particular marker, including antigens, and whether it resides in or on the cell. The mantle-GC region contains both typical macrophages and FDC's. Both can interact with particulate tracers, the former via endocytosis and the latter by some extracellular trapping mechanism. Even lymphocytes endocytose a tracer like HRP. It remains to be determined what factors alter the relative distribution of materials in association with these different cells, and for what duration a marker persists in any one cell.

The physiologic significance of particle retention on FDC's is unclear. Conceivably, as the dogma would have it, extracellular retention could have an important immunogenic function. Alternatively, retention may have a barrier role, i.e., to limit the access of certain materials into the GC proper where they might interact with proliferating lymphoblasts and/or TBM's. We obtained evidence that soluble HRP gained rapid access to all cells in the GC whereas colloid penetration appeared to be slowed at the mantle-GC junction where extensive association with FDC's was noted. Finally, retention may be a secondary

phenomenon, the primary event being that FDC's generate large numbers of processes as they interact directly with other cell types in the follicle.

Origin of FDC's:

The third set of findings in this paper evolved from our studies with colloidal tracers and relates to the origin of the various cell types in the GC, particularly TBM's and FDC's. The former probably exist as tissue macrophages before GC development, since they contain hemosiderin granules and are labeled with endocytic markers if the markers are administered before an antigenic stimulus. The origin of the FDC is of great interest to us. By both cytologic criteria and the property of colloid trapping, the FDC's are not readily identified in sections of unstimulated spleen (31). So they must arise de novo during GC development. We do not yet know when this happens. Conceivably, the FDC's arise by activation of the novel dendritic cells that we have identified in vitro in spleen from unstimulated mice (28). Like the FDC's, these cells can be stellate in shape, have large, irregularly shaped nuclei, and contain identical cytoplasmic organelles. Unlike the FDC's, dendritic cells in vitro have more heterochromatin and do not exhibit such complex cell shapes or particle binding (28, 29, 31). However, dendritic cells are the only cells isolated in vitro that resemble FDC's. We are trying to establish additional markers and/or properties to resolve these possibilities, and we are trying to prepare cell suspensions from isolated GC's to further characterize the FDC's.

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