Phenotypic Expression of B-Lymphocytes

2. Immunoglobulin Expression of Germinal Center Cells

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With a sensitive and specific immunohistochemical technique, immunoglobulin expression was examined in germinal centers with frozen and paraffin-embedded tissues. The majority of cells positive for surface immunoglobulin (IgM and IgD) were confined to the dark zone of the germinal center, while the cells positive for cytoplasmic immunoglobulin (IgM and IgG) were found in the light zone. There were considerable numbers of germinal center cells without identifiable immunoglobulin. The results, in conjunction with previous *in vitro* and *in vivo* B-cell activation studies, indicate that centroblasts (noncleaved cells) are formed during early stages of B-cell differentiation. Centroblasts further mature into centrocytes (cleaved cells). (Am J Pathol 1984, 114:396–402)

THE GERMINAL CENTER is a two-zone structure, the light zone and dark zone. The light zone is composed of mainly cleaved cells (centrocytes), and the dark zone, of noncleaved cells (centroblasts). There has been controversy as to whether centrocytes transform into centroblasts or vice versa.[1,2]

Recently, an *in vitro* study[3] with mitogen activation has shown that B-lymphocytes in early stages of transformation bear surface immunoglobulin (sIg), and in the later stages become positive for cytoplasmic Ig (cIg). The mitogen-activated B cells *in vitro* and the germinal center (GC) cells in tissues may not be morphologically comparable. Nevertheless, the phenotypic changes associated with B-cell activation *in vitro* would be similar to those of GC cells in tissues, because GC cells are also considered to be actively proliferating B cells.

Both frozen sections and paraffin-embedded tissues can be used to study the sIg and/or cIg expression of GC cells. The antigens are maximally preserved in the frozen tissues but correlation with cytologic features is best achieved in paraffin-embedded sections. The findings will be useful in investigating the relationship between cleaved and noncleaved cells.

Materials and Methods

Materials

Formalin-fixed, paraffin-embedded human tonsils were prepared by prewashing the specimens in normal saline for 2 hours before fixation in 10% buffered for-

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malin for 10 hours. The tissues were dehydrated through graded alcohol and xylene as in routine processing. Frozen tissues were embedded in OCT (Miles), snap-frozen in a dry ice-2-methylbutane mixture (−70 C). Four-micron sections of paraffin and frozen sections were then cut and used for immunohistochemical staining.

Reagents

Biotin-labeled mouse anti-human κ and λ, and mouse anti-human IgA and IgD were obtained from Becton-Dickinson (Sunnyvale, Calif). Mouse anti-human IgG and IgM were obtained from Coulter Immunology (Hialeah, Fla). Biotin-labeled goat anti-human IgG, IgM, IgA, κ, and λ, and goat anti-human IgD were obtained from Tago (Burlingame, Calif). Labeling reagents including biotin-conjugated horse anti-mouse, IgG, rabbit anti-goat IgG, and avidin-biotin-peroxidase complex (ABC) (Vectastain, ABC Kit, PK 4002) were obtained from Vector Laboratories (Burlingame, Calif).

Staining Procedures

The staining procedure has been described elsewhere in detail.[4-6] Briefly, frozen sections were fixed in acetone for 5 minutes and quickly transferred to Tris-buffered saline, 0.05 M, pH 7.4, and then immersed in 1% normal horse serum buffer for 5 minutes. The primary antibodies (monoclonal and polyclonal antibodies) were used at 1-2 μg/ml, followed by biotin-labeled secondary antibody (1:400). Avidin-biotin-peroxidase complex was then applied, followed by developing in DAB-Ni-H_2O_2 solution. When biotin-labeled antibodies were available, the reaction procedures were shortened to two steps by omission of secondary antibody. The sections were countersigned with methyl green. For paraffin sections, the tissues were deparaffinized and rehydrated as in routine processing, and only polyclonal antibodies were applied. The staining procedures were identical to that for frozen sections.

Controls

Controls for method specificity were performed by omission of primary antibody or replacement of primary antibody with nonimmune serum, BALB/c mouse serum, or mouse ascites fluid at an equivalent protein concentration.

Morphologic Evaluation of Germinal Centers

Evaluation of staining was related to three types of GC, which relate to sequential stages of GC development as determined by animal experiments.[7,8] To avoid differences related to the plane of sectioning, only GCs with eccentric lymphoid cuffs were examined.

1) Type 1 C: The GC cells were predominantly large or medium-sized centroblasts with or without tingible body macrophages. The follicular cuff was usually narrow.

2) Type 2 C: The GC had distinct dark and light zones. The extracellular reaction in the light zone could be delicate or coarse. The dark zone contained numerous centroblasts and tingible body macrophages. Staining with anti-IgG revealed that cIgG$^+$ cells constituted less than 10% of the total cell population in GCs.

3) Type 3 C: The GC may have distinct dark and light zones, or the dark zone is very small or not conspicuous. The cIgG$^+$ cells in Type 3 GCs were generally numerous (>10%).

Distinction Between Extracellular Reaction and Membranous Staining

The so-called extracellular reaction (dendritic pattern) in GCs was characterized by an irregular, branching staining around a group of 2-5 cells. The cell-associated (membranous) staining, however, showed a complete dark rim around individual cells.

Table 1—Immunoglobulin Expression in the Germinal Centers

		Type 1	Type 2	Type 3
IgD	Dark zone	60-80%	10-30%	5%
	Light zone	–	Rare (<5%)	Rare (<5%)
IgM	Dark zone	>90%	30-60%	10-20%
	Light zone	–	10-20% (cytoplasmic staining in 20-40%)	20-30% (cytoplasmic staining in 20-30%)
IgA	Dark zone	Absent	5-10% (sIgA$^+$)	5-10% (sIgA$^+$)
	Light zone	–	5-10%	5-10%
IgG	Dark zone	Absent	5-10%	Rare
	Light zone	–	5%	10-30%

Unless otherwise stated, staining in dark zone (centroblasts) is primarily membranous and staining in light zone (centrocytes) is primarily cytoplasmic. The results are expressed as the percentage of centroblasts and/or centrocytes stained. The three types of GC represent sequential stages of development.[7,8] They are described in detail in the text.

The distinction between extracellular reaction and membranous staining is generally feasible. The evaluation of cell-associated staining was further facilitated by the examination of prewashed paraffin sections, which showed minimal extracellular reactions.

Results

Immunoglobulin Expression in the Germinal Centers

The staining results with anti-IgD, IgM, IgA, and IgG in these three types of GCs are summarized in Table 1. In general, Type 1 GCs composed of large noncleaved cells showed abundant sIgD$^+$ and sIgM$^+$ cells; sIgA; sIgG or other cytoplasmic Ig$^+$ cells were rarely seen. In Type 2 GCs, sIgD$^+$ and sIgM$^+$ cells comprised about 10–30% and 30–60% of cells in the dark zone, respectively. A few cIgA$^+$ and cIgG$^+$ begin to appear in the light zones. In Type 3 GCs, sIgM$^+$/sIgD$^+$ cells in the dark zone further decreased accompanied by an increase of cIgG$^+$ cells in the light zones. In all GC except for type 1, a significant proportion of GC cells did not express or contain sIg or cIg.

Differences of Staining Patterns in Paraffin and Cryostat Sections

Both cytoplasmic and sIg could be detected in formalin-fixed tissues by polyclonal antibodies. The

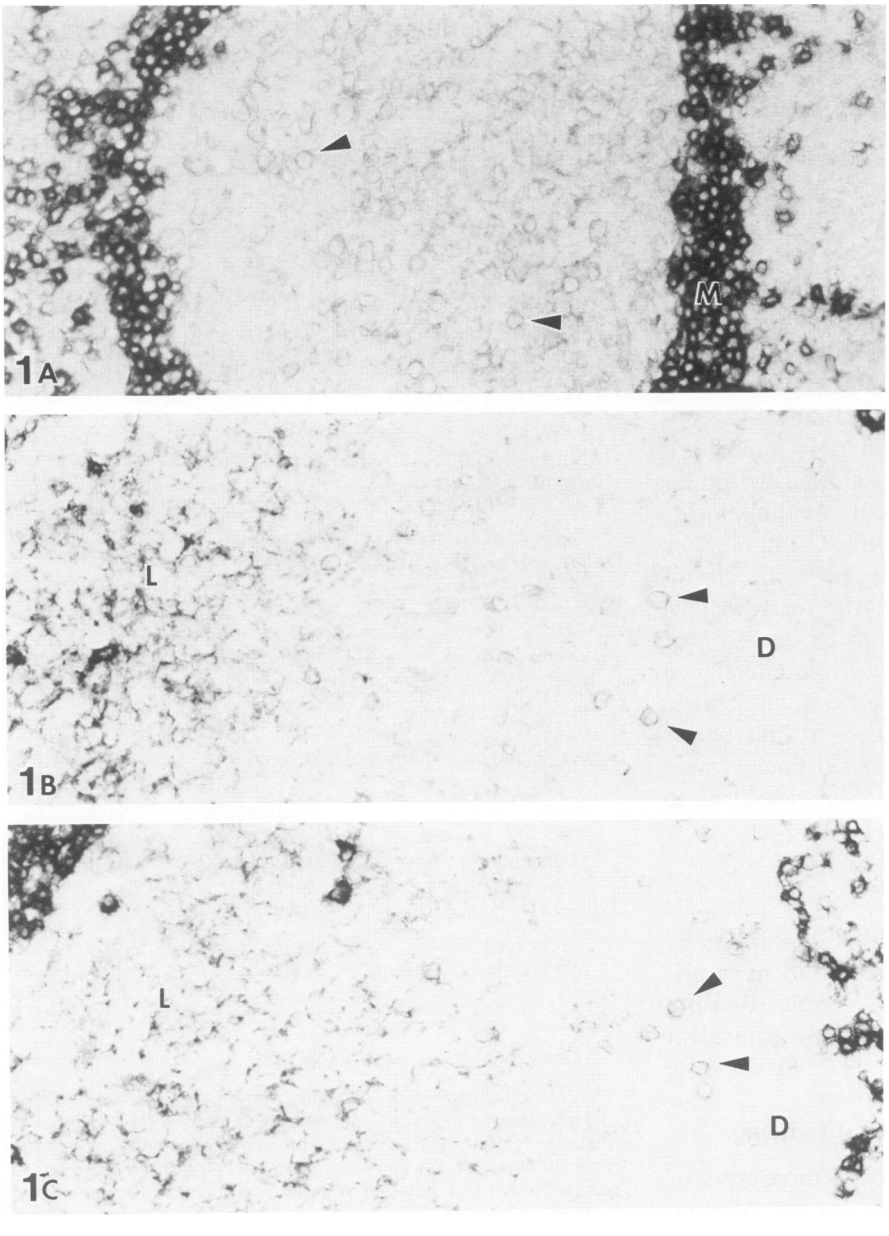

Figure 1 – IgD expression in germinal centers. **A**–Type 1. **B**–Type 2. **C**–Type 3. Numerous sIgD$^+$ centroblasts (*arrows*) can be seen in Stage 1 GC. The number of s-IgD$^+$ cells decreases as GCs proceed to Types 2 and 3. The sIgD staining of GC cells is generally weaker than that of MZ lymphocytes. Cells in the light zone do not stain for sIgD, although extracellular staining is present (**B** and **C**). *L*, light zone; *D*, dark zone. (Frozen sections, ×250)

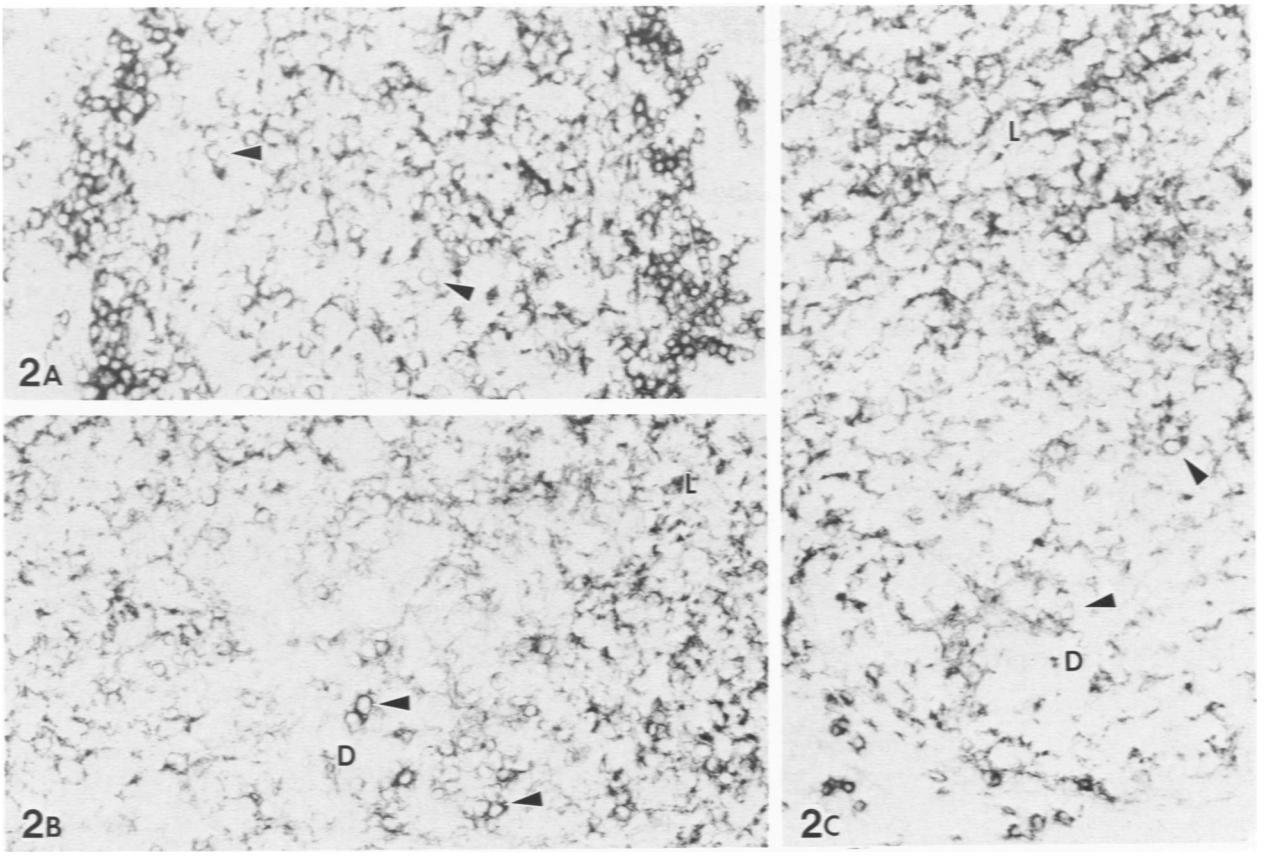

Figure 2 – IgM expression in germinal centers. **A** – Type 1. **B** – Type 2. **C** – Type 3. The number of sIgM$^+$ cells (*arrows*) decreases from Type 1 to Type 3. The majority of sIgM$^+$ cells are confined to the dark zone of the GC. In the light zone there is irregular, branching extracellular reaction present that should not be mistaken for cell-associated staining, although numerous cIgM$^+$ cells can be seen in light zones of Type 3 GCs. (Frozen sections, ×250)

results obtained with monoclonal and polyclonal antibodies in frozen sections were very similar to those with polyclonal antibodies in formalin-fixed tissues. There were, however, some noticeable differences:

1) Cytoplasmic staining in frozen sections was unexpectedly weak. The cytoplasmic reactions obtained in formalin-fixed tissues were very intense. Conversely, there were no differences in staining intensities between membranous and cytoplasmic reactions in frozen sections. Sometimes the cytoplasmic reactions were even weaker than membranous reactions. It was also noted that some of the plasma cells showed diffuse staining around the cells. The findings indicated that the frozen tissue technique is inferior to paraffin tissues for the evaluation of cytoplasmic reactions.

2) Frozen tissues have the advantage that membranous antigens are maximally preserved. The membranous reactions in paraffin sections were generally weak. For example, IgD membranous staining could be seen in many GC cells in frozen sections, but not in paraffin sections.

3) Extracellular staining in GCs was sometimes intense in frozen sections. This may interfere with the observation of GC cell reactions, especially in the light zone. In prewashed formalin-fixed sections, extracellular staining was weak and minimal, allowing more precise evaluation of GC cells.

4) Paraffin section techniques allow a better evaluation of cell size and nuclear morphology, whereas frozen sections, in general, were more difficult to interpret cytologically. Because staining with monoclonal and polyclonal antibodies produces similar findings in both paraffin and frozen sections, our results were then obtained from the observations based on a combination of both types of tissues, with emphasis on the cytoplasmic reactions in paraffin sections and membranous staining in frozen sections.

Discussion

Three types of GCs can be identified, and these relate to sequential stages of development.[7,8] We have studied Ig expression in relation to these morphologic variants in order to better understand sequential

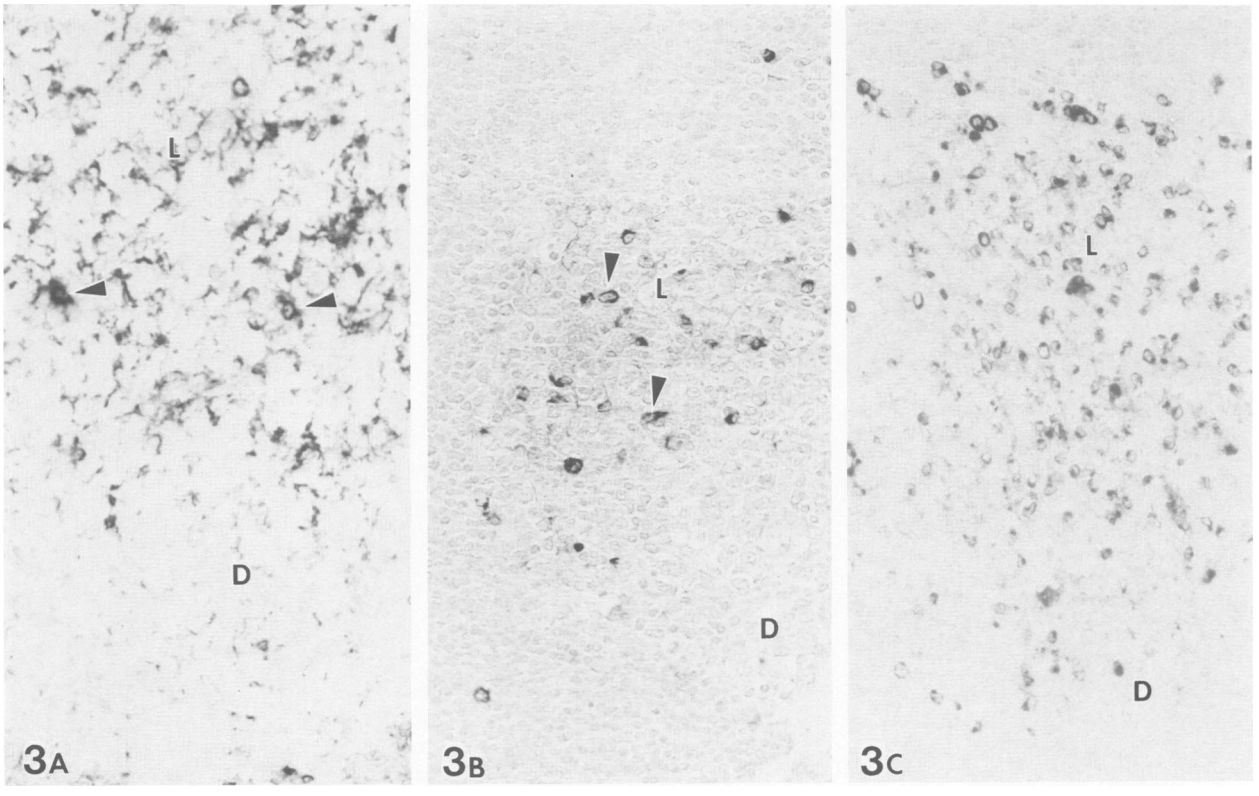

Figure 3 — IgG staining in the germinal centers. **A** — Type 2, frozen section. **B** — Type 2, paraffin section. **C** — Type 3, paraffin section. The Type 2 GCs show similar cell-associated staining in frozen and paraffin sections. Few peripheral IgG-stained cells (*arrows*) in the light zone can be identified. In frozen sections, but not in paraffin sections, an intense extracellular reaction is also present. In Type 3 GCs numerous peripheral (cytoplasmic or surface) IgG$^+$ cells are present in the light zone. (×250)

changes in Ig expression in GC cells. Our study has shown that in the early stage of germinal center development sIgM$^+$ and sIgD$^+$ cells (centroblasts) are frequently seen. With further development, sIg$^+$ centroblasts in dark zone decrease, and this is accompanied by an increase in cIg$^+$ (IgM or IgG) centrocytes and plasma cells in the light zone. A significant proportion of GC cells do not express or contain sIg or cIg. Noteworthy is the fact that the majority of sIg$^+$ cells localize in the dark zone, while the cIg$^+$ cells localize in the light zone of the follicles. Although cIg$^+$ cells in general comprise only 20–30% of total cells in the light zone, the proportion of cIg$^+$ B cells in the light zone is significantly higher because non-B lymphoid cells, such as T-lymphocytes and natural killer (Leu 7$^+$) cells, constitute up to 20–40% of cells in the light zone.[4] Among sIg$^+$ cells in the dark zone of GCs, the majority express sIgM or sIgD, whereas the majority of cIg$^+$ cells in the light zone express cIgG.

Our findings are generally in agreement with previous immunohistochemical studies.[9–13] However, there are some noticeable differences. In previous studies, the Ig expression of GC cells has been very difficult to evaluate. With polyclonal antibodies, Curran et al[10] had not been able to conclude the staining profile of GCs because of an intense extracellular staining. Recently, Poppema et al[11] and Bhan et al[12] concluded that the GC contains sIgM as well as sIgG by using the peroxidase-antiperoxidase (PAP) technique. They could not identify sIgD$^+$ cells in GCs. However, previously we had shown that the nonspecific and/or high background staining derived from the PAP and indirect conjugate methods probably produced an inconclusive result.[5] Conversely, the ABC method used in this study is highly sensitive and specific. Thus, a precise evaluation of GC staining can be obtained.[5] The ability to stain sIgD$^+$ cells in GCs is probably due to a high sensitivity of the ABC method. Both monoclonal and polyclonal reagents were used, and no cross-reactivity was observed with other immunoglobulins or B-cell-associated antigens. It should also be noted that IgD$^+$ cells are frequent only in Type 1 or early GCs. This fact might also account for their failure to be identified in earlier studies.

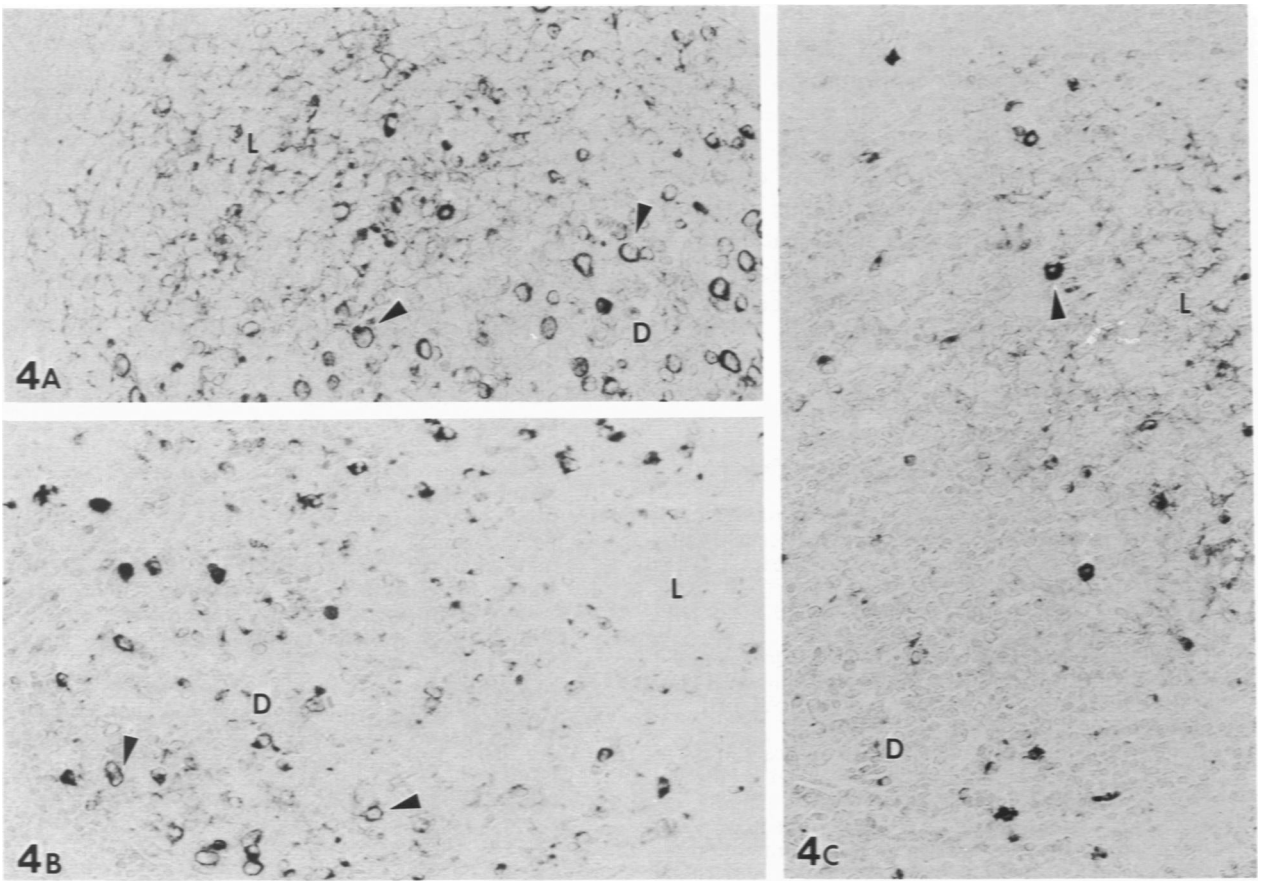

Figure 4—IgA staining in the germinal centers. **A**—Type 2, frozen section. **B**—Type 2, paraffin section. **C**—Type 3, paraffin section. In Type 2 GCs, many peripheral IgA$^+$ cells (centroblasts) and few cIgA$^+$ cells (centrocytes) are present in the dark and light zones, respectively. In the majority of GCs the number of IgA$^+$ cells was far less than shown in these examples. Cytoplasmic IgA$^+$ cells are observed in the light zone of a Type 3 GC. (×250)

The findings of Ig expression among GC cells provide an insight as to the relationship between centrocytes and centroblasts. On the basis of cytophotometric and autoradiographic investigations, Lennert[1] and Koburg[14] had concluded that centroblasts transformed into centrocytes, and the cellular proliferation in GCs is primarily a function of centroblasts. Opposing views have been expressed by Lukes and Collins[2] and supported by Taylor.[15] The results of this study, in conjunction with the results obtained from a previous B-cell mitogen activation study,[3] favor the former view. In this B-cell activation study, Stashenko et al had found that sIg$^+$ cells occurred prior to the appearance of cIg$^+$ cells. The early phase activated B cells expressed both sIgM and sIgD. By days 4 and 5, sIgD disappeared, and this was accompanied by the development of intracytoplasmic IgM. The acquisition of sIgG and cIgG appears to be a later event. The presence of sIgM or sIgD on some centroblasts would indicate that centroblasts occur in the early stage of B-cell proliferation.

The phenotypic changes associated with cells in culture may not be exactly identical to what happens in tissues, and there are some differences between our observations regarding GC staining and Ig expression associated with *in vitro* activation. First, there are a considerable number of GC cells, especially centroblasts, in Type 2 and Type 3 GCs, that have no sIg or cIg. In *in vitro* activation, loss of sIgD and the appearance of cIgM or IgG appears to be a continuous process, so that all B cells have either surface or cytoplasmic Ig, and there is no demonstrable stage of B-cell differentiation *in vitro* lacking Ig. Second, sIgG$^+$ cells are scanty in GCs. It is possible that the sIgG$^+$ cells also produce cIgG, so that the surface reactivity may not be readily detectable. Alternatively, the sIgG$^+$ stage may be very brief; and, thus, rare sIgG$^+$ cells can be found. It is also interesting to note that many sIgA$^+$ cells appear to be large centroblasts. The findings imply that heavy chain switching can go directly from IgM to IgA, or that due to the brevity of the IgG$^+$ stage, it is rarely seen.

Our study, not a dynamic one, can answer neither whether the maturation of B cells into plasma cells has to go through stages of centroblasts and centrocytes, nor whether centrocytes are derived entirely from centroblasts. However, the staining results are important to clarify the immunoglobulin expression in the GC. The lack of Ig markers in many of the GC cells seems to be consistent with our recent discovery that at least one-third (11 of 35) of follicular lymphomas do not express detectable surface or cytoplasmic Ig (manuscript in preparation).

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