STAGES OF B CELL DIFFERENTIATION
IN HUMAN LYMPHOID TISSUE*

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In the last two decades, anatomic compartmentalization of T and B cells within lymphoid tissues has been demonstrated in a wide variety of species, including man (1-5). T cells are located in the paracortical or interfollicular regions of lymph nodes and in the lymphoid tissues of the intestine and tonsil, whereas in the spleen, T cells are found primarily in the periarteriolar region of the Malpighian corpuscles. In contrast, the majority of B lymphocytes are found in lymphoid follicles. Until recently, studies in man have been severely limited by the lack of specific reagents to define T and B lymphocytes and their subpopulations. This situation has improved with the development of monoclonal antibodies against specific populations of lymphocytes. We have reported on studies using a panel of T cell-specific monoclonal antibodies in which the exact anatomic location in the human lymph node of subpopulations of T lymphocytes was determined (6).

The present study was undertaken to investigate the distribution of B lymphocytes in various stages of differentiation within human lymphoid tissue. Lymphocytes of B cell origin have been shown to express a large number of cell surface determinants. Human B cell differentiation has been studied through the identification of several cell surface markers, including the heavy chain isotypes of immunoglobulin (7-8), HLA-D related Ia-antigens (9-10), receptors for the C3 component of complement (11, 12), receptors for the Fc portion of IgG (13, 14), and receptors for murine and monkey erythrocytes (15, 16). Although these markers have provided some insight into the sequence of B cell differentiation in man, their utility has been limited because they are also expressed on cells of other lineages. Recently, we have reported on the development and characterization of two monoclonal antibodies that define B cell-specific differentiation antigens (17-20). These antigens, termed B1 and B2, have been shown to be distinct from previously described B cell surface determinants. Moreover, they are distinct from each other, as shown by their molecular weights and their restricted expression on normal and malignant B lymphocytes (17-20). In pokeweed mitogen-induced B cell differentiation, B2 and then B1 antigens are sequentially lost from the B cell surface (19).

In the present study, the cellular localization of the B cell-specific antigens, B1 and

B2, was examined in frozen sections of normal human lymphoid tissues. In addition, the cellular expression of heavy chain isotypes (IgM, IgD, and IgG), Ia, and T10 antigens was investigated. These studies demonstrate distinctive B cell differentiation states within lymphoid follicles. The relationship of the B cell compartments to the distribution of T cell subsets was analyzed.

Materials and Methods

Five lymph nodes, four tonsils, and two appendices were obtained from surgical procedures performed for diagnostic or therapeutic purposes. All specimens showed follicular and diffuse hyperplasia. The specimens were snap frozen in OCT compound (Ames Co., Div. of Miles Laboratories, Inc., Elkhart, Ind.) and stored at −70°C.

Monoclonal Antibodies.   The production and characterization of the monoclonal antibodies anti-B1 and anti-B2 have previously been reported (17, 18). The antibodies were obtained as ascitic fluid from mice injected with hybridoma cells. Control ascitic fluid was obtained from mice injected with nonsecreting hybridoma cells. The reactivity of anti-B1 and anti-B2 with suspensions of normal human cells from several sources is summarized in Table 1.

In addition, monoclonal antibodies directed against human IgM, IgD, and IgG were used (prepared by V. Raso and L. Nadler, Sidney Farber Cancer Institute, Boston, Mass.). Ia-like antigens were detected by the use of anti-I1 and anti-I2 monoclonal antibodies, which recognize nonpolymorphic regions of human Ia-like antigens (21, 22).

T cells and T cell subsets were identified by the use of the following monoclonal antibodies: anti-T1 and anti-T3 (all T cells), anti-T4 (helper/inducer T cells), anti-T8 (supressor/cytotoxic T cells), and anti-T10. In addition to reacting with significant proportions of activated T cells, anti-T10 also reacts with a minority of resting peripheral T cells, B cells, and monocytes. The production and characterization of these antibodies has been described in detail elsewhere (23).

Immunoperoxidase Procedure.   A four-step immunoperoxidase technique was used to stain 4-mμ thick, acetone-fixed, frozen tissue sections (6, 24). The sections were incubated with 1:100–1:500 dilutions of monoclonal antibody containing ascitic fluid. Control sections were incubated with phosphate-buffered saline or control ascitic fluid. All sections were incubated further in sequence with rabbit anti-mouse immunoglobulin (DAKO-immunoglobulins A/S, Copenhagen, Denmark), swine anti-rabbit immunoglobulin, and peroxidase-rabbit-anti-peroxidase complex (DAKO-immunoglobulins A/S, Copenhagen, Denmark). The sections were stained by incubation of slides in a solution of 3-amino-9-ethyl carbazol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and hydrogen peroxide, and coverslipped with Elvanol (Dupont Co., Wilmington, Del.).

In addition, sections were also stained by using another peroxidase substrate, 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) (25). Further darkening of the reaction product was obtained by incubation of slides in 1% osmium tetroxide (Polysciences, Inc., Warrington, Pa.) for 2 min.

TABLE I

Reactivity of Anti-B Cell Monoclonal Antibodies as Detected in Cell Suspension by Indirect Immunofluorescence*

Monoclonal antibody	Peripheral blood mononuclear cells	Bone marrow	Thymus	Lymph node	Tonsils	Spleen
Anti-B1	9‡	<5	0	36	64	35
Anti-B2	6	0	0	28	45	26

* Data from references 17 and 18.
‡ Percentage of reactivity (approximate).

Results

Distinct staining patterns were clearly discernible after the use of the monoclonal antibodies with the four-step immunoperoxidase technique and were easily distinguishable from the faint background staining obtained with control ascitic fluid. The patterns of staining were similar in lymph nodes, tonsils, and appendices (Table II).

All lymphoid follicles stained with the anti-IgM antibody. Most cells in the primary follicles and the mantle zones of secondary follicles showed intense staining in a peripheral pattern that was consistent with membrane staining (Fig. 1). Most of the stained mantle-zone cells were located on the capsular or mucosal side of the follicle. The cells within the germinal centers also stained, but in a different pattern, which appeared to reflect not only staining of surface IgM, but of cytoplasmic and intercellular material as well (Fig. 1). The surface staining for IgM appeared to be fainter than in the mantle zone.

In contrast to the expression of IgM throughout the lymphoid follicles, the expression of IgD was more compartmentalized. Anti-IgD resulted in intense peripheral staining of most cells in primary follicles and in mantle zones of secondary follicles, but there was no staining of cells within the majority of germinal centers (Fig. 2); however, a rare IgD-positive cell was seen in some germinal centers. In the interfollicular and paracortical regions, scattered cells expressed surface IgM and IgD, and a few showed cytoplasmic staining for IgM.

Staining for IgG showed yet another pattern; within primary follicles and mantle

TABLE II
*Phenotype of B Cells in the Secondary Follicle**

Monoclonal antibody	Mantle zone	Germinal center
Anti-IgM	Peripheral staining of a majority of cells‡	Weak peripheral and intense cytoplasmic and intercellular staining throughout most of the germinal center
Anti-IgD	Peripheral staining of a majority of cells	Only rare cells with peripheral staining in a few germinal centers
Anti-IgG	Peripheral staining of very few scattered cells	Peripheral and cytoplasmic staining of a majority of cells with some intercellular staining
Anti-I1, anti-I2	Intense peripheral staining of a majority of cells	Less intense peripheral staining of most cells and perhaps some cytoplasmic and intercellular staining
Anti-B1	Peripheral staining of almost all cells	Peripheral staining of a majority of cells with some intercellular or cytoplasmic staining
Anti-B2	Faint peripheral staining of a large number of cells, intense staining around a small number of cells, which may be intercellular	Intense staining throughout the germinal center, much of which may be cytoplasmic or intercellular, in addition to peripheral staining
Anti-T10	No staining	Faint peripheral staining of a majority of cells; intense cytoplasmic staining of a variable number of cells

* Staining of frozen tissue sections performed by the peroxidase-anti-peroxidase technique (see Material and Methods).

‡ Peripheral staining indicates membrane staining. Cytoplasmic staining reflects diffuse staining; with this pattern, it cannot be determined whether membrane staining is also present. Statements comparing the relative intensity of staining refer to differences between the mantle zone and germinal center with the same antibody.
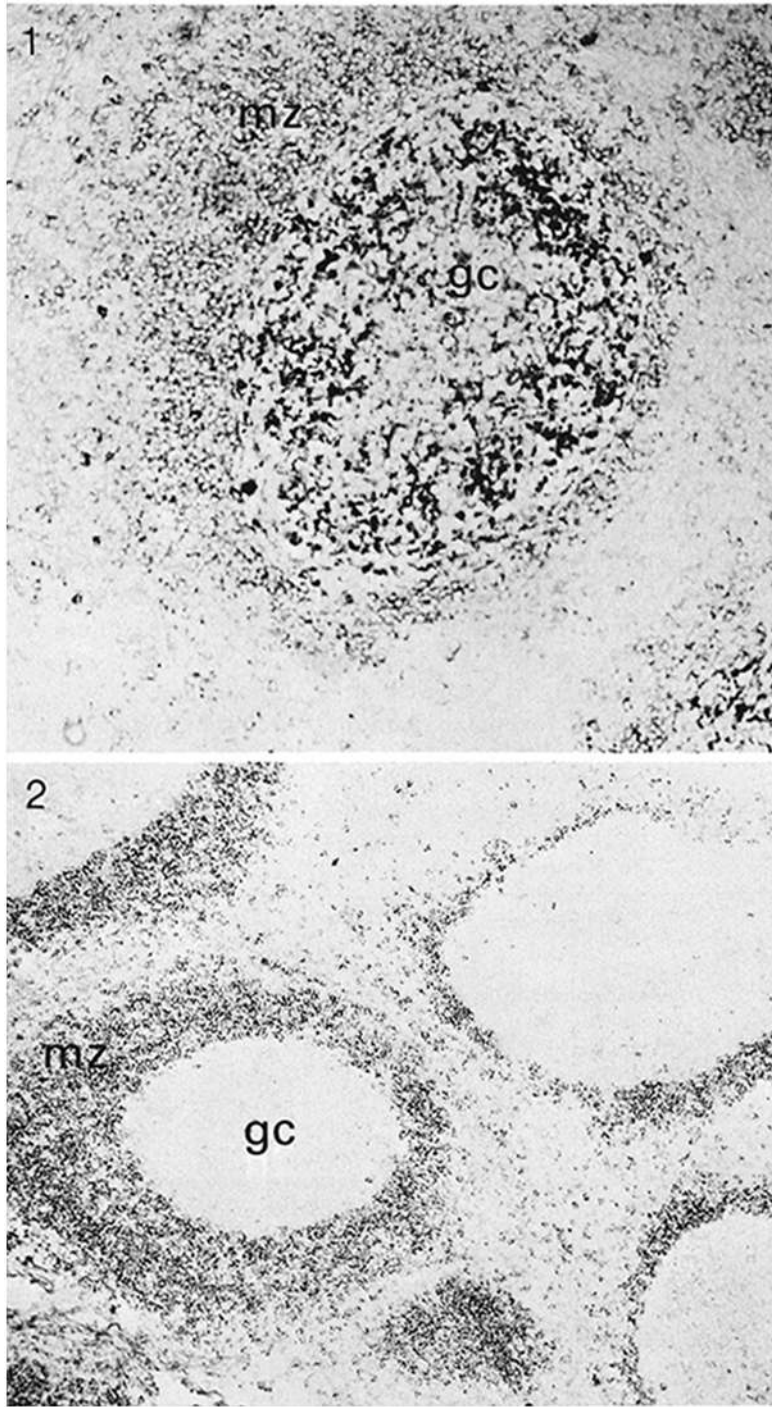
Fig. 1. A section of tonsil stained with anti-IgM antibody. A majority of the small lymphocytes in the mantle zone (mz) show peripheral staining. There are more stained cells on the left and upper side of the secondary follicle. The germinal center shows weak peripheral and strong cytoplasmic and intercellular staining. × 200.

Fig. 2. Tonsil stained with anti-IgD antibody. There is intense peripheral staining of a majority of lymphocytes in the mantle zones (mz) of secondary follicles. In contrast, except for an occasional cell with peripheral staining, the germinal center (gc) areas do not stain. Scattered IgD-positive cells are also present in the interfollicular areas. × 100.

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zones of secondary follicles, only a small number of cells were stained, in a peripheral pattern. However, most if not all cells within germinal centers showed membrane and possibly cytoplasmic staining; in addition, there was some intercellular staining (Fig. 3). Thus, the germinal center staining pattern of IgG was similar to that of IgM, although there appeared to be less intercellular staining. In addition, small numbers of cells scattered in the paracortical and interfollicular areas showed peripheral staining for IgG. In extrafollicular regions, cytoplasmic staining with anti-IgG was seen in plasma cells.

The monoclonal antibodies anti-I1 and anti-I2, which recognize nonpolymorphic regions of human Ia-like antigens, showed peripheral staining of most of the cells within primary and secondary follicles (Fig. 4). Most of the cells in the mantle zone stained more intensely than in the germinal centers. Intensely stained large cells with cytoplasmic extensions, considered to be interdigitating reticulum cells, were seen in the paracortical and interfollicular regions. In addition, scattered cells with a similar type of staining were seen in the surface epithelium of the tonsil; these probably represent Langerhans cells. Although most lymphocytes in the paracortical and interfollicullar areas were unstained, a variable number showed peripheral staining with anti-I1 and anti-I2.

Staining with anti-B1 and anti-B2 was primarily restricted to lymphoid follicles, although small numbers of cells in paracortical and interfollicular areas were also stained. With anti-B1, there was peripheral staining of cells throughout primary and secondary follicles (Figs. 5 and 6); in addition, there appeared to be some intercellular or cytoplasmic staining in germinal centers. With anti-B2, the majority of the
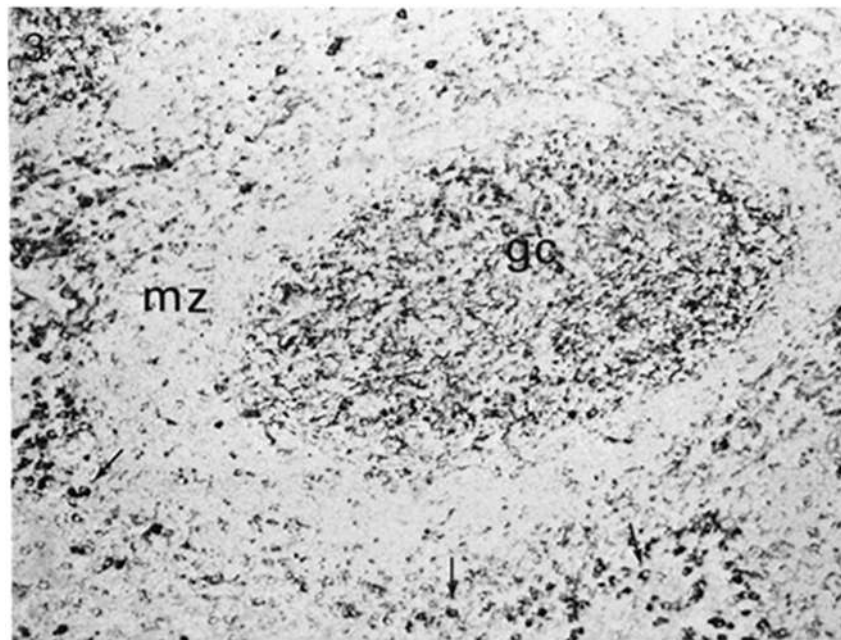

Fig. 3. A section of tonsil stained for IgG. The germinal center (gc) shows predominantly peripheral and cytoplasmic staining of a majority of the cell, as well as some intercellular staining. Only a few cells in the mantle zone (mz) demonstrate peripheral staining. Plasma cells, outside the secondary follicle (arrows) show intense cytoplasmic staining. × 160.
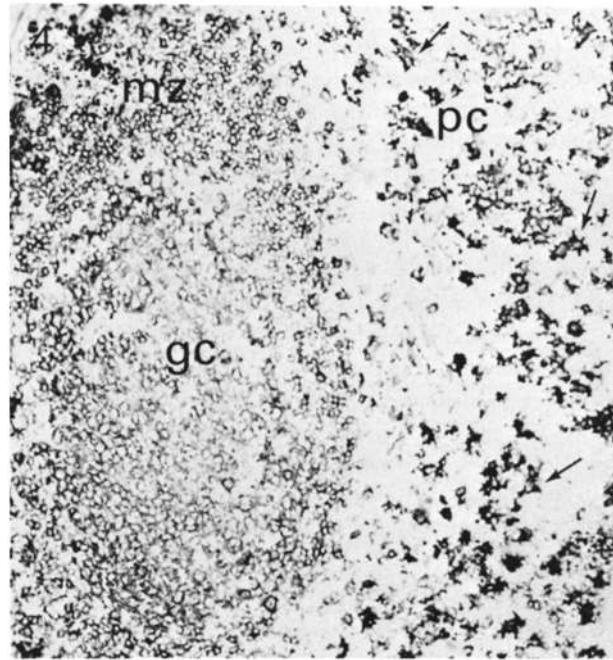
FIG. 4.   A section of lymph node stained for Ia-antigen (I2). A majority of the cells in the mantle zero (mz) and germinal center (gc) show peripheral staining. Most of the mantle zone cells are intensely stained. Large cells with cytoplasmic extensions, considered to be interdigitating reticulum cells (arrows), are diffusely stained. In addition, a small number of cells in the paracortical area show peripheral staining. × 160.

peripheral cells in primary follicles and in the mantle zones of secondary follicles were stained only faintly (Figs. 7 and 8). However, apparently intercellular, intense staining was seen in some areas of the mantle zone, especially adjacent to the germinal centers, and in primary follicles. Within germinal centers there was intense staining for B2, and in most germinal centers this appeared to be principally either cytoplasmic or intercellular (Figs. 7, and 8); because of this pattern it was difficult to evaluate the degree of peripheral staining. However, in some germinal centers in which there was less intercellular staining, it was possible to see intense peripheral staining. There were some cells showing peripheral staining for B2 in the interfollicular or paracortical areas, but the number appeared to be considerably fewer than those showing peripheral staining with anti-B1, anti-IgM, or anti-IgD. Plasma cells did not stain with anti-B2 or anti-B1.

Sections treated with the anti-T10 monoclonal antibody revealed intense staining of the cytoplasm of plasma cells in the subcapsular, paracortical, and medullary regions of lymph nodes, in the mucosa of the appendix, and in the subepithelial and interfollicular regions of the tonsil. Most cells within germinal centers showed faint peripheral staining with anti-T10, and a variable but small number of cells demonstrated intense cytoplasmic staining (Fig. 9).

The distribution of subpopulations of T cells was similar in lymph nodes, tonsils, and appendices. With all the monoclonal antibodies against T cell-specific antigens (T1, T3, T4, and T8), only peripheral (membrane)-type staining was seen. Most T
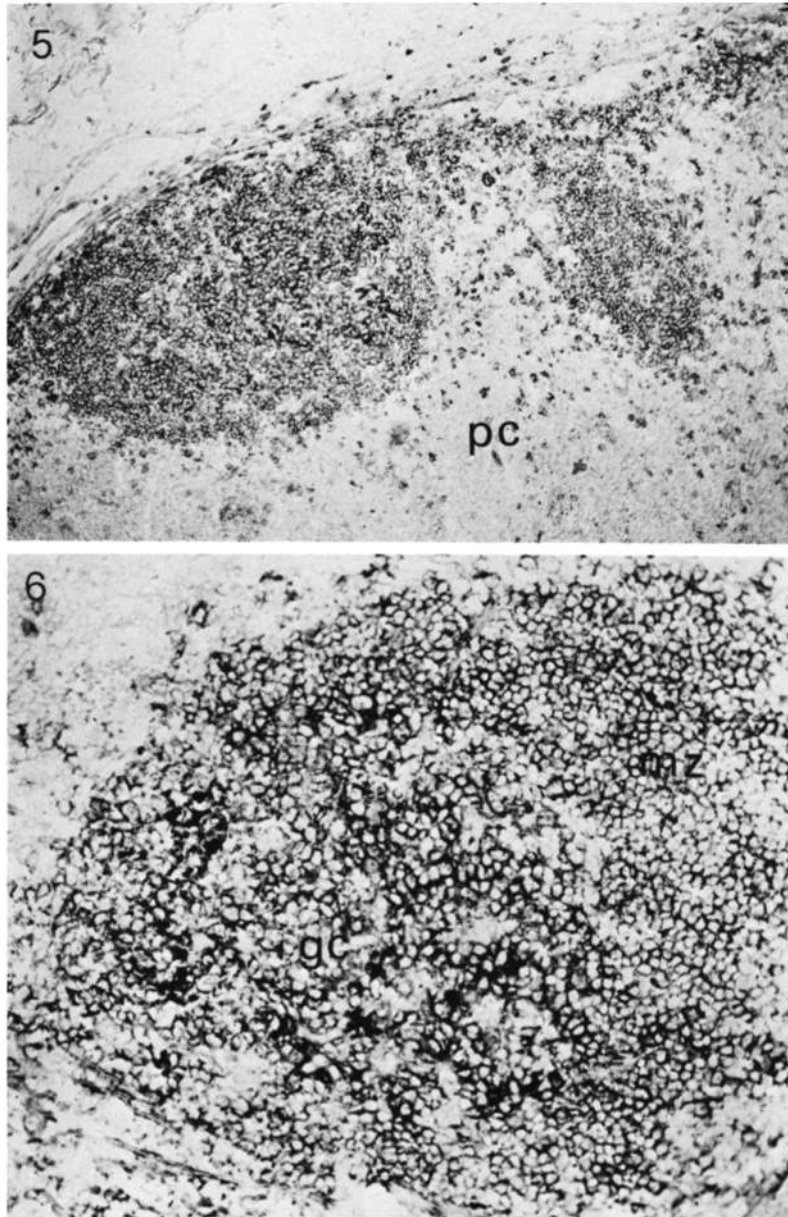
Fig. 5. Lymph node stained with anti-B1 antibody. Most of the cells in the mantle zone and in the germinal center of the secondary follicles are stained. Scattered positive cells are present in the paracortical area (pc). × 100.

Fig. 6. A higher magnification of a secondary follicle from a lymph node shows peripheral staining of most of the cells in the mantle zone (mz) and the germinal center (gc) with anti-B1 antibody. In addition, there is some intercellular and cytoplasmic staining in the germinal center. × 256.

cells were located in the paracortical or interfollicular regions (Fig. 10). The vast majority of cells reacted with anti-T4, whereas only a minority of cells were T8 positive. A small number of cells in the primary follicles and mantle zone of the
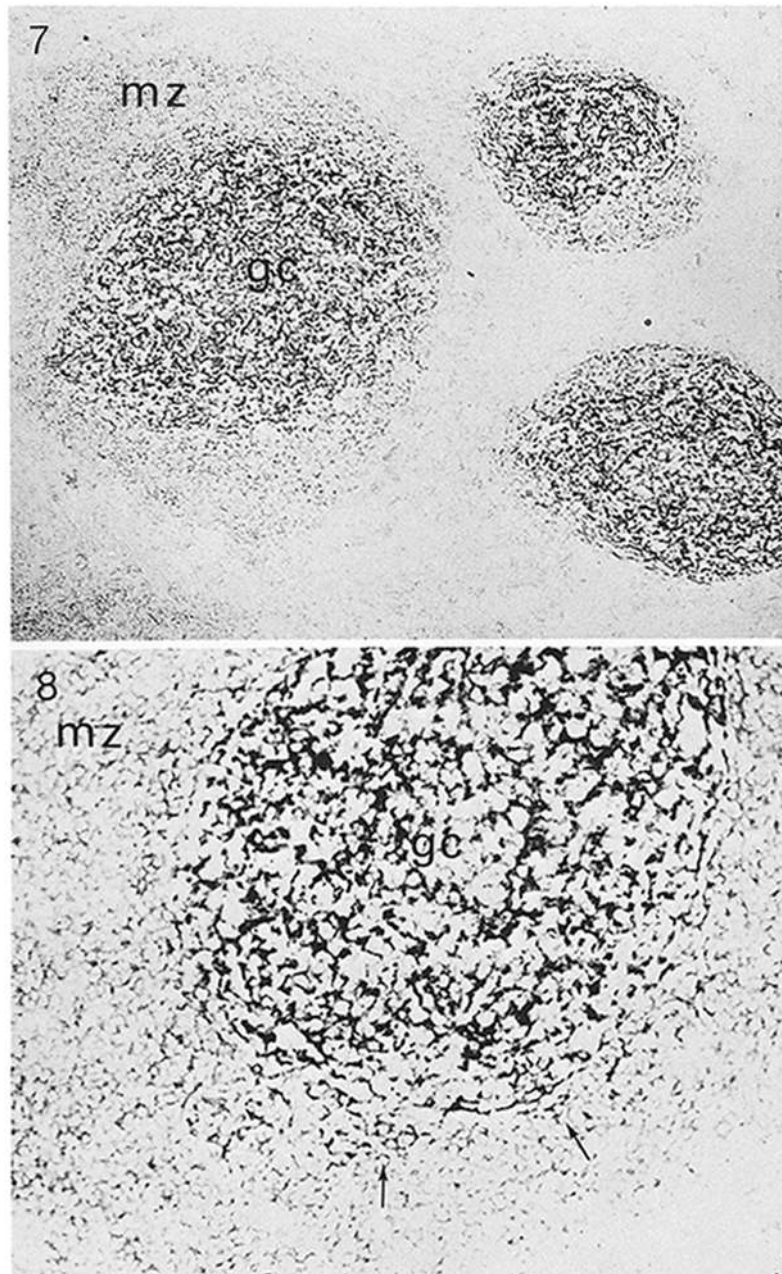
Fig. 7.   A tonsil stained with anti-B2 antibody. There is intense staining of the germinal center (gc) of secondary follicles, whereas most of the cells in the mantle zone (mz) are stained faintly. However, some mantle-zone cells immediately adjacent to germinal center are also strongly stained. The intense staining in the germinal center and in some areas of the mantle zone appears to be intercellular or cytoplasmic. × 100.

Fig. 8.   A higher magnification of a secondary follicle from a tonsil stained with anti-B2 antibody. A distinctive pattern of staining is seen in the germinal center (gc) and in some parts (arrows) of the adjacent mantle zone (mz). This type of staining probably represents intercellular material, but some may be cytoplasmic. Most of the mantle zone cells show relatively faint membrane staining. × 256.
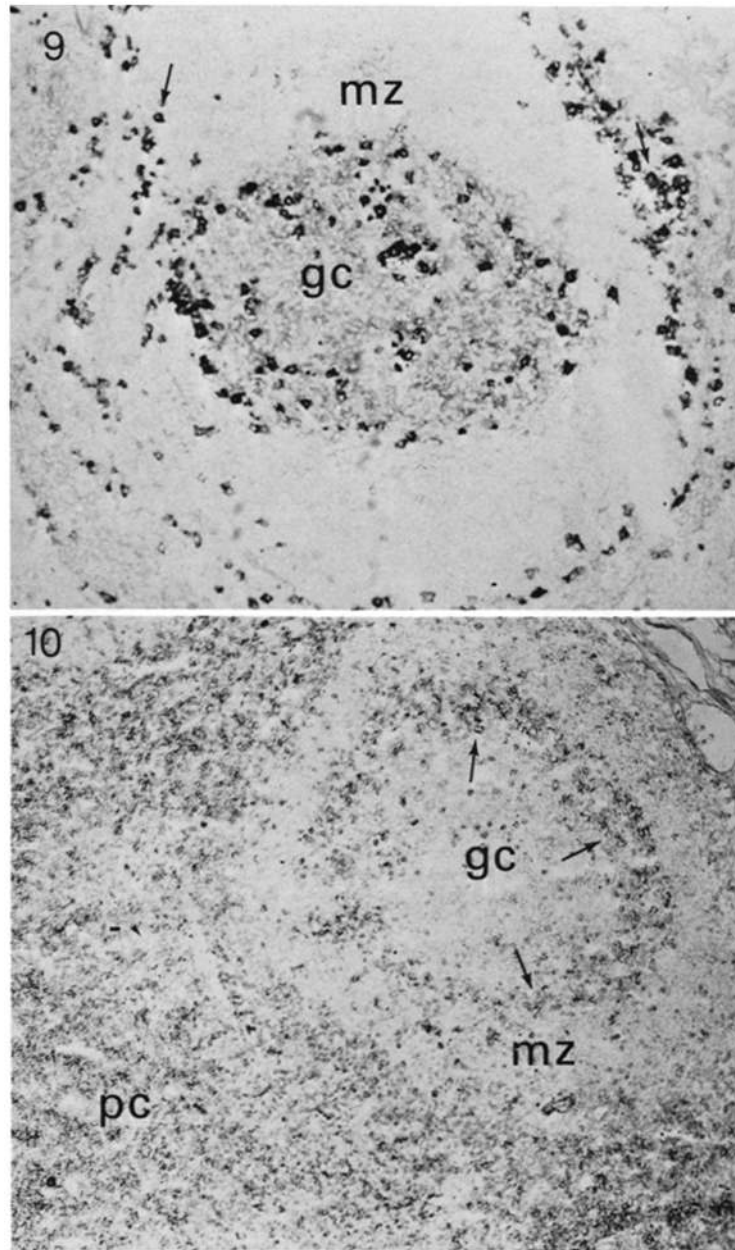
FIG. 9. Section of tonsil stained with anti-T10 antibody. Although most of the germinal center (gc) cells show weak peripheral staining, a small number of cells demonstrate intense cytoplasmic staining. Plasma cells, located outside the follicle (arrows), also show intense cytoplasmic staining. The mantle-zone (mz) cells are not stained. × 160.

FIG. 10. Lymph node stained with anti-T3 antibody. The majority of cells located in the paracortical area (pc) are stained. In addition, some positive cells are seen in the mantle zone (mz) and in the germinal center (gc) as scattered cells or arranged in groups (arrows) at the junction of mantle zone and germinal center. × 100.

secondary follicles stained with anti-T4 and anti-T8. Most of the T cells in germinal centers were T4 reactive, with only a rare cell expressing T8. A variable, generally small number of T4-positive cells were scattered throughout germinal centers. In some secondary follicles, the T4-positive cells were arranged in a clearly defined crescent at the junction of the mantle zone and the germinal center. A small number of T8-positive cells were seen beneath and in the surface epithelium of the appendix and tonsil.

Discussion

In the present report, the anatomic compartmentalization of human B cell differentiation antigens was examined. Using a panel of B cell-reactive and -specific monoclonal antibodies, frozen tissue sections of normal human lymph nodes, tonsils, and appendices were stained using a four-step immunoperoxidase technique. Monoclonal antibodies reactive with human heavy chain isotypes IgM, IgD, and IgG, as well as monoclonal antibodies to T cell and T cell subsets, gave results similar to those previously described in human lymphoid tissue (6, 26). Most cells in the primary follicles and mantle zones of the secondary follicles demonstrated peripheral (membrane) staining with anti-IgM and anti-IgD. In contrast, germinal center cells stained with IgM and IgG, but almost totally lacked IgD. The staining pattern of the germinal center indicated that cytoplasmic and intercellular IgM and IgG were also present, probably as the result of being locally secreted or trapped.

Ia and T10 antigens, although not lineage specific, have provided insight into human B cell differentiation (19, 27). Ia-like antigens were expressed on the majority of cells within primary and secondary follicles. The germinal center cells also stained with another monoclonal antibody, anti-T10. This antibody has previously been shown to react with the majority of cortical thymocytes, activated T cells, a minority of circulating B cells and monocytes (23, 24), and a majority of acute leukemias of T and non-T cell origin (28). Most germinal center cells demonstrated faint peripheral staining with anti-T10. In addition, a small, variable number of germinal center cells showed intense cytoplasmic staining with anti-T10; these probably represent early plasma cells. Plasma cells located outside the follicle also showed cytoplasmic staining with anti-T10.

Further characterization of B cells was carried out using the B cell-specific monoclonal antibodies, anti-B1 and anti-B2. The findings support the interpretation that the maturation of a primary follicle to a secondary follicle is associated with phenotypic changes. Primary follicles and mantle-zone B cells display a cell surface phenotype previously demonstrated for peripheral blood B cells (18–20), with generally intense staining for IgM, IgD, B1, and Ia, and weak staining for B2. In contrast, the germinal center cells express IgM, IgG, and B1, but not IgD, and they stain intensely for B2 and acquire a new antigen, T10. The staining for B2 in germinal centers was striking and appeared to show considerable intercellular material; the significance of this is unknown. Plasma cells were totally unreactive with anti-B1 and B2.

Somewhat similar stages of B cell differentiation have been shown by in vitro studies performed on human B cells (20). After stimulation with pokeweed mitogen, peripheral blood B cells lose B2 (days 4–5) and subsequently lose B1 (days 6–7). However, an increase in B2 expression (days 2–3) occurs before B2 disappearance.

The loss of B2 antigen in vitro appeared to be associated temporally with the loss of surface IgD, the expression of T10 antigen, and development of intracytoplasmic IgM. The loss of B1 antigen was correlated with the acquisition of surface IgG and the appearance of intracytoplasmic IgG. The findings in the present study of strong staining for B2 antigen in the germinal center cells with absence of staining for surface IgD would appear to be in conflict with the in vitro experiments (19). However, as indicated above, a transitory increase in B2 expression is seen before its disappearance during activation of peripheral B cells and it may be that there is a brief stage during which B2 persists, even though IgD has disappeared. Furthermore, the in vitro studies were performed with peripheral B cells, and it is possible that a better correlation between the phenotypic characteristics observed in tissue sections and the phenotype alterations of B cells after in vitro stimulation would be obtained by studying single cell suspensions obtained from lymph nodes or tonsils.

In the present study, it has not been possible to identify precisely various stages of B cells differentiation within the germinal center. Interpretation of the staining pattern is complicated by abundant amounts of extracellular immunoglobulins (both IgM and IgG), B2, and possibly B1. However, it is clear that the germinal center cells differ from mantle-zone cells, which are identical to peripheral blood B cells in staining pattern. The phenotypic difference appears to reflect maturation and differentiation toward immunoglobulin-secreting cells in the germinal center, as indicated by the lack of surface IgD, the presence of surface and cytoplasmic IgG, and also by intense staining of the cytoplasm of some of the germinal center cells by anti-T10 antibody.

The development of germinal centers in primary follicle results from antigen stimulation, and in the early stages may represent multiplication of B lymphocytes specifically reactive with antigen. The presentation of antigen to B cells is probably facilitated by the binding of antigen to the surface of dentritic reticulum cells located in the germinal center area (29). The finding in our previous (6) and present study of T4-positive helper/inducer cells in the germinal center and, in particular, at the junction of germinal center and mantle-zone, provides evidence for a role of T4-positive cells in the generation of secondary follicles. In support of this, nude mice, which lack a thymus, show germinal center formation in lymphoid follicles only after transfer of syngeneic thymus cells (30). The T4-positive cells in the germinal center may also play an important role in the perpetuation of B cell proliferation.

Further insights into the stages of B cell differentiation may be provided by the study of malignant lymphomas both in cell suspension and in tissue sections. It is believed that most B cell neoplasms represent frozen stages of B cell differentiation (31). The cellular architecture of these tumors and their surface phenotype may allow for additional insight into B cell differentiation.

Summary

Monoclonal antibodies reactive with B cell-specific differentiation and other antigens were used to investigate stages of B cell maturation in human lymphoid tissue, using an immunoperoxidase technique on frozen tissue sections. Lymphoid follicles, which represent the major anatomic compartment of B cells, demonstrated cellular antigenic expressions that appear to reflect differentiation of B cells. The majority of cells in the primary follicles and the mantle zones of secondary follicles expressed

surface antigens similar to those of circulating B cells, namely IgM, IgD, Ia, B1, and B2. In contrast, the germinal center cells of secondary follicles stained for IgM, IgG, B1, B2, and Ia antigens, but not for IgD, and furthermore, acquired the T10 antigen. The germinal centers stained much more intensely than mantle zones with anti-B2, whereas no such striking difference in the staining intensity was observed with anti-B1. Plasma cells, which represent the end stage of B cell differentiation, showed intense cytoplasmic staining with the anti-T10 antibody. The results indicate that the generation of germinal center cells in primary lymphoid follicles involves phenotype changes that correspond largely to those previously observed after both antigenic and mitogenic activation of B lymphocytes.

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