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Morphological and Cytochemical Identification of Lymphoid Cells

D. Catovsky, A. Enno

M.R.C. Leukaemia Unit, Royal Postgraduate Medical School, London

Summary

This paper reviews the contribution of light and electron microscopy, surface structure and cytochemistry (acid phosphatase, B-glucuronidase, α-naphthyl esterase, and PAS reactions) in the recognition and classification of normal and abnormal lymphocyte populations

In recent years important advances in the methods for recognition of different populations of lymphocytes have taken place (1). At the same time new techniques in cell morphology and cytochemistry have been applied successfully to the study of lymphoid cells. The two main problems in the identification of lymphocytes are:

1. Distinguishing lymphoid from nonlymphoid cells, particularly when the features of differentiation and maturation are not obvious. This is apparent in the problem of diagnosing and classifying leukemias (2) and lymphomas in order to establish with certainty the nature of the malignant process. Frequently, studies of normal mononuclear cells, separated by density gradient techniques, require the clear identification of lymphocytes from other mononuclear cells such as monocytes.
2. Identifying the various lymphocyte populations (B, T, K, "Null") by means other than the surface markers. This whole area is at a very early stage of development, but the promises of the initial observations with scanning electron microscopy (1) have, however, not been fulfilled (3, 4, 5, 6).

We will review the contributions of morphology, surface structure, and cytochemistry for the identification of lymphoid cells.

Morphology – Light and Electron Microscopy (TEM)

Morphological features of lymphocytes may depend on the state of transformation and on the degree of differentiation. The latter may be demonstrated better by surface markers. "Mature" lymphocytes can readily be distinghished from other leukocytes, but it is not possible to distinguish between T and B lymphocytes with either light microscopy or transmission electron microscopy. Normal antigenically stimulated lymphocytes can occasionally be difficult to distinguish from malignant cells. Transitional forms between B lymphocytes and plasma cells such as seen in Waldenström's macroglobulinemia are characterized by marked basophilic cytoplasm and increased amounts of rough endoplasmic reticulum (Fig. 1).

The importance of lymphocyte morphology and its accurate description is particularly highlighted in the field of non-Hodgkin lymphomas. In these lymphomas the terms "well differentiated" (morphologically mature) and "poorly differentiated" (resembling lymphoblasts) have quite different prognostic significance. In our experience examination of the nuclear chromatin condensation of lymphoid cells under TEM permits a more accurate and objective definition of the terms "well differentiated" and "poorly differentiated."

Lymphoblasts have almost no chromatin condensation; mature lymphocytes, have a predominance of coarse chromatin and intermediate forms such as the prolymphocyte can readily be recognized (7). Follicular centre B cells which have a cleft (cleaved) or rounded (noncleaved) nucleus (8) also vary in morphological appearances depending their degree of transformation. In follicular lymphoma with

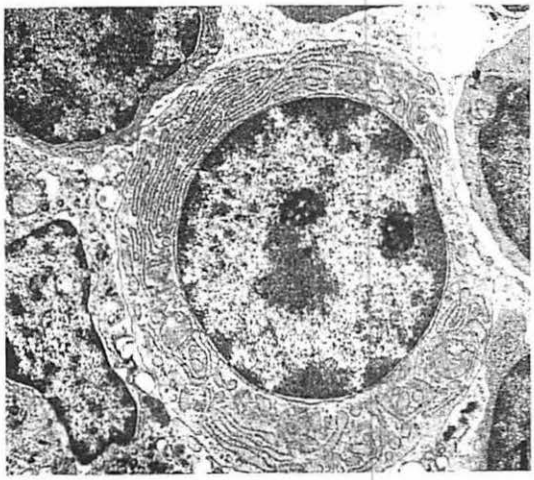

Fig. 1 Lymphoid cell intermediate between a B lymphocyte and a plasma cell, with relatively abundant rough endoplasmic reticulum. From a lymph node of a case of Waldenström macroglobulinemia (x9,000).

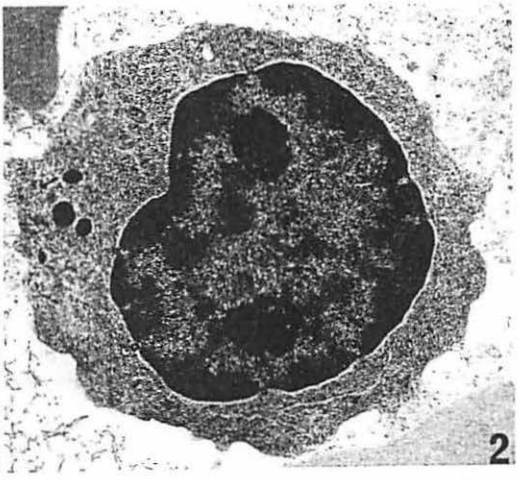

Fig. 2 Lymphocyte with electron-dense granules from a case of T-CLL (x 11,000).

associated peripheral blood lymphocytosis, the cells often have cleaved or notched nuclei (9). TEM studies suggest that the cell seen in follicular lymphoma are similar to those seen in reactive follicles, although nuclear folding is more marked in the neoplastic cleaved follicular centre cells (10). Large noncleaved blast cells with a finely stippled and homogenous nucleus and deep basophilic (pyroninophilic) cytoplasm with many vacuolations containing lipid droplets are characteristic of African (Burkitt's) lymphoma. These cells are seen in the rare cases of sporadic non-African (non-Burkitt's lymphoma which present as or develop acute leukemia (L3 of the FAB classification) (2). Selective involvement of the germinal centers has been demonstrated (11) in this latter group; the cells have been found to have B-lymphocyte characteristics (11, 12). These cells also appear to be larger than those of the African type (8).

Changes seen in disease suggest that T lymphocytes may often be characterized by the presence of numerous azurophilic granules on Romanowsky stains or seen as electron dense granules under TEM (Fig. 2). These features are seen in reactive T lymphocytosis, particularly in infectious mononucleosis (13) and in a rare form of chronic lymphocytic leukemia (CLL) of the T-cell type (T-CLL) (14). Ultrastructure of these cytoplasmic inclusions shows that they are membrane-bound and consists, in some cases, of parallel arrays of microtubule-like structures (13). These structures are also seen in a high proportion of infectious mononucleosis lymphocytes (13) and in some T-CLL cells (15), but rarely in normal lymphocytes.

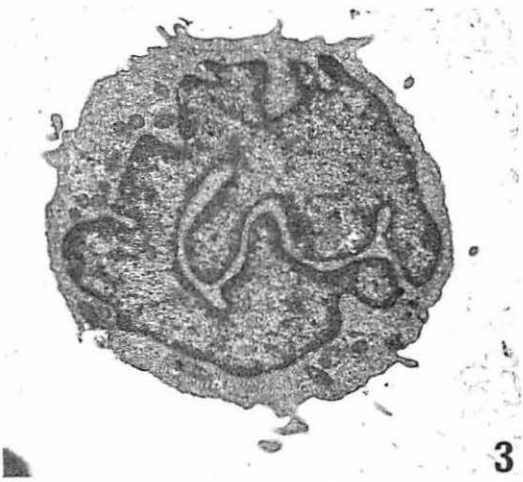

Fig. 3 Sezary cell from a peripheral-blood buffy coat, with a highly convoluted nucleous (x 10,000).

Another type of neoplastic T lymphocyte, the Sezary cell, characteristically has a highly irregular, convoluted, or cerebriform nucleus which is most prominent on TEM (16, 17) (Fig. 3). A small variant of the Sezary cell is seen in cases with a high leucocyte count and may suggest CLL. Sezary cells are less well recognized in paraffin-embedded sections (16). Convoluted lymphoblasts have also been described in a malignant lymphoma originating in thymic cells (8, 18). This disease more often presents as a leukemia and is characterized by T-lymphocyte markers (18, 19, 20). When diagnosed as an acute leukemia, the name "T acute lymphoblastic leukemia" (T-ALL) is commonly used, although the convoluted nucleus initially regarded as typical may not always be present. Other morphological features of the acute lymphoblastic leukemia (ALL) cells and methods for distinguishing them from acute myeloid leukemia (AML) cells are described elsewhere (2).

Hairy cells, characteristic of hairy cell leukemia (leukemic reticuloendotheliosis) have attracted much interest because of the possibility of their being an atypical lymphoid cell (7). Morphologically, the main features of these hairy cells are their long cytoplasmic villi, paucity of lysosomal (electron-dense) granules, and the presence of a cytoplasmic structure known as ribosome-lamella complex in half the cases. This structure is also observed, although rarely, in the cells of other B lymphoproliferative disorders (7).

Surface Structure

Scanning electron microscopy (SEM) has provided a three-dimensional view of the cell surface and its processes. Although most lymphocytes can readily be distinguished from other leukocytes (granulocytes and monocytes 3, 6), except probably eosinophils (21), the evidence for surface differences between T and B lymphocytes has been controversial. Knowledge of the artefacts which can arise from preparation techniques is important because the cell membrane is highly susceptible to many external factors, e.g., temperature (22, 23). More recently, immediate fixation of the cells in suspension and critical-point drying techniques have improved the preservation of the lymphocyte surface morphology (5, 6, 23). All these factors were, however, not considered in the initial studies (3).

Lymphocytes are seen by SEM as spherical cells with numerous short microvilli randomly distributed over the whole cell (23). Initial studies by *Polliack* and co-workers (2, 14) suggested that T lymphocytes had a smoother surface and B lymphocytes, a villous one. The finding of a villous surface in B-CLL and a smooth surface in thymocytes lends further support to this hypothesis. Later, however, it became clear, even using the early techniques, that about 20% of B lymphocytes were indistinguishable from T lymphocytes (24). Studies on rosetted cells (B or T) also conflicted (25) with some reports that B cells were smother than T cells (26). It has now been shown conclusively that smooth cells were artefacts resulting from the contact of unfixed cells with the silver membranes (23). Under optimum conditions most lymphocytes are found to have short microvilli (4, 5, 6); the study of purified populations of B and T lymphocytes has revealed no significant differences (23).

In the formation of RBC rosettes with lymphocytes, the contact between the RBC and lymphocyte occurs via the microvilli. This raises the question of the significance of these structures in cell recognition and communication (23). The lymphocytes in CLL tend, in general, to have fewer microvilli than normal lymphocytes (23, 24), and the blast cells in ALL often have a smooth surface. No differences are found between Null and T-ALL (23, 27) or between B-CLL and Sezary cells (5). Hairy cells when viewed with SEM can have abundant microvilli such as in lymphocytes, ridged and ruffled membranes such as in monocytes (28, 29, 30, 31), or a combination of the two (28, 29).

Cytochemistry

Acid Phosphatase. This lysosomal enzyme can be demonstrated in smears or tissue sections by several cytochemical methods (32, 33). Granulocytes in all stages of development, lymphocytes, plasma cells, monocytes, mega-

karyocytes and histiocytes have all been shown to contain acid phosphatase. In sections of lymph nodes and spleen of human beings and various rodents, *Tamaoki* and *Essner* (34) have shown a positive reaction in the lymphocytes of the T-dependent areas and a negative reaction in the lymphocytes of the B-dependent areas. *Wehinger* and *Möbius* (35), although not able to demonstrate a clear difference between T and B lymphocytes, found a higher proportion of T than B lymphocytes with a positive reaction; *Barr* and *Perry* (36) were unable to confirm these findings. Thus, it would appear that a clear distinction between normal peripheral-blood T and B lymphocytes cannot be made using the acid phosphatase reaction. This variation in results may, however, be a reflection of the different separation techniques employed.

Human fetal thymocytes have been shown to be strongly positive for acid phosphatase (18); this corresponds with the findings of *Tamaoki* and *Essner* in rodent thymus lymphocytes (34). They also noted a difference in the intracellular distribution of acid phosphatase granules between thymic and nonthymic lymphocytes in the mouse, the rat, and the guinea pig. Thymic lymphocytes usually contained one or a few coarse particles of positivity or an aggregate of small particles at one pole of the cytoplasm; lymphocytes in the lymph nodes and spleen possessed many particles scattered throughout the cytoplasm.

Mitogen-stimulated T lymphocytes and infectious mono-nucleosis cells known to be T-derived have also been shown to have a strong acid phosphatase activity (37). A low acid phosphatase activity was reported in CLL cells (38). In our experience most B lymphoproliferative disorders, ie., B-CLL, prolymphocytic leukemia (B-PL), B-ALL, as well as Null-ALL, have only a weak or moderate acid phosphatase reaction in a small proportion of cells, usually less than 30% (39). These findings in B-cell neoplasia are contrasted with those in T cell neoplasia where strong acid phosphatase activity in a majority of cells has been reported in T-CLL (14), T-PL (39), T-ALL (18, 20, 27, 36, 39, 40) and Sezary's syndrome (41) (Table 1). The acid phosphatase reaction in T-ALL is almost always localized to a small paranuclear zone, similar to that observed in thymocytes of rats, mice, and guinea pigs (34).

A double-blind study of the value of acid phosphatase reaction in immunological classification of ALL and its relationship to prognosis, on material from the M.R.C. UKALL trials, has shown in a preliminary analysis, that the characteristic strong localized reaction is found almost exclusively in T-ALL and not in Null-ALL cases (42).

Electron microscopy of T-ALL cells has shown that most of the acid phosphatase activity is localized to the membranes of the Golgi apparatus, but enzyme activity is also present in the few lysosomal granules (27). This characteristic pattern was also noted in some normal T lymphocytes forming rosettes with sheep RBC and in a relatively higher proportion of PHA-transformed T lymphocytes (43). In contrast to T-ALL, the acid phosphatase activity seen at the ultrastructural level in T-CLL is, confined predominantly to the numerous lysosomal granules (Fig. 4) with little or no reaction in the membranes of the Golgi zone.

Table 1 Cytochemistry of the Lymphoid Cell Leukemias.

Cytochemical Reaction	T-ALL	Null-ALL	T-CLL; Sezary	B-CLL
Acid Phosphatase	+++	±	+++	±
β-Glucuronidase	++	++	+++	±
Acid-α-Naphthyl Esterase	+	–	Not reported	–
PAS	+	++	+/++	++

± Weak positivity, minority of cells reacting
+/++ Moderate reaction +++ Strong reaction and most cells positive

A strong acid phosphatase reaction can also be found in hairy cells. This enzyme is L (+) tartrate resistant, and it corresponds to the isoenzyme 5 on disk acrylamide gel electrophoresis (44). Tartrate-resistant acid phosphatase activity is, however, not specific for hairy cells, as it has been demonstrated in Gaucher's cells (45), PHA-transformed lymphocytes, lymphocytes of infectious mononucleosis (45), B-PL (46), an occasional case of T-PL (47), and in T-CLL (42).

β-Glucuronidase

Lymphocytes, in contrast to granulocytes and monocytes, have a granular rather than a diffuse reaction pattern for this enzyme (4). It has been suggested that the reaction is useful for distinguishing AML from ALL. Lymphoblasts react with a granular pattern in contrast to the myeloblasts which often to not react, or occasionally show a diffuse reaction (48, 49).

Barr and *Perry* (36) described two patterns of granularity in peripheral blood lymphocytes: type α: large blocks of reaction; type β: multiple small granules. They found that T lymphocytes were almost always positive and half the cells had type α reaction. Less than three quarters of the B lymphocytes were positive, but they always exhibited the type β reaction. β-glucuronidase has also been shown to be present in the lymphocytes of the T-cell dependent areas and absent from the lymphocytes of the B-cell dependent areas in the spleen and lymph nodes of human beings and rats (34). In contrast to the findings with acid phosphatase, *Tamaoki* and *Essner* (34) found no β-glucuronidase activity in thymus lymphocytes of most rodents with the exception of rats.

While the morphologically well differentiated B-cell neoplasms, i.e., B-CLL and B-cell lymphomas, have been shown to have a low β-glucuronidase activity (50, 51) the well-differentiated T-cell neoplasms, i.e., T-CLL (14) and Sezary cells (52), have a strong β-glucuronidase activity (Table 1).

Although the reaction pattern in ALL has not been studied in great detail, *Brouet* et al. (20) found two-thirds of the cases from both T-ALL and Null-ALL to be equally reactive. It would appear from this study that the β-glucuronidase reaction may not be as useful as the acid phosphatase reaction in distinguishing between the various types of ALL.

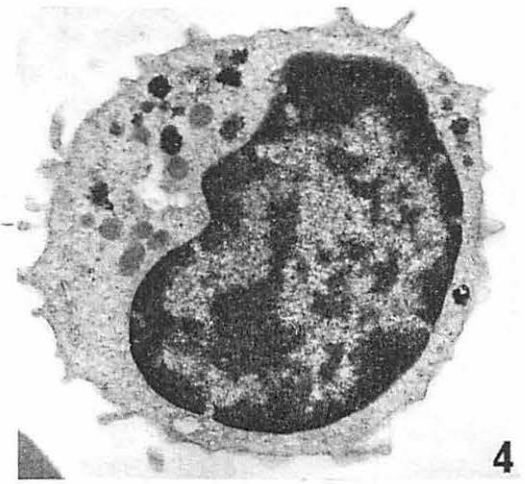

Fig. 4 Acid phosphatase reaction localized to the cytoplasmic granules in a lymphocyte, from a case of T-CLL (x 15.000).

α-naphthyl Acetate Esterase

The α-naphthyl acetate esterase reaction, in conjunction with NaF inhibition, can be used to differentiate monocytes and monocyte precursors from myeloid cells (53). Using a modified technique, *Mueller* et al. (54) demonstrated acid α-naphthyl esterase activity in lymphocytes of the T-cell dependent areas of rat lymph nodes and spleens, but no activity in the lymphocytes of the B-cell dependent areas. More recently, *Kulenkampff* et al. (55) used this technique on human lymphocyte preparations from blood and tonsils and demonstrated two specific patterns: 1) a localized strong reaction (T-like) in a majority of T-lymphocytes; 2) a weak localized reaction (thymus-like) in a thymocyte subpopulation. Most thymocytes and all B lymphocytes did not react. Mitogen-activated human T lymphocytes showed a decrease in activity; B-CLL cells and Null-ALL cells were negative (Table 1). T-ALL cells were positive with the "thymus-like" re-

action. This reaction may help to distinguish T-ALL from Null-ALL, but it would seem more useful for distinguishing normal T from B lymphocytes. In some respects the pattern of reaction for this enzyme in T cells may be similar to the β-glucuronidase reaction, i.e. a strong reaction in mature T lymphocytes and a weak reaction in thymocytes. In contrast, the acid phosphatase activity would appear to be stronger in thymic cells and weaker in mature T lymphocytes.

PAS Reaction

A positive reaction in blood cells usually denotes glycogen; this can be confirmed by the sensitivity to diastase (33). Normal lymphocytes contain much less staining material than granulocytes; but a few fine, or even coarse, granules may often be demonstrated. A positive, granular PAS reaction has been considered characteristic of lymphoid cells. *Astaldi* and *Verga* (29, 56) and *Quaglino* and *Hayhoe* (57) demonstrated that the strong PAS staining in CLL cells was due to an increase in glycogen. In a study of a large number of cases of acute leukemia, *Hayhoe* et al. (58) found that PAS staining (granules and blocks) was the most reliable characteristic of ALL. It seems that the PAS reaction may not be as specific for ALL as originally felt. PAS positivity has been demonstrated in the blasts of acute monocytic leukemia and erythroleukemia (58, 59); "blocks" were even noted in acute monocytic leukemia (60).

Although some normal lymphocytes contain a few PAS-positive granules, it has been difficult to determine whether these cells are B or T lymphocytes because of the rapid changes in intracellular glycogen when incubated. We have observed (39) that Null-ALL, B-CLL, and B-PL cells generally have a greater PAS reactivity than T-PL and T-ALL cells (Table 1); B-ALL cells have usually been found to be negative. Other workers have noted that ALL cases with a strong paranuclear acid phosphatase reaction in the blast cells and clinical features suggestive of T-ALL had smaller amounts of PAS-positive material than the acid phosphatase-negative ALL's (61). However, this was not confirmed by *Brouet* et al. (20) who found no difference between T-ALL and Null-ALL with regard to the PAS reaction.

PAS positivity may not necessarily represent a difference in lymphocyte subpopulations but may indicate a difference in proliferative activity. Using tritiated thymidine uptake studies, *Löffler* has shown that a larger number of PAS-negative blasts were in the S-phase than PAS-positive blasts (61). Additional evidence (62) suggests that T-ALL has a greater proliferative activity, i.e., higher mitotic and labelling index, than blasts from Null-ALL. This may explain why T-ALL cells may have a lower glycogen content than Null-ALL cells.

Taswell and *Winklemann* (63) first noted that Sezary cells contained granules of PAS-positive material. More recently (41), it was noted that only 75% of the cases have PAS-positive cells. The PAS material in these cells is often seen forming coarse granules or clumps.

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D. Catovsky, M.R.C. Leukaemia Unit, Royal Postgraduate Medical School, London W 12 OHS