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CELLULAR COMPOSITION OF FOLLICLES OF FOLLICLE CENTRE CELL LYMPHOMAS IN RELATION TO GERMINAL CENTRES OF REACTIVE LYMPH NODES. A MORPHOMETRICAL ELECTROMICROSCOPICAL STUDY

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SUMMARY

The cell spectrum of neoplastic and benign reactive germinal centres was determined ultrastructurally. The degree in which the cell composition found in reactive germinal centres is maintained in analogous structures of follicular lymphomas was investigated by pattern recognition methods and discriminant analysis based on the frequencies of the various lymphoid and non-lymphoid cell types. The follicular lymphomas included lymphomas with predominantly centrocytes (FCCL, Cb-cc) and lymphomas with predominantly centroblasts (FCCL, Cb).

Pattern analysis of FCCL Cb, FCCL Cb-cc and reactive germinal centres indicates that FCCL Cb follicles resemble reactive germinal centres in more aspects than follicles of FCCL Cb-cc.

Clear statistical differences were encountered between the frequencies of the lymphoid cell types and of the follicular dendritic and histiocytic reticulum cells in follicles of FCCL Cb-cc and FCCL Cb and reactive germinal centres.

Application of a discriminant analysis using a combination of the frequency of centrocytes and follicular dendritic cells demonstrated that both types of neoplastic follicles and reactive germinal centres were correctly classified on the basis of their cell spectrum. For the three groups the most potent discriminator was the centrocyte, whereas the small and large centroblast were of less value. For discrimination between Cb-cc follicles and reactive germinal centres again the centrocyte was the most potent discriminator. Discrimination between FCCL Cb follicles and reactive germinal centres of FCCL Cb-cc follicles can be easily achieved using the frequencies of small centroblasts or centrocytes on their own.

These findings indicate that (1) follicles of both FCCL Cb and FCCl Cb-cc differ greatly in the cellular composition and not only with respect to their content of centroblasts but also in their content of follicular dendritic cells and (2) they may be considered as neoplasms representing different developmental phases of germinal centres.

KEY WORDS—Germinal centre, follicle centre cell lymphomas, ultrastructure, morphometry

INTRODUCTION

The differentiation between follicular hyperplasia and follicular lymphoma is often a difficult problem in the histopathology of lymphoid tissue. Histologic criteria for differentiation have been defined by Rappaport *et al.*,[1] Lennert[2] and Nathwani *et al.*[3] Most of these criteria refer to changes in lymph node architecture, though the absence of prominent phagocytosis and the uniformity of the population of the follicle centre cell population have to be taken into consideration. Apart from these morphological criteria the immuno-histochemical demonstration of immunoglobulin light chain restriction is probably the most reliable criterion for differentiating between reactive and neoplastic follicles.

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The relationship of follicular lymphomas with normal germinal centres has been stressed by histological,[4] and immunohistological studies.[5,6,7] Ultrastructurally a remarkably wide spectrum of lymphoid cells and of non-lymphoid cells is encountered in normal germinal centres.[8] Follicular lymphomas can be classified according to the relative proportion of germinal centre cells (centrocytes or centroblasts) and the size of the proliferating cells.[1,2,9,10] In most follicular lymphomas the lymphoid cells in the follicular structures can be related to normal germinal centre cells[11,12] but data referring to the degree in which this spectrum is maintained in follicular lymphomas containing varying numbers of centroblasts are lacking. In this study we compare the cell composition of reactive and neoplastic follicles using morphometrical electron microscopy in combination with pattern recognition methods.

MATERIAL AND METHODS

Lymph nodes

Fresh lymph node biopsies were processed for multidisciplinary studies according to the protocol described by Van der Putte *et al.*[13] This protocol included routine histology, cytology, immunohistochemistry and enzyme histochemistry on frozen sections and enzymatically prepared cell suspensions, and electron microscopy.[14]

Fifteen lymph nodes with follicular lymphoma and eleven lymph nodes with follicular hyperplasia were selected for our biopsy files on the basis of histopathological diagnosis and immunohistochemistry. Histologically twelve cases were categorised as follicle centre cell lymphoma centroblastic centrocytic, follicular (FCCL Cb-cc) and three as follicle centre cell lymphoma centroblastic, follicular (FCCL Cb).

Morphometry

The lymphoid and non-lymphoid cell types were identified in reactive germinal centres on the basis of their ultrastructure. The relative frequency of these cell types was determined on low power electron micrographs. For this purpose three follicular structures in each biopsy were selected in 1 μm sections. In ultrathin sections a set of six electron micrographs (magnification 3700 $\times$) were randomly taken from each follicular structure. These micrographs represented a total area of 14 000 μm^2 per biopsy.

Data analysis

The data were analysed by pattern recognition methods and several statistical procedures.

The data of the frequencies of the various cell types were subjected to a cluster analysis: a pattern recognition method which aims to reveal the most striking pattern in a set of data (in this investigation the distinct biopsies characterized by a number of parameters). Cluster analysis is a non-supervised method: i.e. grouping of data is achieved by the method without intervention of the investigator. The method, using Biopat,[15] thus classifies the data into the most relevant groups. The outcome of the cluster analysis is depicted in a dendrogram. A dendrogram shows the similarity level at which the respective objects (here biopsies) and groups of objects are combined into new groups (i.e. clusters). Cluster analysis is explained in more detail by Sneath and Sokal.[16]

The statistical procedures attempt to find differences in individual parameters between groups which are predefined (i.e. supervised) by the investigator. The differences in frequency of the various cell types between reactive and lymphomatous follicular structures were assayed by the Wilcoxon test. This procedure was applied at a probability level of 5 per cent.

Although the data of an individual parameter (cell type) may differ significantly between the histopathological groups, this does not imply that the biopsies can be classified correctly on the basis of this statistical difference. This problem can be resolved by subjecting the data of the various parameters to a discriminant analysis; a method that analyses the data of several parameters concomitantly.

RESULTS

In reactive germinal centres a large number of cell types can be identified at the ultrastructural level. A number of these lymphoid and non-lymphoid cell types are illustrated in Fig. 1.

The following cell types were encountered: Immunoblast, large ($>12\ \mu$m in diameter) and small centroblast ($<12\ \mu$m in diameter), centrocyte, multilobated cell, centroplasmacytoid cell, lymphocyte, plasma cell, follicular dendritic cell, histiocytic reticulum cell, fibroblastic reticulum cell and dark reticulum cell.

The ultrastructural distinction of the above men-

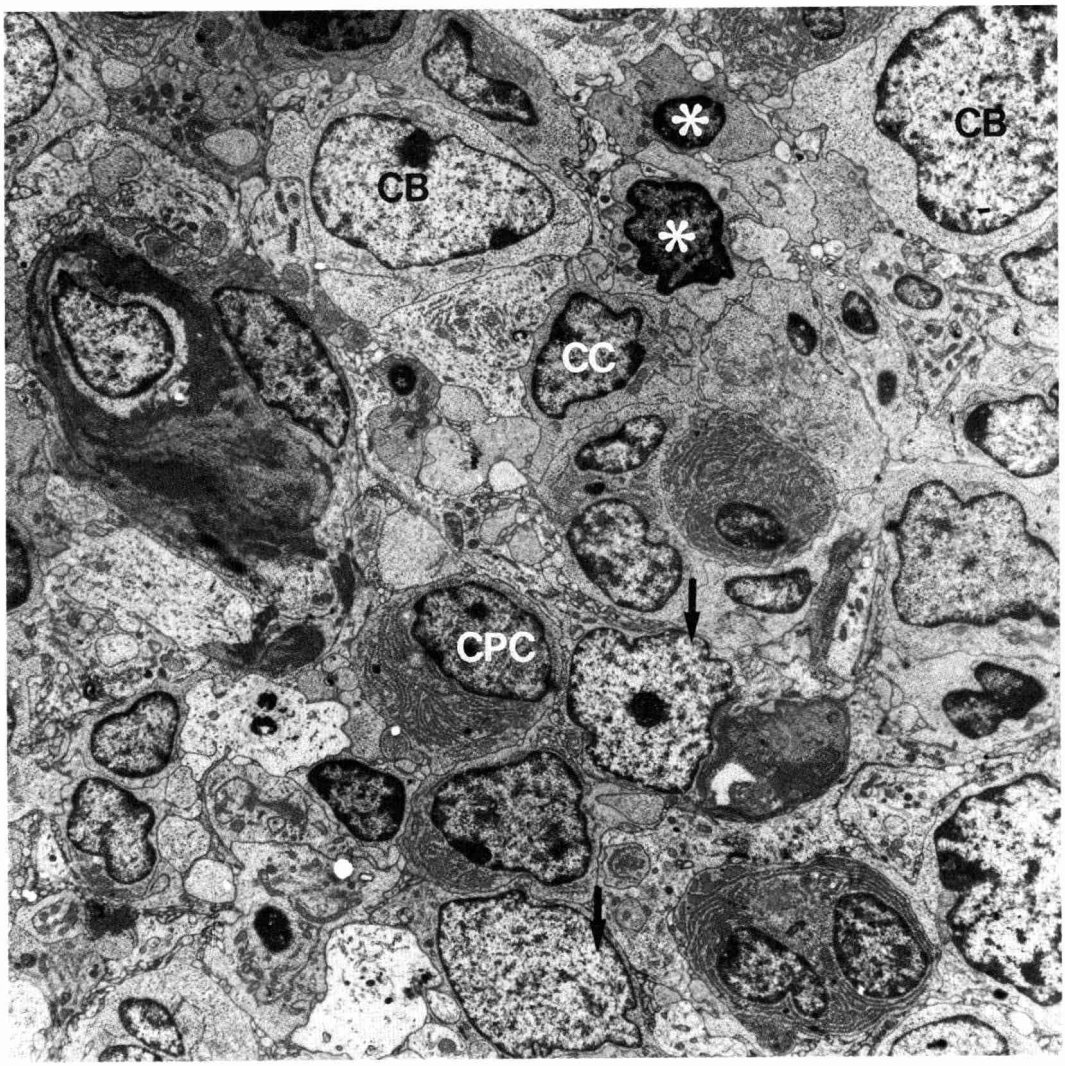

Fig. 1—Electron micrograph of a reactive germinal centre (biopsy R5) showing the extensive web of cytoplasmic extensions of follicular dendritic cells (FDC) around the lymphoid cells. The lymphoid cell population is heterogenous; large centroblasts (CB), centrocytes (CC) and centroplasmacytoid cells (CPC) are indicated. Note the similar chromatin pattern of the latter two cell types. Asterisk—lymphocyte; arrows—nuclei of FDC. × 4000

tioned cell types was based for most cell types on descriptions by Lennert and Müller-Hermelink[8] and Lennert.[2]

Multilobated cells[13] have marked nuclear lobulations, a heterochromatic chromatin pattern and a cytoplasmic organization of organelles comparable with those of centrocytes.

Centroplasmacytoid cells also resemble centrocytes, but have a well developed rough endoplasmic reticulum and Golgi complex. These cells probably represent the Ig-producing centrocytes previously reported by Lennert and Stein.[17]

Lymphocytes are defined as round small cells. Their nucleus contains heavy marginal and central chromatin condensations. Cell organelles are sparse in the cytoplasm; only occasionally clusters of electron dense granules occur.

Dark reticulum cells[18] are cells of various shapes with an electron dense cytoplasm. In our material the majority of these cells represent degenerating follicular dendritic cells, in few cases histiocytic and fibroblastic reticulum cells.

In neoplastic follicles most of the above mentioned cell types could be recognized on the basis

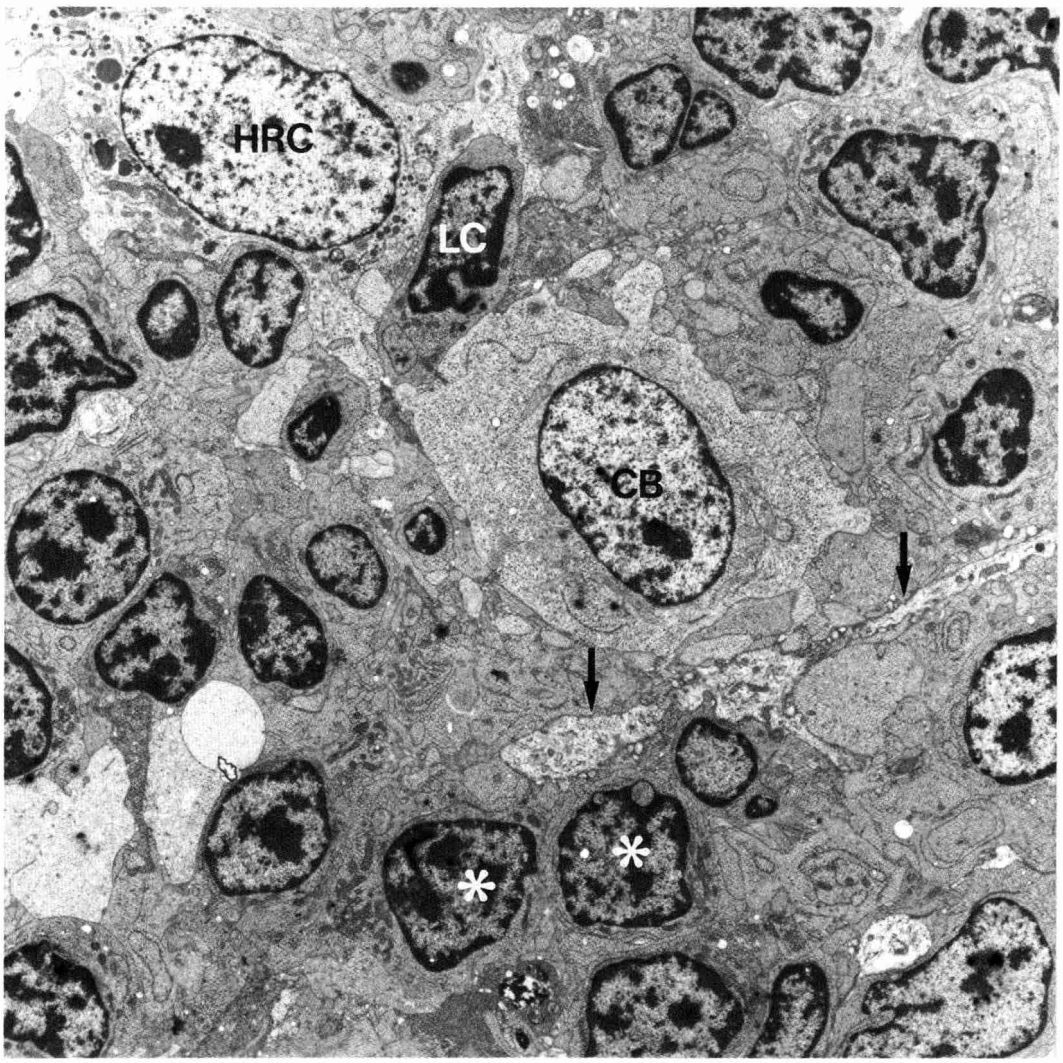

Fig. 2—Centroblastic-centrocytic follicular lymphoma (biopsy CB-CC 6). Neoplastic follicle with predominantly centrocytes (asterisk); CB: centroblast; HRC: histiocytic reticulum cell; LC: Lymphocyte; Arrows—broad cytoplasmic extensions of FDC. ×4000

of their ultrastructural characteristics (Fig. 2). In FCCL Cb-cc the malignant centroblasts and centrocytes often had a strong resemblance to their benign counterparts, whereas in other cases these cells were more irregular and had more pronounced chromatin condensations. In two cases nuclear pockets were frequent in the nuclei of centrocytes. In all these cases the centrocytes were smaller compared with their analogues in reactive germinal centres. The histiocytic reticulum cells and follicular dendritic cells were morphologically different from those in reactive germinal centres as described previously.[14]

In FCCL Cb the centrocytes were larger and also the centroblasts showed pronounced nuclear indentations. However, such cells could be identified without difficulty (Fig. 3).

Ultrastructural analysis of the cell population in follicular structures

No clear polar distribution of germinal centre cells with respect to the light and dark zones as described by Lennert[2] was observed by light microscopy in most of the reactive germinal centres. Only in a few cases was a slight peripheral accumulation of centroblasts observed. A polar dis-

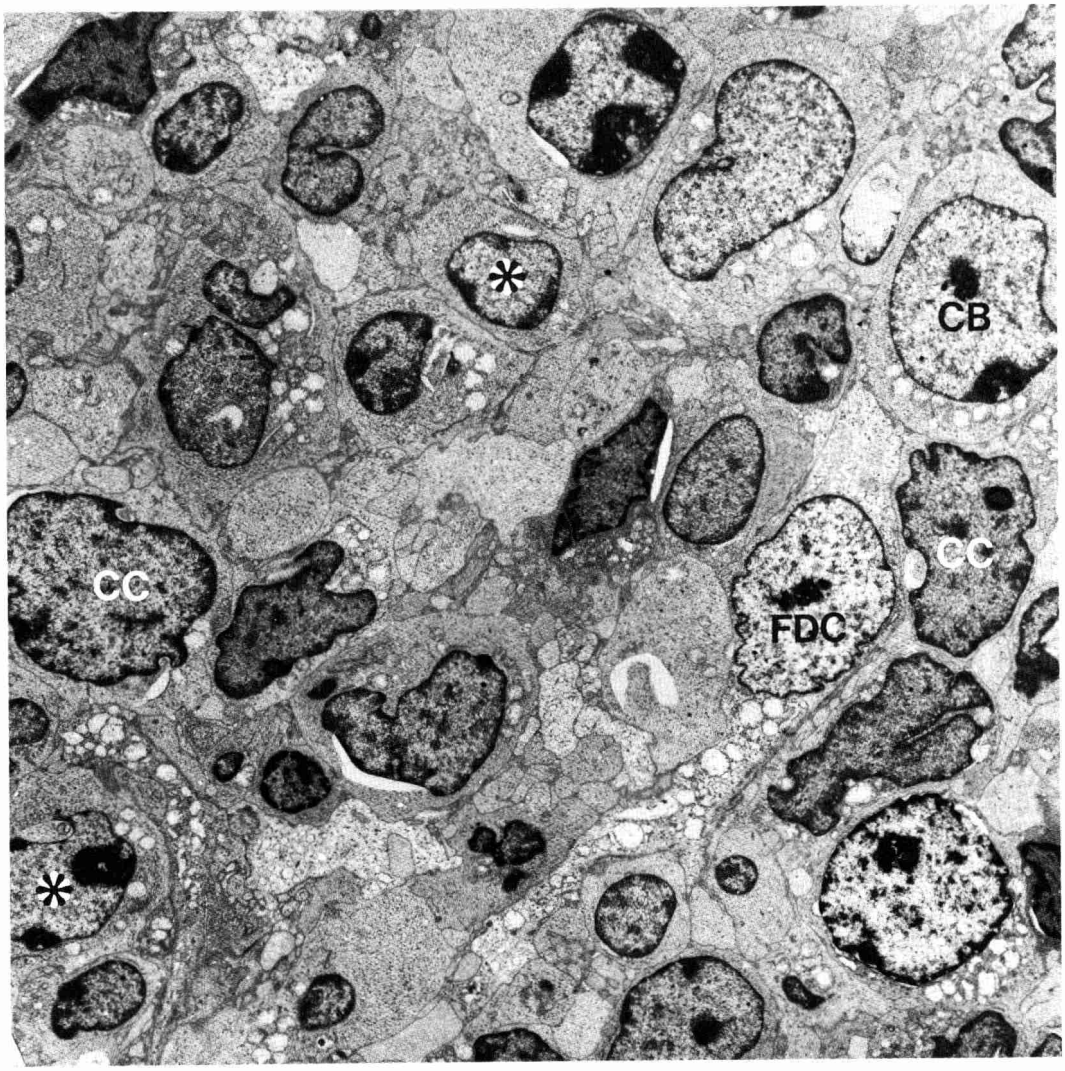

Fig. 3—Centroblastic follicular lymphoma (biopsy CB 14); neoplastic follicle. Small (asterisk) and large centroblasts (CB) are abundant. Centrocytes (CC) are of larger size than usual. Note FDC with broad cytoplasmic extensions. ×4000

tribution with respect to histiocytic reticulum cells was also not evident in semithin sections. For the analysis of the cell population therefore the samples represented randomly intermingled types of germinal centre cells.

The data obtained after morphometrical analysis of reactive germinal centres (R) of each biopsy are given in Table 1a. The data on neoplastic germinal centres are presented in Table 1b.

Cluster analysis

The data, i.e. the 26 biopsies characterized by their respective cell counts, were subjected to agglomerative cluster analysis with Ward's criterion.[19] Note that the histopathological class of the biopsies is not taken into account in this (non-supervised) method: the biopsies are grouped on the basis of their cell counts only. This analysis yields the dendrogram depicted in Fig. 4. The dendrogram clearly shows 3 groups (clusters) which largely correspond to the 3 histopathological groups (reactive, FCCL Cb and FCCL Cb-cc). Only germinal centres of the reactive lymph nodes R1 and R6 are placed 'incorrectly', in the FCCL Cb-cc cluster. These two biopsies have a relatively high centrocyte count. The fact that this non-supervised method yields the

Table 1a—Relative frequency (%) of distinct cell types in normal germinal centres of reactive (R) lymph nodes

	R 1	R 2	R 3	R 4	R 5	R 6	R 7	R 8	R 9	R 10	R 11
Immunoblast	0·6	1	1	0·7	0·1	0·2	4	4	3	1	0·3
Large centroblast	3	14	3	7	3	0·5	10	8	5	8	6
Small centroblast	7	16	13	17	9	6	15	7	21	14	20
Centrocyte	70	63	60	59	44	81	52	60	46	66	44
Centroplasmacytoid	5	1	6	3	14	1	4	3	7	2	0
Multilobated cell	1	1	0·5	0·7	3	0·5	0·2	1	1	0·6	0·9
Lymphocyte	6	0	0·5	4	14	0	4	7	4	1	15
Plasma cell	0	0	1	0·2	0·5	0	0·2	1	0·5	0	0
Follicular dendritic cell	2·8	1	5	4	8	7	6	4	3	2	7
Histiocytic reticulum cell	2	2	7	4	3	4	4	4	7	4	4
Fibroblastic reticulum cell	0	0	0	0	0	0	0·2	0	0·3	0	0
Dark reticulum cell	2	0	0	0	0	0	0	1	0	0·3	0
Mitosis of lymphoid cells	1	1	3	2	1	1	0·4	1	0·3	0·9	1
Total number of cells/ $14{,}000\mu m^2$	503	450	441	426	406	399	397	391	365	333	338

Table 1b—Relative frequency (%) of distinct cell types in malignant follicles of follicular lymphomas of the centroblastic-centrocytic (Cb-cc) and centroblastic type (Cb)

	CB-CC 1	CB-CC 2	CB-CC 3	CB-CC 4	CB-CC 5	CB-CC 6	CB-CC 7	CB-CC 8	CB-CC 9	CB-CC 10	CB-CC 11	CB-CC 12	CB 13	CB 14	CB 15
Immunoblast	1	2	1	1	0	3	0·8	0·2	1	0·8	0·8	1	0	0	0·5
Large centroblast	0·9	5	2	0	0·2	2	0·5	2	0·9	0·8	3	1	0	13	30
Small centroblast	1	4	3	3	1·2	3	3	4	2	5	6	3	57	45	34
Centrocyte	87	76	92	92	94	77	92	85	91	92	83	83	22	23	9
Centroplasmacytoid cell	0	0·4	0	0	0	6	0	0	0·7	0·8	2	0	7	0	0
Multilobated cell	0	2	1·5	0	0·2	3	0	0	0·7	0	0·3	3	2	6	8
Lymphocyte	8	7	1	2	0	1	1	1	0	1	2	1	7	15	6
Plasma cell	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Follicular dendritic cell	0	0·8	0	0	3	0·3	1	0·2	2	1	2	3	2	6	4
Histiocytic reticulum cell	1	3	0·5	2	1·2	0·6	0·3	2	0	1	2	5	0	1·5	2
Fibroblastic reticulum cell	0·7	0	2	0	0	1	0	0	0·9	0	0	0	0	0	0·5
Dark reticulum cell	0·3	0	0	0	0	0·6	1	6	0	0·8	0	1	0	0	0
Mitosis of lymphoid cells	0	0	0	0	0·2	0·2	0	1	0	0	0·6	1	0	0·5	0
Total number of cells/ 14,000μm^2	647	524	514	492	488	468	468	468	462	400	369	367	384	200	207

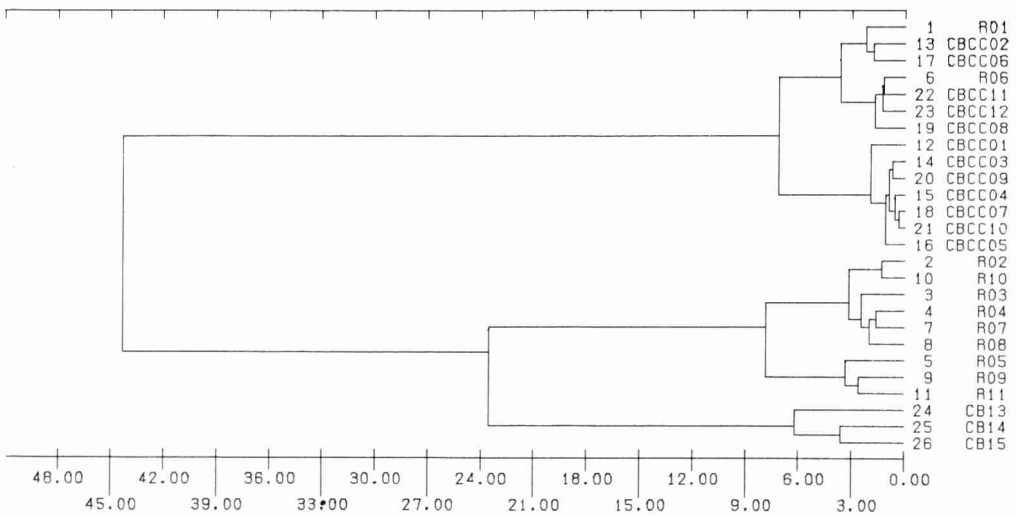

Fig. 4—Dendogram of an agglomerative cluster analysis of reactive germinal centres and neoplastic follicles. Vertical axis: germinal centres of the 26 lymph node biopsies characterized by 13 parameters and grouped according the similarity of their data. Horizontal axis: dissimilarity level at which the objects are grouped. This level is determined by Wards criterion[19] and based on the mean city block distance.[23] The 3 histopathological classes of follicles can be recognized in this diagram.

3 groups distinguished beforehand leads us to conclude that the 3 groups differ clearly from each other in cell composition. These differences between the groups were investigated by statistical (supervised) methods.

Statistical analysis

The mean values, range and the results of the statistical evaluation of the differences between the data of each group are listed in Table II.

Statistical analysis of the data with the Wilcoxon test of the distinct cell types revealed differences between the groups. Highly significant differences in the frequencies of the various parameters were observed between the reactive germinal centres and FCCL Cb-cc follicles: the frequency of centrocytes in the FCCL Cb-cc follicles were significantly higher compared with the reactive-group, whereas the numbers of centroblasts, centroplasmacytoid cells, follicular dendritic cells and histiocytic reticulum cells were significantly lower. This was also apparent for the data on mitotic figures.

In follicles of FCCL Cb the frequencies of immunoblasts, centrocytes, histiocytic reticulum cells and mitotic figures were significantly lower, in comparison with the reactive germinal centres whilst the figures obtained for multilobated cells and small centroblasts were higher. Compared with the FCCL Cb-cc follicles the FCCL Cb follicles showed significantly higher values for small centroblasts, multilobated cells, lymphocytes and follicular dendritic cells. The data for centrocytes were significantly lower.

In addition, significant differences were observed in the total number of cells/area between the three histopathological groups indicating differences in cell size, since no gaps were present between the cells.

Discriminant analysis

Discriminant analysis, using a subset of characters (cell types), of the biopsies divided into the 3 histopathological groups, allows a 100 per cent successful classification of all biopsies. We first checked the importance of each parameter individually. It appeared that if we only consider the centrocyte count of each biopsy 25 (out of 26, i.e. 96 per cent successful classification) biopsies are classified correctly (the reactive node R6 is again misclassified to belong to the FCCL Cb-cc group).

It thus appears that knowledge of the centrocyte count only is almost (ie. for 96 per cent) sufficient for classifying any biopsy into its correct group. The second best is the small centroblast which classified 23 (89 per cent) of the biopsies correctly (R1, R6 and R8 are misclassified). All other cell types are of no value for classification of the biopsies. Next a combination of parameters was tested: i.e. we

Table II—Mean values and range of the frequency of cell types occurring in follicular structures of hyperplastic lymph nodes (R) and follicular lymphomas of the centroblastic-centrocytic type (Cb-cc) and centroblastic type (Cb).

	Group 1 'R' n = 11		Group 2 'CB-CC' n = 12		Group 3 'CB' n = 3		Wilcoxon test		
	$\bar{x}$	Range	$\bar{x}$	Range	$\bar{x}$	Range	1 vs 2	1 vs 3	2 vs 3
Immunoblast	1·4	0– 4	1·0	1– 3	0·2	0– 0·5	–	–	–
Large centroblast	6·1	0·5–14	1·5	0– 5	14	0–30	+*	–	–
Small centroblast	13·2	6–21	3·2	1– 6	45	34–57	+*	+*	+*
Centrocyte	59	44–81	87	76–94	18	9–23	+*	+*	+*
Centroplasmacytoid cell	4·2	0–14	0·8	0– 6	2·3	0– 7	+*	–	–
Multilobated cell	0·9	0·5– 3	0·8	0– 3	4·6	2– 6	–	+*	+
Lymphocyte	5·1	0–15	2·1	0– 8	10	7–15	–	–	+
Plasma cell	0·3	0– 1	0	–	0	–	–	–	–
Follicular dendritic cell	4·5	1– 8	1·1	0– 3	4·0	2– 6	+*	–	+
Histiocytic reticulum cell	4·1	2– 7	1·6	0– 3	1·2	0– 2	+*	+*	–
Fibroblastic reticulum cell	0·1	0– 0·3	0·4	0– 2	0·2	0– 0·5	–	–	–
Dark reticulum cells	0·3	0– 2	0·8	0– 6	0	–	–	–	–
Mitosis of lymphoid cells	1·2	0·3– 3	0·2	0– 1	0·2	0– 0·5	+*	+	–
Total number of cells/14,000 μm^2	404	333–514	476	367–647	263	200–384	+	+	+

$\bar{x}$: mean value; n: number of biopsies; + : significant difference ($P < 0{\cdot}05$); *$P < 0{\cdot}01$.

tested the centrocyte count in combination with every other parameter. It turned out that only the combination of centrocyte and follicular dendritic cell, although the latter cell type has no discriminating value of its own, gives a 100 per cent correct classification.

Discriminant analysis of the reactive germinal centres versus FCCL Cb-cc follicles also indicates that the centrocyte and small centroblasts are the best discriminators giving a correct classification of 22 (96 per cent) and 20 (80 per cent) out of 23 biopsies respectively; however the same mistakes mentioned above are made. The centrocytes and small centroblasts are followed in order of importance by histiocytic reticulum cells, mitoses, follicular dendritic cells, centroplasmacytoid cells and large centroblasts. Only with a combination of centrocytes and follicular dendritic cells is a 100 per cent successful classification obtained.

Analysis of the reactive germinal centres versus FCCL Cb follicles shows that both small centroblasts and centrocytes on their own allow successful (100 per cent) classification of the biopsies. Also FCCL Cb-cc follicles versus FCCL Cb follicles can be reliably classified (100 per cent correct) by the small centroblast or centrocyte counts on their own.

DISCUSSION

In the present study the cellular composition of the follicles from FCCL Cb-cc and FCCL Cb lymphomas was compared with that of reactive germinal centres. The cluster analysis separates the data into 3 groups (FCCL Cb, reactive and FCCL Cb-cc respectively). The dendrogram (Fig. 4) shows that follicles of FCCL Cb and reactive germinal centres are combined before (i.e. at a lower dissimilarity level) they combine with the Cb-cc group. This suggests that follicles of FCCL Cb are more closely related to reactive germinal centres than follicles of FCCL Cb-cc.

Using discriminant analysis we were able to estimate the importance of each parameter for the separation of follicles of FCCL Cb, FCCL Cb-cc and reative germinal centres. For discrimination between these three types of follicles most of the parameters had some discriminating capacity, The centrocyte and centroblast were most important. However, biopsy R6 was incorrectly placed among the Cb-cc follicular lymphomas. Correct classification of R6 was only achieved using a subset of the parameters centrocyte and follicular dendritic cell. After checking up the clinical background, it appeared that R6 was the initial lymph node biopsy of a patient who developed in the next 5 years persistent generalized lymphadenopathy and symptoms of AIDS.

Statistical analysis of each parameter separately shows that FCCL Cb-cc are a rather homogenous group in which centrocytes predominate. In contrast the FCCL Cb appear to have a more heterogenous cellular composition.

The cellular composition of reactive germinal centres may range between a relative high number of centroblasts and a predominance of centrocytes (Table 1a). Lennert[2] described four phases of germinal centre development: (i) a phase lasting about 24 hours with predominantly medium sized centroblasts; (ii) a phase, lasting one to three weeks, with predominantly centroblasts and 'starry sky' macrophages; (iii) a phase lasting one to three months with an occasional zonal distribution and the occurrence of immunoblasts, centroblasts, centrocytes, plasma cells, follicular dendritic cells and histiocytic reticulum cells, and (iv) a phase in which only centrocytes and follicular dendritic cells remain. This phasic development accounts for the large variance in cell frequencies observed in reactive germinal centres. Most of the reactive germinal centres studied can be considered as third phase germinal centres of various stages; the germinal centres (biopsies R1 and R6) with high centrocyte counts as fourth phase germinal centres. According to the cluster analysis these reactive germinal centres have a cell spectrum which is comparable with that of the FCCL Cb-cc. However, the follicular dendritic cells, present within the germinal centres of these two biopsies, have the ultrastructural characteristics which are specific for reactive germinal centres.[14] Moreover, discriminant analysis with a subset of parameters, classifies these germinal centres correctly.

Consequently, the different types of follicular lymphomas might be regarded as a malignant deviation of different developmental stages of germinal centres. FCCL Cb-cc might represent the malignant counterpart of an 'end stage' (of fourth phase) germinal centre whereas FCCL Cb may represent a malignant analogue of an early phase, presumably a second or third phase, germinal centre.

As discussed before[14,21] we regard the non-lymphoid follicular dendritic cells as non-malignant cells which develop within the neoplastic germinal centre as a result of factors mediated by the lym-

phoid tumour cells. In the three cases examined follicular dendritic cells were present in higher numbers in the follicles of FCCL Cb than in the follicles of FCCL Cb-cc. This suggests that the differentation of follicular dendritic cells from precursor cells might be facilitated in FCCL Cb in comparison with FCCL Cb-cc. From studies on rodent systems it is known that follicular dendritic cells differentiate during germinal centre development.[22] Studies in our laboratory (Rademakers *et al.*, to be published) suggest that dark zones of germinal centres, which are composed mainly of small to medium sized centroblasts, are important locations for differentiation of follicular dendritic cells during the expansion of germinal centres.

Ultrastructural analysis of the cell spectrum of follicular structures combined with powerful patterns recognition methods demonstrated that the classical FCCL Cb-cc and FCCL Cb are different entities and may be considered as neoplasms reflecting different phases of germinal centre development. The follicles of FCCL Cb have a structural organization which shows more resemblance to benign reactive germinal centres than to the follicles of FCCL Cb-cc.

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