ELECTRON MICROSCOPIC AUTORADIOGRAPHY
OF GERMINAL CENTER
CELLS IN MOUSE SPLEEN

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ABSTRACT
The fine structure of tritiated thymidine-labeled cells in antigen-stimulated mouse spleen germinal centers is described. In studies on the ultrastructural level, two labeled cell types found in germinal centers are observed. Large lymphocytes are characterized by their very numerous free ribosomes, a paucity of endoplasmic reticulum, relatively few mitochondria, and a poorly developed Golgi region. The nuclei are large and vesicular, and large nucleoli are present. A second labeled cell type appears to contain more mitochondria and has a higher development of the Golgi area. The nucleus contains large, numerous blocks of chromatin, indicative of a more differentiated cell type. Reticular cells, both phagocytic and non-phagocytic, were not observed to be labeled in the germinal centers.

INTRODUCTION
Recent autoradiographic studies using tritiated thymidine have described the kinetics of proliferating cells in white pulp germinal centers in normal rat (11) and antigen-stimulated mouse spleen (13). A steady state was described for the cells in non-immunized animals. Early disruption of the steady state was demonstrated in spleen germinal centers of immunized animals. An approximate generation time of 5 to 7 hours with a DNA synthesis (S) period of 4 to 5 hours was reported for these cells. In most mammalian tissues so far investigated, the length of the S period is 5 to 10 hours (8, 18, 19, 34). From these data it is apparent that rapidly proliferating cells of the germinal center are approaching the lower observed limits of time in which mammalian cells perform the required processes in preparation for division.

Germinal centers have been considered to function (a) in the formation of lymphocytes following Flemming's (10) original observations, (b) as reaction centers (15), and (c) in immunologic phenomena (6; see 5 for earlier references). No adequate ultrastructural description of germinal centers exists, possibly owing to difficulties of orientation and also to the cyclic activities and changes which these structures undergo (7). Cyclic activities have been described as being dependent to some degree on antigen stimulation (16, 28, 36). Since the degree of non-specific antigenic stimulation in normal animals is unknown, we have chosen to study the synchronized cellular response in an antigen-stimulated animal. The proliferative capacity and functional role attributed to cells of germinal centers warrant further investigations of these cells on an ultrastructural level.

As observed by light microscopy, the characteristic large cell of the germinal center has a basophilic cytoplasm. The purpose of this report is to describe the fine structure of these rapidly proliferating cells labeled with tritiated thymidine in antigen-stimulated mouse spleen germinal centers.
MATERIALS AND METHODS

Adult (101/Cum 9 X C3H/Anf Cum 8) F1 male mice were injected intravenously with 2 X 10^8 sheep erythrocytes (SRBC) in Tyrode's solution, and, 1 hour later, with 100 µc of H3-thymidine (0.9 c/mmole specific activity, New England Nuclear, Inc.). Three hours after the isotope injection, two animals were killed with ether anesthesia and the spleens were removed. One half of each spleen was fixed in Bouin's fixative for light microscopic autoradiography. The other half was cut into small pieces and fixed for 1 hour in ice cold 2 per cent OsO4 (pH ~ 7.4) buffered with acetic acid-veronal (29); 3-µm sections of spleen tissue were prepared for light microscopic autoradiography. Slides of tissue were coated with Kodak NTB-2 liquid emulsion, exposed for 14 days, developed, fixed, and stained with hematoxylin and eosin. OsO4-fixed tissue was embedded in Epon 812 (20) and thin sections were cut. Ultrathin sections were picked up on uncoated 300-mesh copper grids, the grids were affixed to glass slides, and a thin film of Ilford L-4 emulsion was applied to the surface containing the sections. The method is a slight modification of that described by Caro and van Tubergen (4). Sections were exposed for 70 days, developed, fixed, and washed, and the emulsion was removed in 0.05 N NaOH. After three washes, the grids were stained in saturated aqueous uranyl acetate, washed, dried and examined in a Siemens Elmiskop I microscope.

Since removal of the emulsion and staining with uranyl acetate might influence the number of silver grains observed during electron microscope examination, grids were also examined without NaOH treatment and staining with uranyl acetate. No detectable differences in the pattern of silver grain distribution were observed to result from the removal of emulsion.

Histochemical tests were made to demonstrate the nature of the basophilic material in the cytoplasm of labeled cells in mouse spleen germinal centers. Two animals were injected with 2 X 10^8 SRBC, and 4 hours later were killed with ether anesthesia. The spleens were removed, frozen, and sectioned on a cryostat. Frozen sections were stained with methyl green–pyronin (33), and control sections were treated with ribonuclease (Armour Co.) for 2 hours and stained by an identical methyl green–pyronin procedure. Sections were also treated by the periodic acid–Schiff (PAS) technique (31) and with Best's carmine stain (21) as tests for glycogen.

RESULTS

An active germinal center of the antigen-stimulated mouse spleen 3 hours after the intravenous injection of tritiated thymidine is shown in Fig. 1. It is composed mainly of large basophilic cells with labeled nuclei and is clearly demarcated from the surrounding lymphocyte mass. Labeled mitotic figures are present as well as dense pyknotic nuclear debris. The identification of germinal centers in thin sections employed in electron microscope study is thus facilitated by the presence of many labeled cells, as well as mitotic figures and the characteristic macrophages containing cellular debris (the tingible bodies of Flemming).

The ultrastructure of the large cells observed in active germinal centers is shown in Figs. 2 to 6. The nuclei contain large nucleoli, and chromatin blocks are located around the periphery. In all five figures, silver grains are associated with nuclear chromatin over labeled cells. In one labeled cell type (Figs. 2 to 5), abundant free ribosomes and a relative scarcity of other organelles characterize the cytoplasm. Methyl green–pyronin treatment of ribonuclease-treated frozen sections and histochemical stains for glycogen demonstrated that the particulate material in the cytoplasm was composed largely of ribonucleic acid, and it was therefore assumed to be ribosomes. Although the majority of the ribosomes are free, clusters and spiral configurations are frequently encountered. No highly organized endoplasmic reticulum was present in these cells. Mitochondria were generally large and swollen in appearance, some with incomplete cristae (Figs. 2 and 3). Centrioles (Fig. 3) were also observed in the region of the Golgi vesicles near the nucleus. These cells are structurally similar to primitive cells seen with the electron microscope by Sorenson (38) in normal rabbit lymph nodes and probably are the "large lymphocytes" of Maximow (23). The term large lymphocyte will be used hereafter in this report to refer to this cell type.

A labeled mitotic figure with the cytoplasmic characteristics of the large lymphocyte described above is shown (Fig. 5) adjacent to an unlabeled reticular cell. The latter cell appears to form the stroma of the germinal center. A cytoplasmic extension of the reticular cell partly surrounds the labeled lymphocyte. A close association of reticular cells with other cells in the germinal center is brought about by extensions and interdigitations of their plasma membranes, but no direct cytoplasmic contact or syncytial arrangement has been observed. The reticular cell cytoplasm contains membranous components, dense bodies...
FIGURE 1 A low magnification photomicrograph showing the intensely labeled cells in a spleen germinal center 3 hours after isotope injection (sheep erythrocytes injected 1 hour previously). Hematoxylin and eosin stain. × 750.

(Fig. 5), and ribosomes, both free and associated with the endoplasmic reticulum. None of the observations made on reticular cells (including phagocytic cells) found in germinal centers at the interval studied revealed DNA labeling of their nuclei. However, labeled nuclear debris was observed in phagocytic cells.

A second labeled cell type is observed in active germinal centers (Figs. 5 and 6). Although this cell has many of the same characteristics as the large lymphocytes described above, some of the ultrastructural features are those of a more differentiated cell. Mitochondria appear more numerous, the Golgi zone is more developed, and the multivesicular bodies are observed more frequently. In the nuclei, scattered clumps of chromatin are present (Figs. 5 and 6). These cells appear to be smaller than the large lymphocytes described above and may correspond to the "medium lymphocytes" of Maximow (23).

DISCUSSION

Considerable evidence can be cited to support the assumption that cells having a high concentration of free ribosomes, in contrast to the relative exclusion of other cytoplasmic organelles, are rapidly proliferating. Palade (30) observed the occurrence of abundant particulate material (ribosomes) unassociated with the endoplasmic reticulum in rapidly proliferating cells (epithelial cells of intestinal crypts, young lymphocytes, and cells of the basal layer of the epidermis). Also, the pattern of cytodifferentiation observed in embryonic development (2, 17, 39) and regeneration of cells (14) is similar to that proposed for lymphatic tissue cells in germinal centers.

Electron microscope observations on lymphatic tissue have in general been interpreted as indicating a reticular cell genesis of lymphocytes and plasma cells (see 25 for references). These studies support the light microscope investigations of Downey and Weidenreich (9), who reported...
Figure 2. An electron micrograph of a labeled large lymphocyte (Pc) of the germinal center. Four silver grains lie over the nucleus. A large nucleolus (No) is present, abundant ribosomes fill the cytoplasm, mitochondria (M) are large and have poorly defined cristae, and infoldings of the plasma membrane (arrows) with adjacent cell membranes are numerous. × 18,000.
intermediate forms between fixed reticular cells and lymphocytes. Numerous transitional and intermediate forms are encountered in the morphologic study of lymphatic tissue.

However, morphologic study alone does not offer convincing evidence of the direction in which cells are transforming. For example, the application of radioautography to the study of lymphoid tissue has led Rieke et al. (35) to the conclusion that reticular cells are not a rapidly proliferating population of cells, and thus do not meet the requirements for stem cells. Since quantitative data based on total populations (35) do not support a reticular cell genesis, the morphologic interpretations given to intermediate cell types should be viewed cautiously.

In the present work, electron microscopy and autoradiography have been combined to correlate functional capabilities predicated on the uptake of tritiated thymidine with the ultrastructure of labeled cells in antigenically stimulated animals. Reticular cells should be expected to show an increased incidence of labeling if they participate by division in the formation of blast cells. This should particularly be true in germinal centers, where antigen injection interrupts a steady state with an increased proliferation of cells (13). Reticular cells in germinal centers as described in the present study were essentially unlabeled. Neither phagocytic nor non-phagocytic reticular cells were observed to incorporate tritiated thymidine in appreciable amounts such as would involve them in the rapidly proliferating cell populations. Schooley (37), in an autoradiographic study using animals which were hyperimmunized, also noted the rarity of labeled reticular cells during later stages of an immune response.

The possibility exists, however, that some reticular cells transform without division into lymphocytes. Thus, a labeled large lymphocyte described in our study might be a transformed reticular cell which no longer possesses the morphologic properties characteristic of the latter cell type. As reported by Ackerman (1), basal endodermal epi-
FIGURE 4  An electron micrograph of another labeled large lymphocyte (Pc) found in the germinal center. Nine silver grains lie over the nucleus. Two nucleoli are present. Free ribosomes, mitochondria (M), and small vesicles are observed in the cytoplasm. The plasma membrane of the cell is irregular and forms small extensions (arrows). × 19,000.
Figure 5 A low magnification electron micrograph showing four labeled cell nuclei and one unlabeled reticular cell (Ret). The labeled mitotic figure (Pc), probably an early prophase, has characteristics of a large lymphocyte. The other three labeled cells appear to be more differentiated. A cytoplasmic process (cyt) is in close contact with the lymphocytes. Dense bodies (Db) are located in the reticular cell cytoplasm. X 10,000.

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FIGURE 6 An electron micrograph of the second type of labeled germinal center cell. Numerous mitochondria (M), a well developed Golgi region (Go), ribosomes, and multivesicular bodies (Mvb) are present in the cytoplasm. Centrioles (Cen) are also shown. The nuclei of these cells contain chromatin blocks. These are the most frequently observed labeled cells in germinal centers. X 15,000.
thelial cells of the bursa of Fabricius underwent a reduction in the amount of rough endoplasmic reticulum with an increase in the number of ribosomes in their transformation into lymphoblasts. An identical cytodifferentiation (or dedifferentiation) might occur in germinal centers in the absence of cell division, leading to the transformation of reticular cells to lymphocytes.

Another possibility is the claim (23) that a special type of reticular cell found in germinal centers was observed in division more frequently than the large lymphocytes. Marshall and White (22) discussed different types of reticular cells in immunized rabbits, and Galindo and Imaeda (12) with the electron microscope indicated two types of reticular cells in mouse splenic white pulp. Weiss (40) has also indicated several types of reticular cells in rabbit spleen white pulp. Our electron micrographs of labeled mitotic figures consistently show them to be lymphocytes rather than reticular cells.

There are alternatives to reticular cells as the source of large lymphocytes. Specifically, in antigen-stimulated cells, as proposed by McGregor and Gowans (24), a transformation of small lymphocytes into larger, primitive-appearing blast cells must be considered. Nossal and Måkelä (26) argued against the transformation of small lymphocytes during a secondary immune response, and Nossal et al. (27) found no evidence for their transformation in a primary response. Since two types of labeled cells in active germinal centers were observed, it is possible that the smaller cells form the large basophilic lymphocytes as was suggested by Maximow (23). Also, as pointed out by Weiss (40), lymphocytes observed by electron microscopy are a morphologically varied group. Distinctions between the usual categories of lymphocytes based on size are not entirely valid in electron microscope observations. Thus, both André-Schwartz (3) and Weiss (40) have suggested from their electron microscope observations that medium or small lymphocytes possess the morphologic criteria for cells which engage in immunologic phenomena.

It is believed on the basis of histologic (5, 6) and autoradiographic (13) data that cells found in germinal centers participate in the formation of large pyroninophilic cells associated with an immune response. The hemocytoblasts described by André-Schwartz (3), which proliferate in regional lymph nodes in response to skin homografts, had clusters of ribosomes in contrast to the freely dispersed ribosomes described in large lymphocytes in this report. The presence of free ribosomes in stimulated large basophilic cells, observed during the very early intervals after antigen administration, is in accord with the belief of Polidard et al. (32) that ribosomes first appear to be free and then form clusters (polyribosomes) in the course of cytodifferentiation of a family of cells which respond to antigen stimulation and lead to the formation of plasma cells.

The present study supports the hypothesis that antigen stimulates a population of rapidly proliferating cells found in germinal centers (although not exclusively) very quickly after administration. Although the precise origin of these cells is in doubt (as is their fate), evidence is presented to show that DNA-synthesizing large and medium lymphocytes found in active germinal centers respond to antigen injections and participate in the histologic manifestation of an immune response. No evidence was obtained in this study that reticular cells are involved in the rapidly proliferating cell populations of the germinal center.

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