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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 R4/23 and Flemming's macrophages with anti-alpha$_1$-antitrypsin and anti-alpha$_1$-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; Brandtzaeg 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.

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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$_1$-antichymotrypsin (ACT) and alpha$_1$-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/H_2O_2

according to Gatter et al. (1984). ABC reactives 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 stains 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.

Fig. 1. IgD + primary follicles in a lymph node. Counterstaining with haematoxylin. × 40

Fig. 2. B1 + secondary tonsil follicles. Counterstaining with haematoxylin. × 100

Fig. 3. OKB 7 + secondary follicles in a lymph node. A light zonal tendency for the staining in central follicle can be observed. Counterstaining with haematoxylin. × 100

Fig. 4. Secondary lymph node follicles marked with OKB 2. Counterstaining with haematoxylin. × 100

Fig. 5. Ileal mucosa. IgA positivity is shown in enteric epithelium and GC, in its nearest portion to the lumen. Counterstaining with haematoxylin. × 100

Fig. 6. Secondary tonsil follicle with good development and typical positivity for IgM. Counterstaining with haematoxylin. × 100

Fig. 7. Two secondary lymph node follicles with IgD + MZ. Counterstaining with haematoxylin. × 100

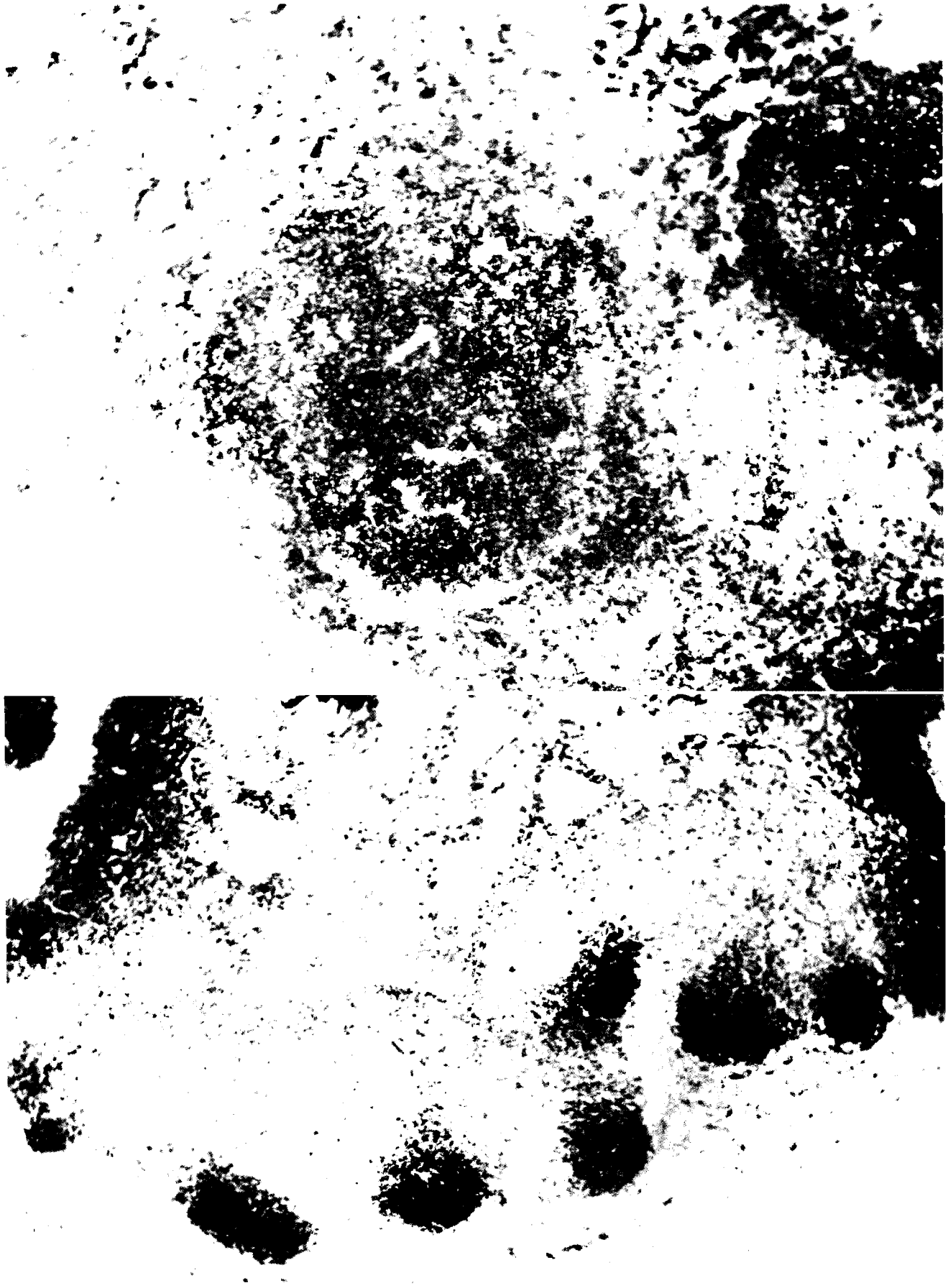
Fig. 8. Secondary lymph node follicles marked with anti-HLA-DR. In T-zone are positive interdigitating reticulum cells too. Counterstaining with haematoxylin. × 100

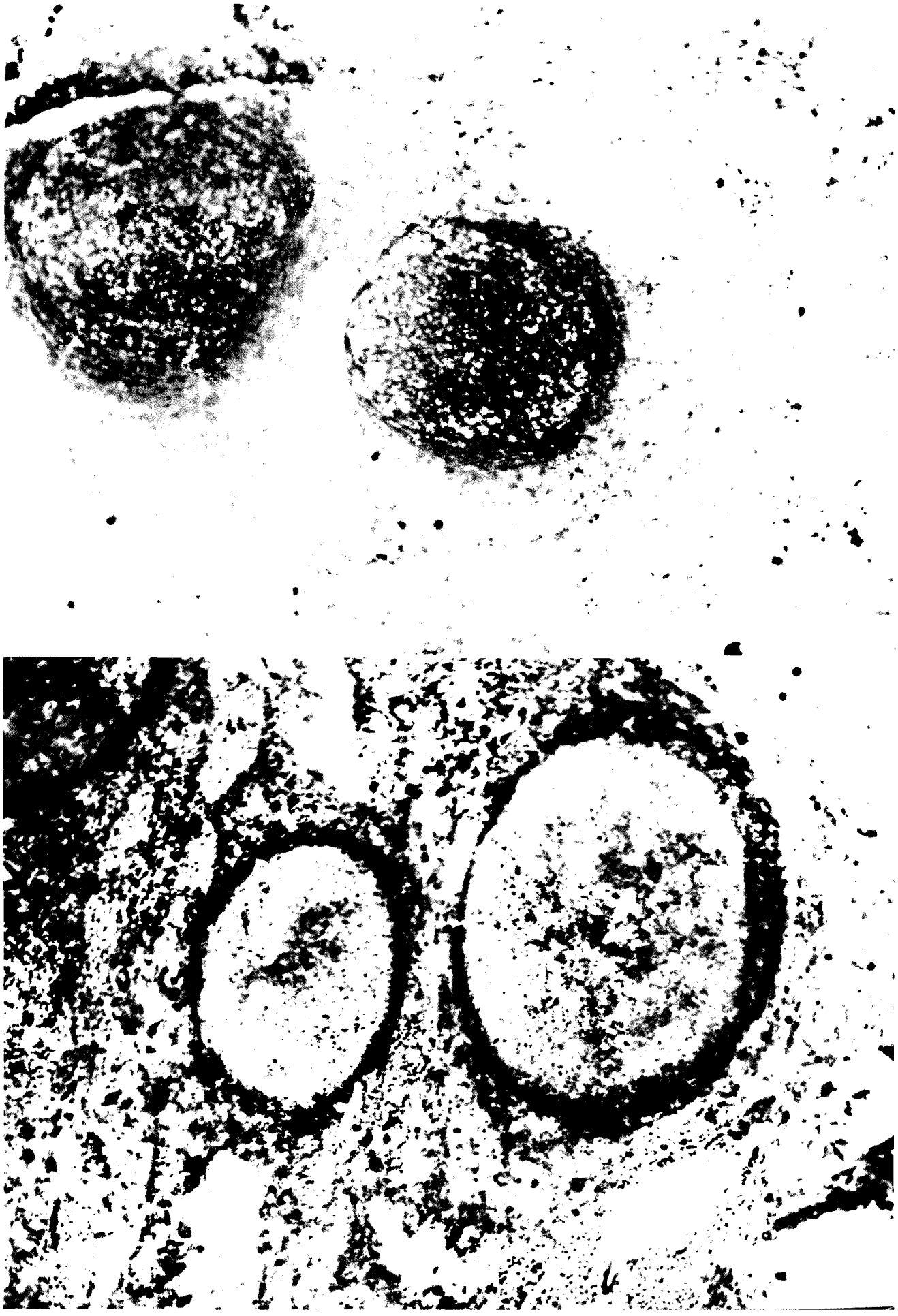
Fig. 9. Lymph node stained with Leu 3a. In center, a secondary follicle with few Leu 3a + cells, mainly in right half of GC. On the right of figure is the T-zone showing intense positivity. Counterstaining with haematoxylin. × 100

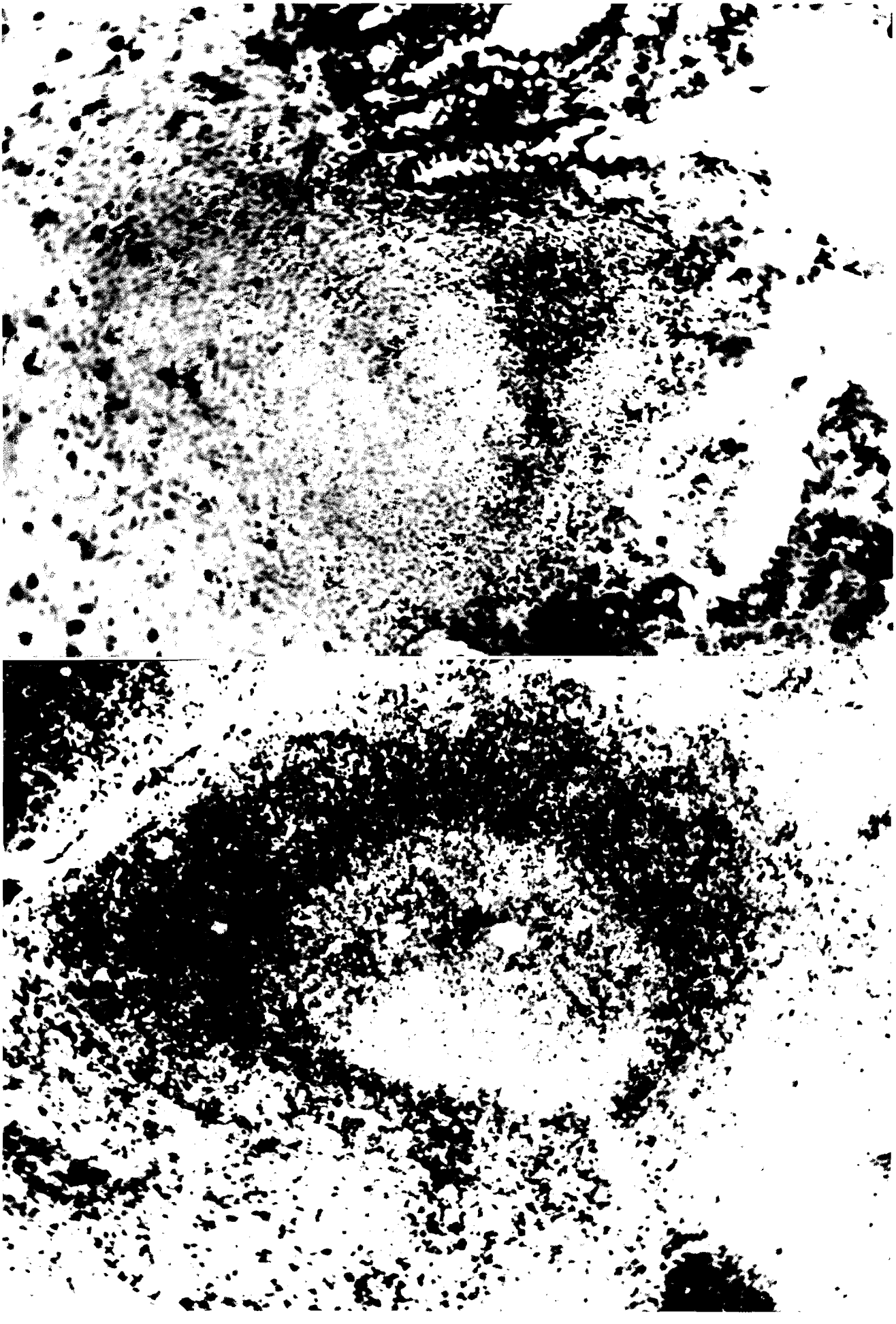
Fig. 10. Lymph node stained with OKT 8. The GC is completely unprovided of Ts-cells. Counterstaining with haematoxylin. × 100

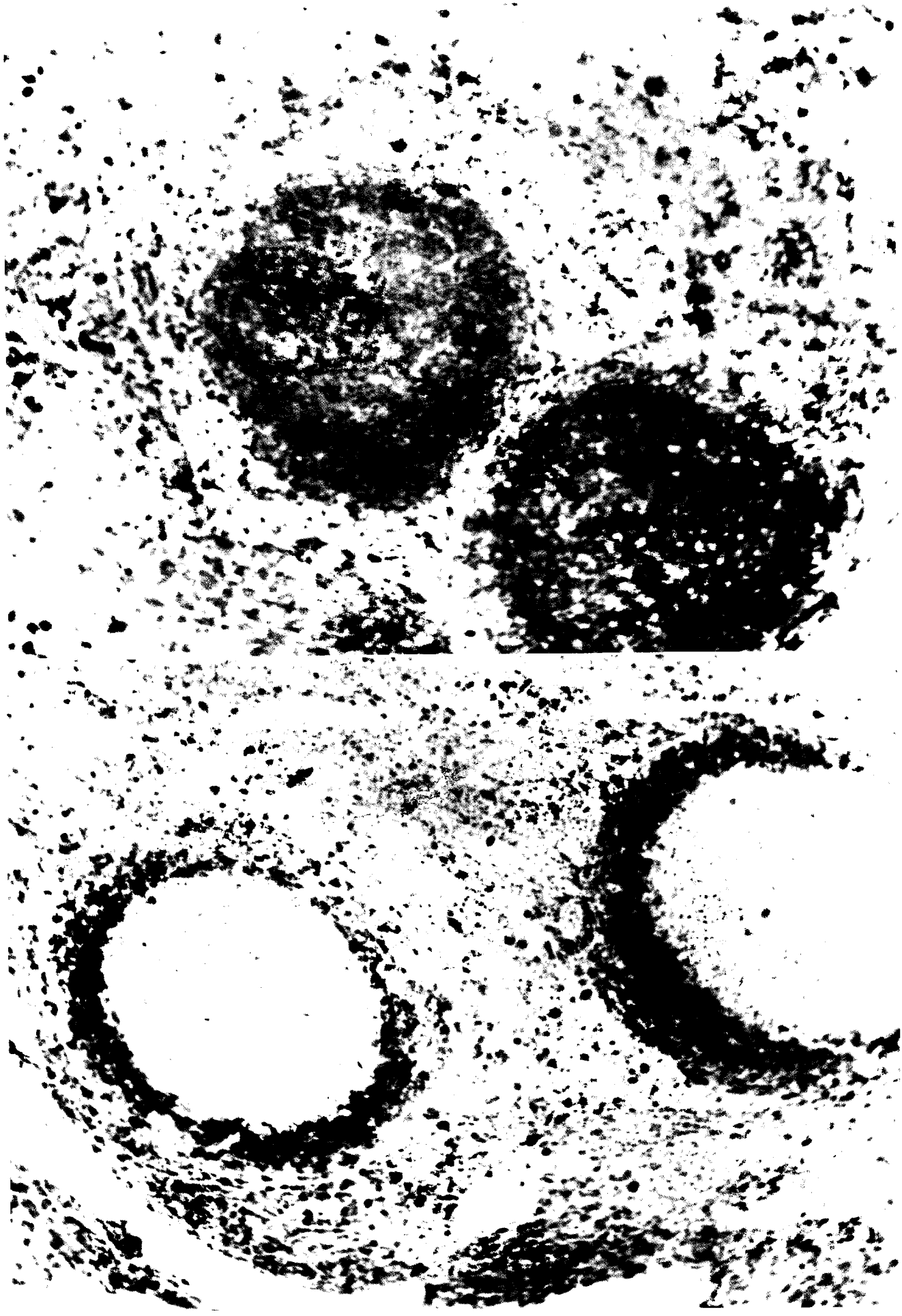
Fig. 11. Leu 7 (HNK-1) + cells in a follicular lymph node GC. Counterstaining with haematoxylin. × 100

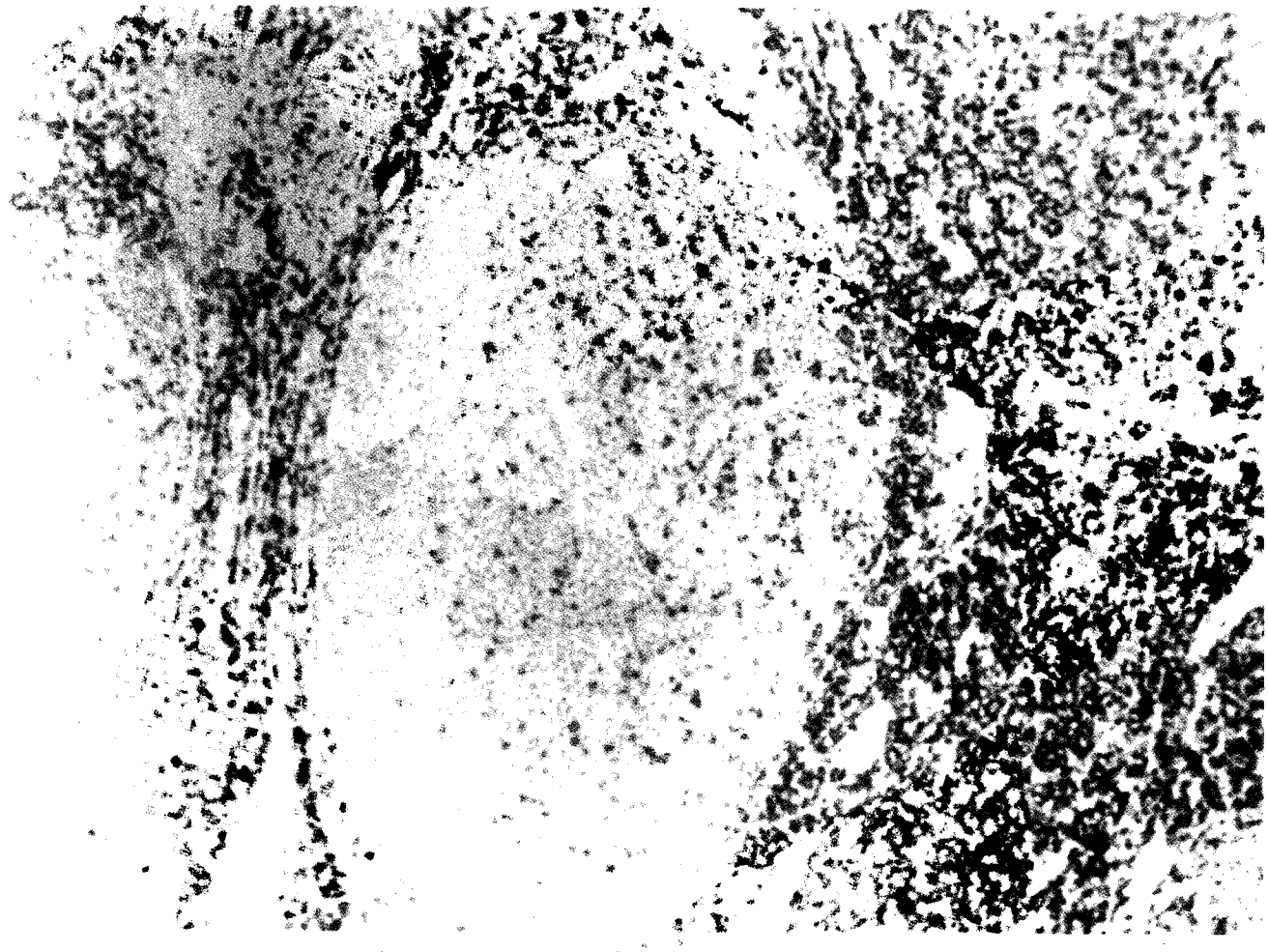
Fig. 12. Secondary lymph node follicles positive for R4/23 showing a network of DRCs. Counterstaining with haematoxylin. × 100

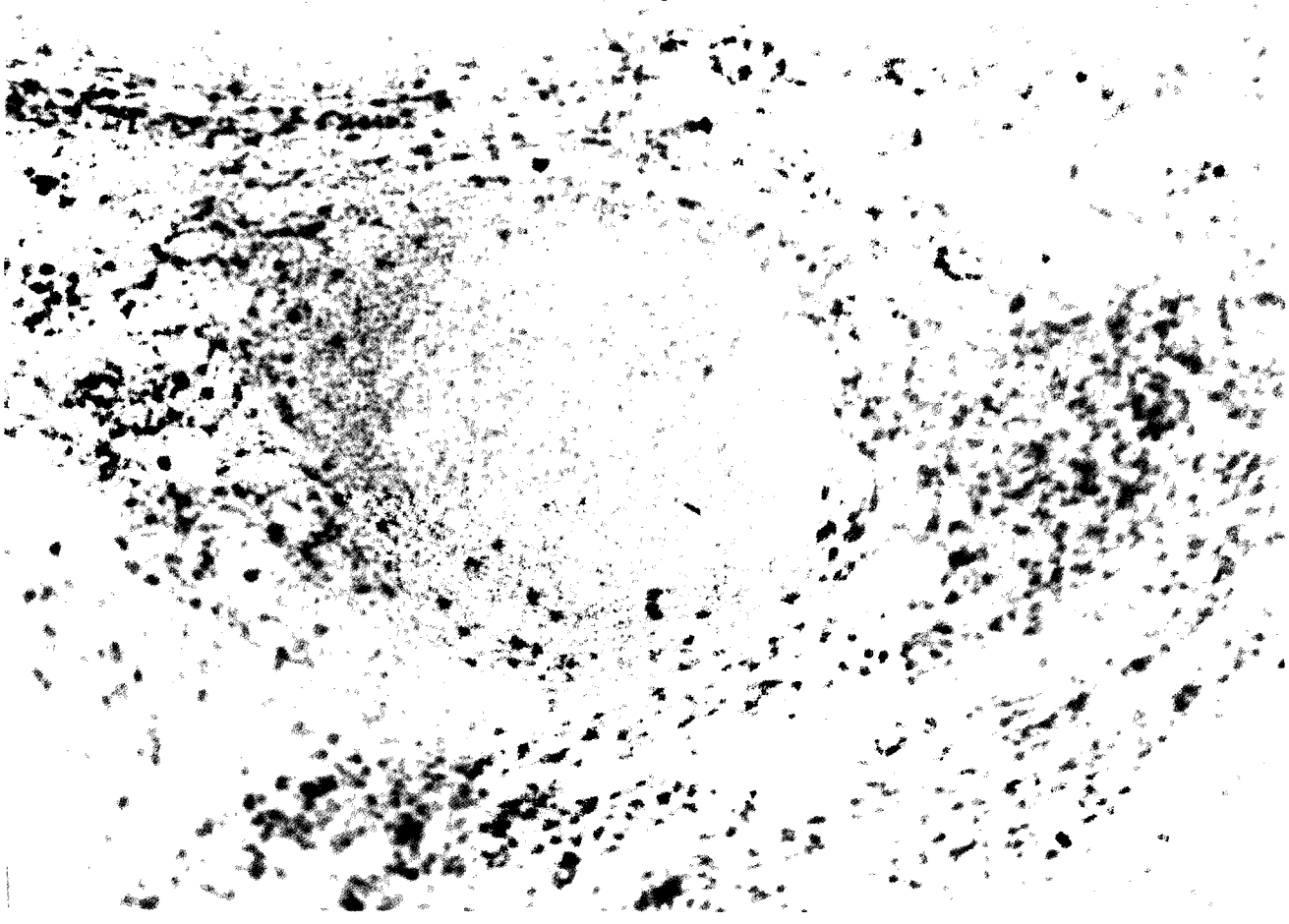












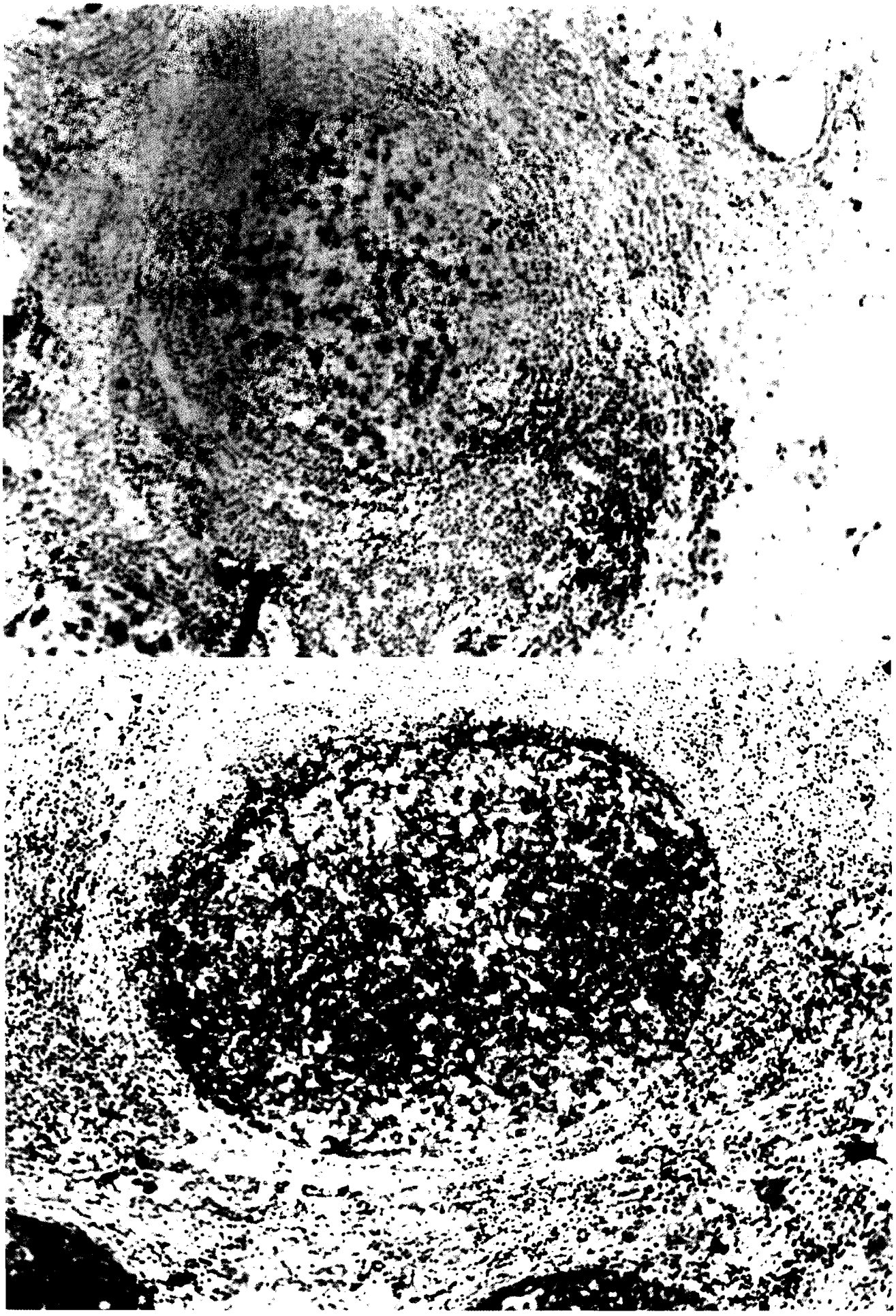


Table 1. Specificity, Optimum Work Concentration and Source of Monoclonal Antibodies.

Antibody	Specificity	Work Concentration	Sources
OKT 6	Thymocytes	1/10	Ortho Diagnostic
OKT 10	Stem Cells, Thymocytes, Prothymocytes, Null Cells, Monocytes (+/−), Activated Antigen	1/10	Ortho Diagnostic
OKT 3	T Cells	1/5	Ortho Diagnostic
OKT 11	E Receptor	1/10	Ortho Diagnostic
T2	T Cells	1/5	Dakopatts
Leu 1	T Cells	1/50	Becton-Dickinson
Leu 4	T Cells	1/50	Becton-Dickinson
Leu 3a	Helper/inducer T-subset	1/50	Becton-Dickinson
OKT 8	Suppressor/cytotoxic T-subset	1/10	Ortho Diagnostic
OKB 2	sIg + B Cells, Squamous Epithelium, Granulocytes	1/10	Ortho Diagnostic
OKB 7	sIg + B Cells	1/10	Ortho Diagnostic
To 15	B Cells	1/10	Dakopatts
B1	B Cells	1/160	Coulter Clone
DK21 (IgD)	Delta Heavy Chain	1/10	Dakopatts
DK20 (IgM)	Mu Heavy Chain	1/10	Dakopatts
SL.3 (IgG)	Fc Fraction of IgG	1/2000	Sera-Lab
GA-1 (IgA)	Alpha Heavy Chain	1/1000	Miles-Yeda
SL.2	Kappa Light Chain	1/2000	Sera-Lab
DK26	Lambda Light Chain	1/10	Dakopatts
Leu 7 (HNK-1)	Large Granular Lymphocytes, NK/K Cells, Some Leu 2 + Cells	1/50	Becton-Dickinson
OKM 1	Monocytes, Granulocytes	1/10	Ortho Diagnostic
OKM 5	Monocytes, Platelets	1/10	Ortho Diagnostic
FHC17	Monocytes, Macropahges, Langerhans Cells	1/2000	Sera-Lab
R4/23	Dendritic Reticulum Cells	1/10	Dakopatts
To 5	C3bR	1/10	Dakopatts
DK22	Beta Chain of HLA-DR Molecules	1/10	Dakopatts
OKT 9	Transferrin Receptor	1/10	Ortho Diagnostic
J5	Common Acute Lymphoblastic Leukaemia Antigen	1/160	Coulter Clone

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

Antibody	Specificity	Work Concentration	Sources
Anti-alpha$_1$-antitrypsin	Monocytes and and Histiocytes (Kerdel et al., 1982)	1/500	Dakopatts
Anti-alpha$_1$-antichymotrypsin	Monocytes and Histiocytes (Kerdel et al., 1982)	1/500	Dakopatts
Anti-S-100 protein	Langerhans Cells, Interdigitating Reticulum Cells (Kahn et al., 1983)	1/400	Dakopatts

Discussion

The secondary lymphoid follicles are phenotypically assymetrical 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 assymetry is mantained 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 assymetric 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 immunosera (Reynes 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 sIg + 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 lymphoma, 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 (Brandtzaeg 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., 1983; Hsu and Jaffe, 1984b), whereas in other ones a significant amount of sIg + 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 sIgD + and sIgM +. However, the GC expresses sIgM mainly in the clear zone and IgD is absent in that structure. We conclude that IgM is predominantly expressed on the centrocytic cell surface, unlike other reports stating that sIg is found only in very scanty GC cells (Hsu et al., 1983), particularly centroblasts (Hsu and Jaffe, 1984b). Nevertheless, our opinion is in agreement with some previous experimental works. So, it has been shown, in enucleated lymphoid follicles from palatal tonsils, that half of GC large lymphocytes are sIg-bearer (Tsunoda et al., 1980). Furthermore, small lymphocytes simultaneously bearing sIgD and sIgM, and large lymphoid cells bearing only sIgM 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 sIg-bearers, whereas centroblasts are sIg-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 immunoglobulinic phenotype corresponding to a mature B cell (sIgM + and sIgD +). Thereafter they completely loose their immunoglobulinic expression, regaining it subsequently like a pre-B cell (sIgM +) (Gugliemi and Preud'Home, 1981).

In relation to cIgs, our results are in concordance with those of other investigators with regard to the scanty presence of cIg + cells in GC, being clearly predominant cIgG (Tsunoda et al., 1980; Matthews and Basu, 1982; Brandtzaeg 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 cIg (Morris et al., 1983). However, Hsu and Jaffe (1984b) report some cIg + centrocytes. We believe that scarce cIg + 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 accompaniing 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 predominace of these in clear zone, mainly being a T-helper population (Si et al., 1983). This finding confirms the assymetric nature of secondary follicles. Their presence corroborates the importance of T-B coopertion (McMillan et al., 1981).

The observation of Leu 7 (HNK-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 Flemming's macrophages, the former is specifically marked with R4/23 MoAb. Since DRCs are positive for FHC17 too, then we should allege for them a mononuclear/phagocytic system origin, such as other writers have suggested (Gerdes et al., 1983). However, they do not shown reactivity for OKM 1 and OKM 5 (MoAbs to soluble antigen presenting monocytic/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 monocytic/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 Flemming's macrophage, this is a monocytic/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 assymetric 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: sIgM + 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.

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