Immunophenotypic characterization of primary and secondary lymphoid follicles

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Summary. The need for an immunophenotypical referential framework relative to lymphoid follicle has led us to apply a panel of monoclonal and polyclonal antibodies, by means of a sensitive immunostaining method. Lymphoid follicle is an immunophenotypically complex structure made up of three lymphoid populations (B, being its bulk, and a few T and NK cells), dendritic reticulum cells (DRCs) and Flemming’s macrophages. Follicular B population is To 15 +, B1 +, OKB 7 +, HLA-DR + and C3bR +. In secondary follicles there are differential characteristic reactivities for each topographic compartment: Mantle zone is positive for OKB 2 and surface IgM (sIgM) and IgD (sIgD); germinal center (GC) clear zone (with centrocytic predominance) for OKT 9, sIgM and weakly for OKB 2; and GC dark zone (with centroblastic predominance) only for OKT 9. In sections, OKT 10 allows one to see immunoblasts and plasma cells, the latter being with lymphoplasmacytoid cells the only intracytoplasmic immunoglobulin holders. 10% of GC lymphocytes are T cells, almost exclusively T-helper (Leu 3a +). Another 10% to 15% of lymphoid cells are Leu 7 (HNK−1) +. In histological sections, DRCs are specifically marked with R4123 and Flemming’s macrophages with anti-alpha,-antitrypsin and anti-alpha,-antichymotrypsin antibodies, both populations being negative to OKM 1 and OKM 5.

Key words: Immunophenotype - Lymphoid follicle - Immunohistochemistry

Introduction.

Lymphoid follicles are topographic domains of lymphoid B population (Weissman et al., 1978). There are many studies about the immunophenotypic characterization of these structures, either by conventional methods (Curran and Jones, 1977, 1978; Brandtzæg et al., 1978; Stein et al., 1980; Tsunoda et al., 1980; Curran et al., 1982; Matthews and Basu, 1982; Morris et al., 1983), or by monoclonal antibodies (MoAbs) (Hsu et al., 1983; Hsu and Jaffe, 1984a, 1984b; Knowles et al., 1984). Nevertheless, there are strong contradictions, specially with regard to the immunoglobulins (Igs) (Curran and Jones, 1977, 1978; Tsunoda et al., 1980; Hsu and Jaffe, 1984b).

In view of the structural complexity of secondary follicle on light microscopy (Muller-Hermelink and Lennert, 1978), it seems very difficult to assign current reactivities by immunohistochemical techniques. Therefore we have applied a wide panel of monoclonal and polyclonal antibodies on peripheral lymphoid tissues in order to clarify, with the greatest accuracy, the distinctive phenotypes of cellular populations that constitute the secondary follicle.

Materials and methods.

Sections of human tissues obtained from reactive lymph nodes, palatal tonsils, appendices, ileal mucosae and spleens were used.

The aforementioned tissues were embedded in O.C.T. Compound and snap-frozen in 2-methylbutane precooled within liquid nitrogen. Six μm sections were cut and placed on gelatinized slides prior to immunostaining.

In order to visualize several intracellular proteins, comprising various Igs, S-100 protein, alpha,-antichymotrypsin (ACT) and alpha,-antitrypsin (AAT), sections from selfsame paraffin-embedded tissues were employed.

Tables 1 and 2 summarize monoclonal and polyclonal antibodies used in this study, and their optimum concentration for immunostaining procedures.

For staining procedures, the avidin-biotin-peroxidase complex (ABC) technique was used as has been described elsewhere in detail (Hsu and Raine, 1984), using the chromogenic reaction with DAB/H2O2.
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according to Gatter et al. (1984). ABC reagents were obtained from Vector Laboratories.

Results

The negative controls displayed only unspecific stainings on sections from fresh-frozen tissues, related with endogenous peroxidase activity in eosinophils. These stainings appeared like a strong intracytoplasmic granulate, masking the nucleus.

According to the positivities obtained on sections from fresh-frozen tissues, three main staining types were seen: 1) Associated with cellular membrane, 2) reticulated or interstitial, and 3) mixed patterns. The third type, only limited to germinal centers (GCs), showed intermediate characteristics between the first and second patterns. When polyclonal antibodies and anti-Ig MoAbs were applied to embedded-paraffin sections only intracytoplasmic cellular stain became apparent.

The primary lymphoid follicles and mantle zone (MZ) of secondary follicles showed the same behaviour when they were marked with different antibodies. Both structures were completely stained with membrane markers to B cells (OKB 2, OKB 7, To 15 and B1). IgG, IgM, IgD, and kappa and lambda light chains (Fig. 1). They were also positive with To 5 (C3bR) and HLA-DR.

In the GC of secondary follicles, the markers for B cells showed a heterogeneous response. A uniform homogeneous staining was obtained with To 15 and B1 (Fig. 2), whereas OKB 7 and OKB 2 MoAbs conditioned different patterns. The GCs were completely stained with OKB 7 drawing a mixed pattern, membranous and reticulated, with stronger intensity of staining in the nearest area to the wide part of MZ (Fig. 3). A similar behaviour was shown by To 5.

While the MZ showed a strong reactivity with OKB 2, in the GC the positivity obtained with this MoAb was weak. The reactivity in GC affected half or two thirds of the GC nearest to the wide part of MZ (Fig. 4).

In sections of fresh-frozen tissues, IgG was the predominant Ig, being positive throughout the MZ and GC. The kappa predominated over lambda light chains. The IgA appeared positive in the nearest area of GC to the wide part of MZ (Fig. 5). This Ig was not revealed in MZ. The IgM was shown positive in MZ and the nearest area of GC to the wide part of MZ (Fig. 6). The IgD appeared only in MZ, while in GC it was sporadic or inexistent (Fig. 7).

Intracytoplasmic Igs (cIgs), studied in embedded-paraffin sections, only appeared in plasmablastic/plasmacytic and very scarce small round cells within GC. The cells cIg + were very scarce and they were not always present in all GCs. The cIg prevailing was IgG, followed by sporadic cIgM + cells and, in abdominal tissues, some cIgA + cells.

The markers related with DR antigenic complex (HLA-DR) stained completely and homogeneously all lymphoid cells in secondary follicles (Fig. 8).

The presence of transferrin receptor (OKT 9 +) was restricted to GC lymphoid cells and to a small amount of MZ and primary follicle lymphocytes (0% to 15%). Also Flemming's macrophages had a higher staining intensity than lymphoid cells.

With regard to T lymphoid cell markers, results were variable too. Whereas OKT 6 was shown to be wholly negative, with OKT 10 a strong staining appeared in 10% of GC lymphoid cells, which had tendency to be grouped. Centroblasts and centrocytes were lightly stained with OKT 10. The common-peripheral T-cell markers (OKT 3, Leu 1, Leu 4, OKT 11 and T2) showed a 10% of positive lymphoid cells. In GC, such cells disclosed a strong zonal distribution being restricted to the nearest area to the wide part of MZ (Fig. 9). In MZ, the ratio between Leu 3a + T-helper cells (Th) and OKT 8 +T-cytotoxic/suppressor cells (Ts) was kept to 2/1. Nevertheless, in GC that ratio raised up to Th/Ts = 12/1 (Fig. 10).

In GC a 10% to 15% of Leu 7 (HNK-1) + lymphoid cells with homogeneous distribution was exclusively revealed (Fig. 11).

With R4/23 and FHC17 MoAbs a follicular network was drawn without lymphocytic membrane pattern, displaying a stronger staining for first of them (Fig. 12). The OKM 1 and OKM 5 MoAbs, in sections of fresh-frozen tissues, and anti-S-100 protein, in embedded-paraffin sections, did not show reactivity in lymphoid follicles. Flemming's macrophages had a cytoplasmic positivity for ACT and AAT in the latter sections. J5 showed negative, or very weakly positive, staining in GCs.
### Table 1. Specificity, Optimum Work Concentration and Source of Monoclonal Antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Work Concentration</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT 6</td>
<td>Thymocytes</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>OKT 10</td>
<td>Stem Cells, Thymocytes, Prothymocytes, Null Cells, Monocytes (+/-), Activated</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>OKT 3</td>
<td>T Cells</td>
<td>1/5</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>OKT 11</td>
<td>E Receptor</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>T2</td>
<td>T Cells</td>
<td>1/5</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>Leu 1</td>
<td>T Cells</td>
<td>1/50</td>
<td>Becton-Dickinson</td>
</tr>
<tr>
<td>Leu 4</td>
<td>T Cells</td>
<td>1/50</td>
<td>Becton-Dickinson</td>
</tr>
<tr>
<td>Leu 3a</td>
<td>Helper/inducer T-subset</td>
<td>1/50</td>
<td>Becton-Dickinson</td>
</tr>
<tr>
<td>OKT 8</td>
<td>Suppressor/cytotoxic T-subset</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>OKB 2</td>
<td>slg + B Cells, Squamous Epithelium, Granulocytes</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>OKB 7</td>
<td>slg + B Cells</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>To 15</td>
<td>B Cells</td>
<td>1/10</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>B1</td>
<td>B Cells</td>
<td>1/100</td>
<td>Coulter Clone</td>
</tr>
<tr>
<td>DK21 (lgD)</td>
<td>Delta Heavy Chain</td>
<td>1/10</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>DK20 (lgM)</td>
<td>Mu Heavy Chain</td>
<td>1/10</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>SL 3 (lgG)</td>
<td>Fr Fraction of IgG</td>
<td>1/2000</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>GA-1 (lgA)</td>
<td>Alpha Heavy Chain</td>
<td>1/1000</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>SL 2</td>
<td>Kappa Light Chain</td>
<td>1/2000</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>DK26</td>
<td>Lambda Light Chain</td>
<td>1/10</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>Leu 7 (HNK-1)</td>
<td>Large Granular</td>
<td>1/50</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>OKM 1</td>
<td>Monocytes, Granulocytes</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>OKM 5</td>
<td>Monocytes, Platelets</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>FHC17</td>
<td>Monocytes, Macrophages, Langerhans Cells</td>
<td>1/2000</td>
<td>Sera-Lab</td>
</tr>
<tr>
<td>R4/23</td>
<td>Dendritic Reticulum Cells</td>
<td>1/10</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>To 5</td>
<td>CD8R</td>
<td>1/10</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>DK22</td>
<td>Beta Chain of HLA-DR Molecules</td>
<td>1/10</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>OKT 9</td>
<td>Transferrin Receptor</td>
<td>1/10</td>
<td>Ortho Diagnostic</td>
</tr>
<tr>
<td>J5</td>
<td>Common Acute Lymphoblastic Leukaemia Antigen</td>
<td>1/160</td>
<td>Coulter Clone</td>
</tr>
</tbody>
</table>

### Table 2. Specificity, Optimum Work Concentration and Source of Polyclonal Antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Work Concentration</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-alpha,-antitrypsin</td>
<td>Monocytes and and Histiocytes (Kerdel et al., 1982)</td>
<td>1/500</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>Anti-alpha,-antichymotrypsin</td>
<td>Monocytes and Histiocytes (Kerdel et al., 1982)</td>
<td>1/500</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>Anti-S-100 protein</td>
<td>Langerhans Cells, Interdigitating Reticulum Cells (Kahn et al., 1983)</td>
<td>1/400</td>
<td>Dakopatts</td>
</tr>
</tbody>
</table>
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Discussion

The secondary lymphoid follicles are phenotype
tically asymmetrical structures made up of a GC and a
surrounding lymphoid ring named mantle zone. The GC
presents a lighter stained area (clear zone), close to the
wide part of MZ with centrocytic predominance, and o
the opposite side a dark stained area with centroblastic
predominance (Muller-Hermelink and Lennert, 1978).
Our findings reveal that surface markers for B cells and
Igs behave heterogeneously, and that a follicular asymm
ancy is maintained when immunohistochemical
techniques are applied. The complexity of this structure
increases with the presence of other T and NK lymphoid
cells and reticular cells.

Whereas with To 15, B1, and anti-DR system
antibodies homogeneous follicular staining is obtained,
the results with To 5, OKB 7 and OKB 2 are different.

Applying To 5 (anti-C3bR) and OKB 7 MoAbs, the
MZ lymphocytes are uniformly stained, but in the GC an
asymmetric and partially reticulated staining is seen.
Other investigators have recognized C3 fraction
receptors in GC lymphoid cells by means of rosetting
techniques (Stein et al., 1980), and in dendritic reticular
cells (DRCs) by immunoserum (Reynsc et al., 1985).
However, with such sera Tsunoda et al. (1980) found the
aforementioned receptors only in 50% of GC lymphoid
cells employing a more specific method than that of
rosetting. The double presence of positivity for lymphoid
cells and DRCs should explain the mixed pattern of GC
staining.

It is well known that OKB 7 and OKB 2 MoAbs are
positive in almost the totality of slg + lymphoid cells
obtained from blood and cellular suspensions from
lymphoid tissues (Mittler et al., 1983). In secondary
follicle, with OKB 7, we have obtained a staining similar
to that of To 5. Therefore, this fact lead us to consider
OKB 7 a marker for lymphocytes and DRCs. Knowles et
al. (1984) corroborate the reactivity for OKB 7 in GC
lymphoid cells, but do not describe this reaction in
DRCs. However, some of our cases diagnosed as
centroblastic-centrocytic follicular lymphomas, whose
malignant cells were OKB 7 unreactive, clearly showed
OKB 7 + DRCs (De Luaces et al., 1987). The greater
density of DRCs in GC clear zone should explain the
stronger staining intensity obtained in this zone with this
MoAb.

In the present study, OKB 2 has shown positivity in
MZ, and a light staining in the clear zone of the GC.
Therefore, this MoAb spares the portion of centroblastic
predominance where the GC proliferative activity is
yielded (Stein et al., 1985). This light staining with zonal
pattern obtained in the space of centrocytic predominance
does not confirm the assertion that OKB 2 is a true pan-B marker (Knowles et al., 1984; Knowles, 1985). Moreover, it is not established that this marker
must stain DRCs.

With regard to Igs, our results have been very
different depending on if the tissues were fresh-frozen or
paraffin-embedded. Although there are some papers
which agree that predominant Igs in the GC are of IgG
type (Brandtzæg et al., 1978; Curran and Jones, 1977,
1978), the interpretation of their cellular specificities is
controversial. There are reports recording GC Igs being
extracellular (Curran and Jones, 1977; Hsu et al., 1985;
Hsu and Jaffe, 1984b), whereas in other ones a significant amount of slg + lymphoid cells is recorded
(Curran and Jones, 1978; Tsunoda et al., 1980).
Most Igs, mainly IgG, probably make up part of
electron-dense material placed between the cytoplasmic
processes of the DRCs (Muller-Hermelink and Lennert.
1978).

The researchers agree that the MZ lymphocytes are
slgD - and slgM +. However, the GC expression report
mainly in the clear zone and slgD is absent in that
structure. We conclude that IgM is predominantly
expressed on the centrocytic cell surface, unlike other
reports stating that slg is found only in very scanty GC
cells (Hsu et al., 1984), particularly centroblasts (Hsu
and Jaffe, 1984b). Nevertheless, our opinion is in
agreement with some previous experimental works. So,
the has been shown, in enucleated lymphoid follicles from
palatal tonsils, that half of GC large lymphocytes are slg-
loser (Tsunoda et al., 1980). Furthermore, small
lymphocytes simultaneously bearing slgD and slgM, and
large lymphoid cells bearing only slgM are seen by means
of separation studies from murine spleen cells (Goodman
et al., 1975). Therefore it does not seem strange that a
wide amount of centrocytes are slg-losers, whereas
centroblasts are slg-negative. This idea is concordant
with the findings obtained in studies about Ig expression
in human lymphoid cell lines. During their development,
these cells attain an immunoglobulin phenotype
corresponding to a mature B cell (slgM + and slgD -).
Thereafter they completely lose their immunoglobulin expression, regaining it subsequently
like a pre-B cell (slgM +) (Gugliemi and Preud'Home, 1981).

In relation to c1gs, our results are in concordance
with those of other investigators with regard to the
scanty presence of c1g + cells in GC, being clearly
predominant c1gG (Tsunoda et al., 1980; Matthews and
Basu, 1982; Brandtzæg et al., 1983; Morris et al., 1983;
Hsu and Jaffe, 1984b). These cells mainly correspond to
the late stages of plasma cell differentiation, whereas
normal immunoblasts lack aforementioned c1g (Morris
et al., 1983). However, Hsu and Jaffe (1984b) report some
c1g + centrocytes. We believe that scarce c1g +
small lymphoid cells are lymphoplasmacytoid ones.
The latter, unlike centrocytes, have copious rough
endoplasmic reticulum (Muller-Hermelink and Lennert.
1978).

With regard to results obtained by means of anti-T cell
MoAbs, two different events occur: OKT 10 positivity
for B cells in GC, and presence of an accompanying T
lymphoid population withing GC.

The OKT 10 antibody has been reported like reactive
for B cell population, when this is maturing towards
plasma cell and losing other surface B markers. OKB and
B1 (Mittler et al., 1983). Previously, OKT 10 + cells has
been described in whole GC, with a greater positivity in clear zone (Hsu and Jaffe, 1984a). Nevertheless, our results denote that OKT 10 is weakly reactive with centrocytes and centroblasts, whereas it is strongly positive for immunoblasts/plasma cells.

In relation to presence of T lymphocytes in GC, there is a strong predominance of these in clear zone, mainly being a T-helper population (Sti et al., 1983). This finding confirms the asymmetrical nature of secondary follicles. Their presence corroborates the importance of T-B cooperation (McMillan et al., 1981).

The observation of Leu 7 (HLA-1) + cells in GC suggests that this population can be related with the control of GC development. This same event has been suggested by other investigators (Swerdlow and Murray, 1984).

Among reticular cells associated to lymphoid follicles, DRCs and Fleming's macrophages, the former is specifically marked with R4/23 MoAb. Since DRCs are positive for FHC I7 too, then we should allocate for them a mononuclear/phagocytic system origin, such as other writers have suggested (Gerdes et al., 1983). However, they do not show reactivity for OKM 1 and OKM 5 (MoAbs to soluble antigen presenting monocyctic/macrophagic system cells (Shen et al., 1983)). The lack of reactivity for OKM 1 and OKM 5 suggests that DRCs may have a mesenchymal origin, unrelated to monocyctic/macrophagic system, which is in agreement with previous ultrastructural studies from developing lymph nodes (Groscurth, 1980; Sakuma et al., 1981; Markgraf et al., 1982).

In relation to Fleming's macrophage, this is a monocyctic/macrophagic system cell with strong phagocytic efficiency (Muller-Hermelink and Lennert, 1978; Lasser, 1983). This cell lacks antigen presenting capacity which is demonstrated by unreactivity for OKM 1 and OKM 5.

To summarize, this study has shown that secondary follicles are structures with complex and asymmetrical immunophenotype, and it has proportioned a referential framework to investigate the immunophenotypical modifications of nodular lymphomas. Moreover, some findings, which were not described or emphasized previously, were shown, for instance: IgM + cells (predominantly centrocytes) are mainly placed in clear zone of GC, but not in dark zone: OKB 2 is not a pan-B cell marker since dark zone of GC is unreactive; and OKB 7 MoAb is reactive with DRCs too.

References


69. 21-25.

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