Surface Features of Cells in Human Lymphoproliferative Disorders. An Immunoelectron Microscopy Study.

M. F. Gourdin, F. Reyes, J. L. Lejonc, P. Mannoni and B. Dreyfus

Unite de Recherches sur les Anémies. INSERM. U. 91 and
C. D. T. S. du Val de Marne. CHU Henri Mondor.
94010 CRETEIL FRANCE

Summary

Peroxidase conjugated antibodies were applied to cell suspensions in order to detect surface associated immunoglobulins. Cell suspensions were fixed prior to incubation with reagents, a procedure avoiding membrane alterations induced by antibodies to surface component. By immunoelectron microscopy an identification of B lymphocytes could be made with simultaneous observation of their surface architecture.

Basic findings were that normal circulating human B lymphocytes had a villous surface. This relationship was not confirmed however by examinating samples from various B and T cell proliferations establishing that surface morphology is not sufficient to categorize cells in disease.

Specimens from hairy cell leukemia were also examined. Despite salient surface characteristics as revealed by the present method, the categorization of cells remains unclear.

Introduction

The recognition of T and B lymphocytes by their surface properties is well documented and has led to numerous studies in humans. Among several available assays the detection of surface immunoglobulins is essential for the chraracterization of B cells (1). Immunoelectron microscopy (I.E.M.) gives the possibility of studying the surface architecture and structure of cells with simultaneous detection of membrane components.

CORRESPONDANCE:
F. REYES
Unité de Recherches sur les Anémies. INSERM U. 91
Hôpital Henri Mondor.
94010 CRETELL FRANCE

Therefore this technic has been largely applied to lymphoid cells. Until recently however no striking differences of the surface morphology were reported between immunoglobulin-bearing (B) lymphocytes and the others, when antibodies conjugated with various markers were applied to live lymphocytes (for review see (2)). Recent data have shown that a clear distinction can be made between B cells and the others on the basis of surface morphology, when normal human blood lymphocytes are reacted with conjugated anti-immunoglobulin reagents as fixed cell suspension (2, 3). These observations have confirmed by immunologic identification previous observations by scanning electron microscopy (S.E.M.) of Polliack and co-workers (4).

In this paper basic findings on normal human B lymphocytes will be recalled and cells from various proliferative disorders described.

Material and Methods

1. *Samples.* The following samples were examined: blood buffy coat from eight normal individuals, ten untreated patients with chronic lymphocytic leukemia (C.L.L.), two with Waldenström macroglobulinemia, four with "prolymphocytic leukemia", two with Sezary syndrom and four with hairy cell leukemia. Splenectomy was performed in two patients of the "prolymphocytic" type. Blood leukocytes were prepared by simple sedimentation at room temperature without other separation procedure and washed in Hank's. Spleen were teased in Hank's with fine forceps, cell clumps discarded and cell suspension obtained after several centrifugations and washings.

2. *Preparation of specimens for I.E.M.* This was carried out as detailled elsewhere (2). Briefly, extensively washed cell suspensions were first glutaraldehyde-fixed. They were then reacted with peroxidase conjugated anti-immunoglobulin reagents, post-fixed with glutaraldehyde and reacted with diaminobenzidine for peroxidase detection. Cell pellets were further post-fixed with osmium and processed for embedding by usual procedures.

3. *Reagents.* Anti-immunoglobulin antibodies were raised in rabbits and sheep, and purified by immunoadsorption (5). They were rendered monospecific for μ, γ, α and $\varkappa$, λ chains (2). Anti-human IgD serum was purchased from C.D.T.S., Bois-Guillaume, Rouen, France; after further absorption on insolubilized whole human serum with a high IgM content, anti-δ antibodies were purified by elution from insolubilized IgD myeloma serum. Anti-F_{ab} antibodies were used as polyvalent anti-immunoglobulin reagent; they were prepared by similar adsorption procedures as previously detailled (6).

4. *Controls.* The specificity of surface labeling was checked by appropriate cellular and serological controls (2).

Results

1. *Normal lymphocytes*

A specific surface staining was found in about 15 % of blood lymphocytes after reacting suspensions with the anti-F_{ab} reagent. IgM-bearing cells were largely predominant over IgG or IgA-bearing lymphocytes, which were uncommon in
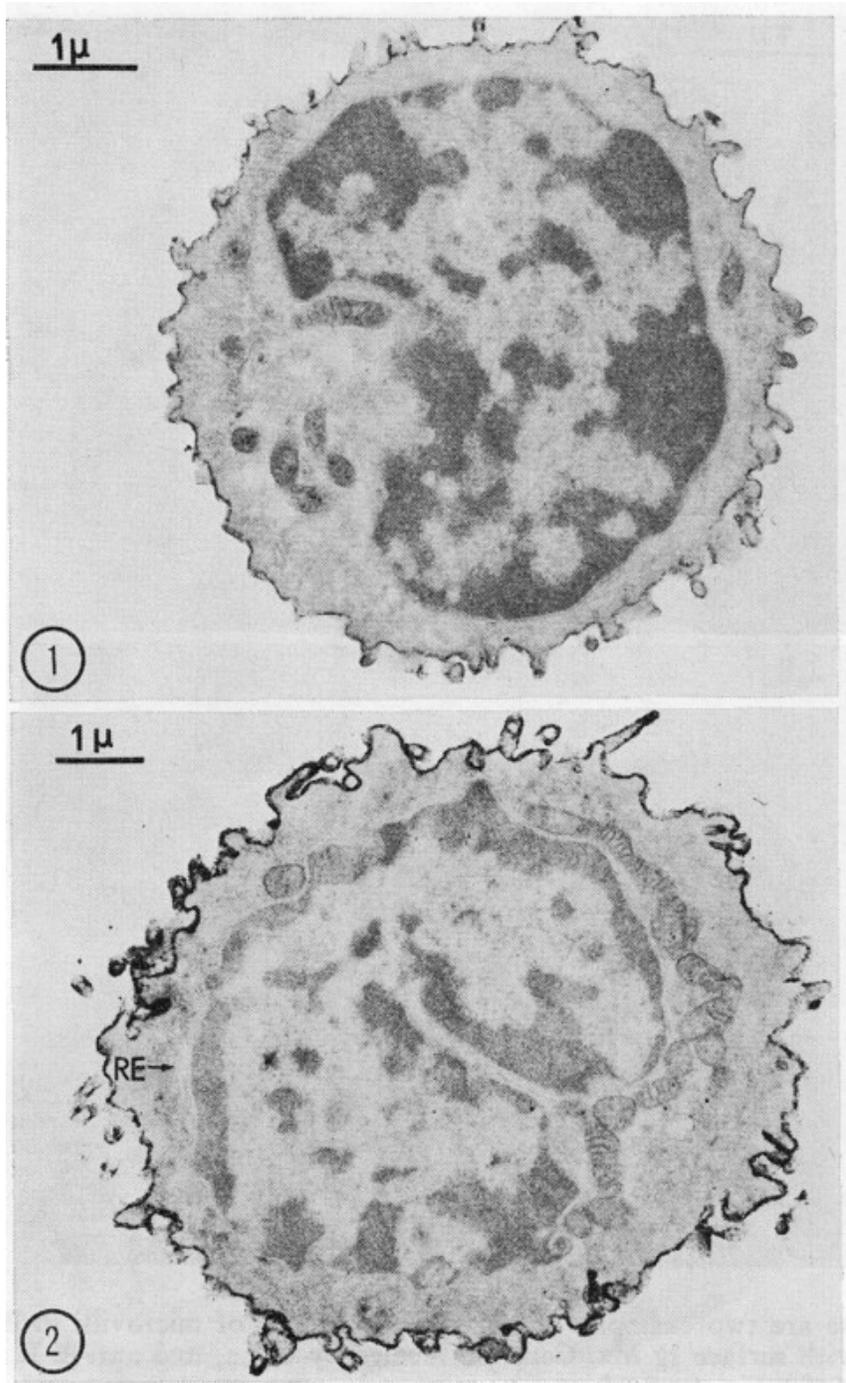
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Fig. 1, 2: Normal human blood B lymphocytes. Villous cells are labeled by peroxidase conjugated anti-Fab. Surface immunoglobulins are revealed by a black reaction product due to the cytochemical detection of peroxidase. Variations of the number and length of microvilli, and of surface staining intensity can be appreciated by comparing these two figures. Short strands of endoplasmic reticulum are appearant. (RE →). Fig. 1 x 13350, Fig. 2 x 11700.

sections. Anti-δ reagent was applied to two samples and IgD-bearing lymphocytes found in sections although less numerous than IgM-bearing cells.

In every sample however the labeling pattern was similar, whatever the s.Ig class detected, in that the surface staining was continuous and diffuse all around the cell. No periodicity or patchy pattern was observed on labeled cells. This did not preclude some variations of the staining intensity in a given sample; IgD
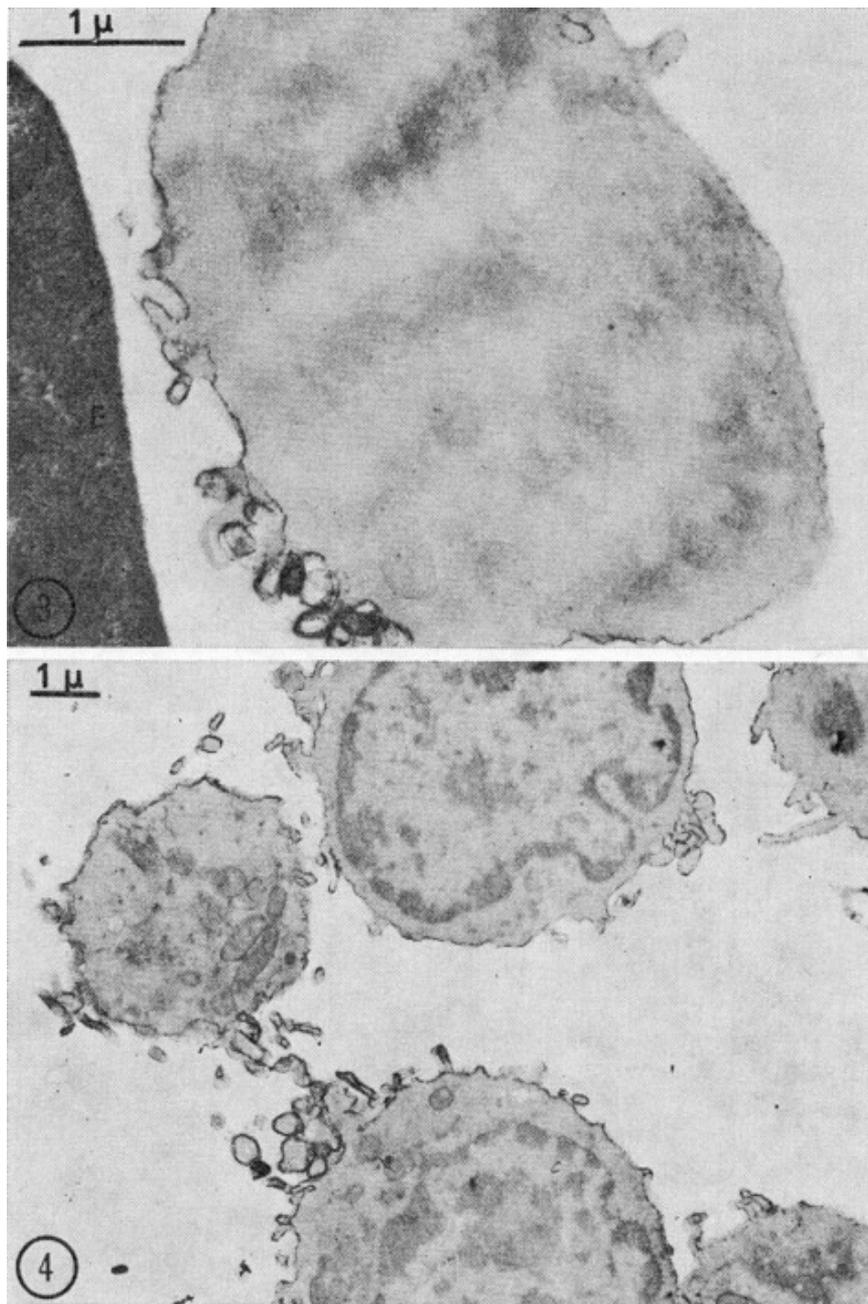
Fig. 3, 4: These are two examples of polar concentration of microvilli in B lymphocytes from C.L.L., with surface Ig Mϰ. Cells are labeled by conjugated anti-μ. E is an erythrocyte. Fig. 3 x 21150, Fig. 4 x 9450.

positive cells for instance were moderately stained when compared to IgM-bearing lymphocytes.

Whatever the class of detected s.Ig, labeled B lymphocytes had a characteristic appearance because of the presence of numerous microvilli (Fig. 1, 2). Non labeled cells had a general smooth surface with sometimes only rare short and spaced microvilli. A few cells were also found which had an intermediate amount of microvilli and a weak surface labeling, therefore considered as B lymphocytes.

2. Lympho-proliferative disorders.

In C.L.L. samples most cells confirmed the relationship between the presence of detectable s.Ig and a villous surface, as previously reported (2). Lymphocytes
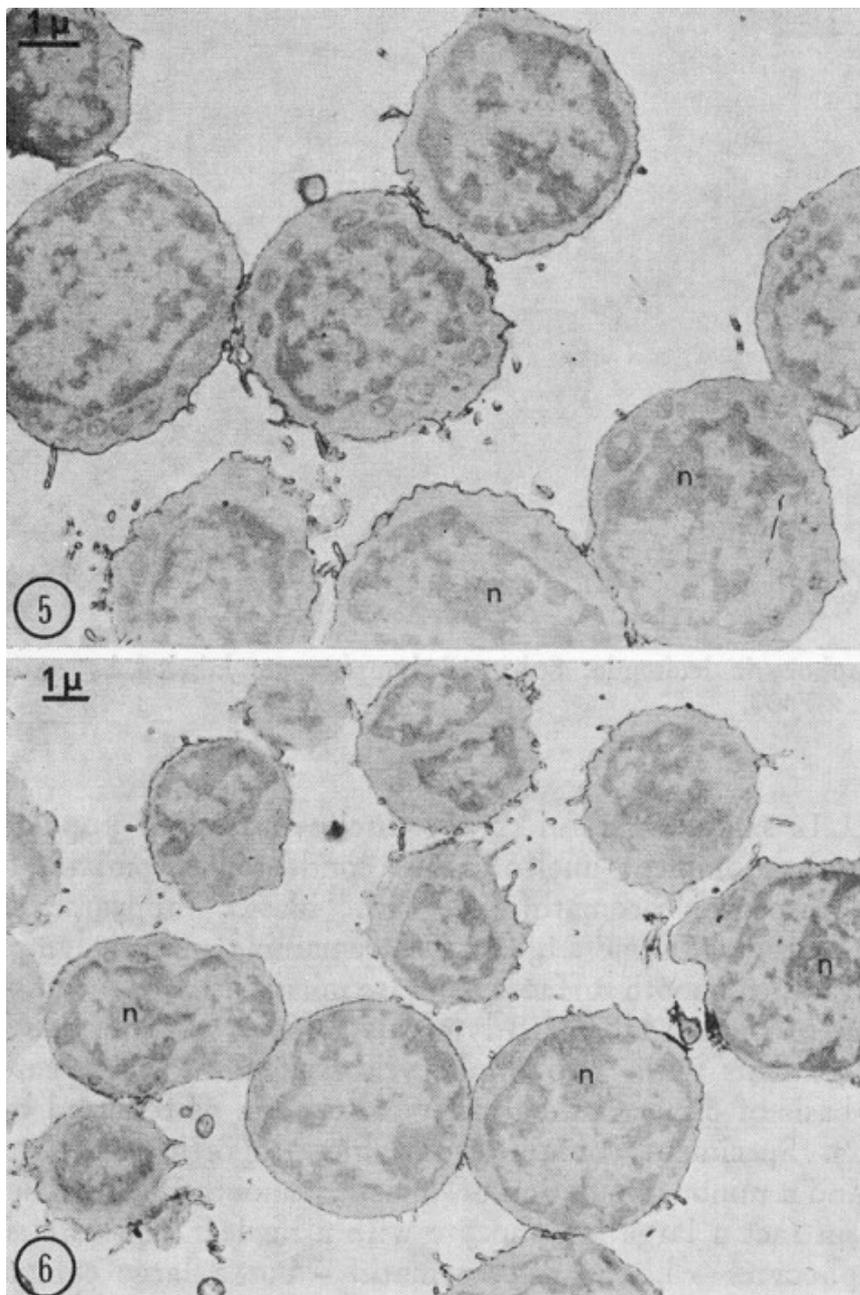
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Fig. 5, 6: Sample from prolymphocytic leukemia with surface Ig M λ. Cells are labeled by anti-μ in Fig. 5, by anti-λ in Fig. 6. Moderately villous and smooth cells can be seen. Nuclei contain coarse chromation. n = nucleolus.
Fig. 5 x 7900, Fig. 6 x 6190.

were identified as B cells by polyvalent anti-Fab reagent and their monoclonal distribution of s.Ig shown by monospecific anti-heavy and light chains reagents (IgM ϰ in 5 cases, IgM λ in 2, IgG ϰ in 1). Among cells classified as mature small lymphocytes on the basis of their ultrastructural appearance, variations of the number of microvilli could be seen paralleled by variations of the staining intensity; therefore in some samples the surface morphology ranged from moderately villous to "hairy" cells. In addition cells were found in some sections exhibiting a polar concentration of labeled microvilli with a remaining smooth surface (Fig. 3, 4).
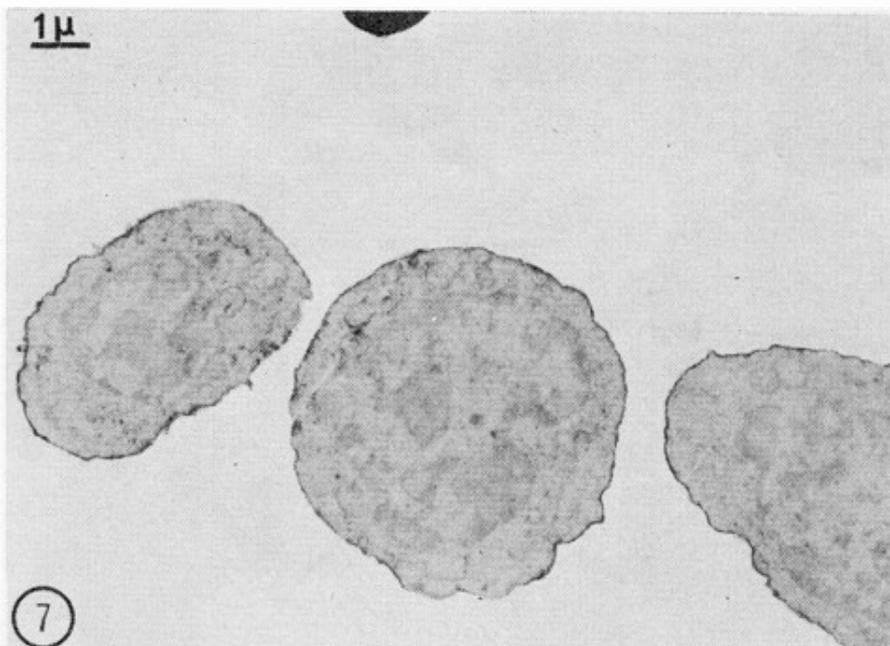
Fig. 7: Prolymphocytic leukemia. Spleen B lymphocytes labeled by anti-μ and with a smooth surface. x 7400.

In some C.L.L. samples careful survey disclosed a minor population of larger cells, either with prominent nucleolus but condensed chromatin, or with large nucleolus and dispersed chromatin (i.e. with "blastic" appearance). These cells were shown to bear the same s.Ig class as remaining mature lymphocytes; they had however a rather smooth surface with rare microvilli.

The presence of smooth labeled B lymphocytes was still more obvious in blood samples from patients with "prolymphocytic leukemia". These cases were diagnosed on the basis of clinical and cytological criteria as reported by Galton and co-workers (7). Specimens contained variable proportions of small villous B lymphocytes and a minor population of "blastic" smooth B cells. The predominant cell type was in fact a large lymphocyte with a nuclear structure similar to that of small lymphocytes – i.e. coarse chromatin – but a large compact nucleolus; cytoplasm also contained some short strands of endoplasmic reticulum. Most of these large cells had few microvilli and their general shape was smooth, although specifically stained by corresponding monospecific anti-immunoglobulin antibodies (Fig. 5, 6). These smooth large B cells were also the predominant feature of splenic suspensions in these patients (Fig. 7).

Blood leukocytes were obtained from two patients with Wäldenström macroglobulinemia. In one patient all lymphocytes were labeled by anti-μ and anti-ϰ reagents; in the other IgM ϰ B cells were only part of circulating lymphocytes (30 %). In both cases a villous surface characterized the mature lymphocytes, as in C.L.L. (Fig. 8, 9). In addition some "intermediate" lymphoid cells were present with some development of endoplasmic reticulum; they were weakly labeled and had a rather smooth surface, but also exhibited a faint specific reaction in the perinuclear space and endoplasmic reticulum lamellae (Fig. 8).

In blood specimens from Sezary syndrom, large abnormal lymphocytes with a typical convoluted nucleus were found. They were regularly free of labeling by
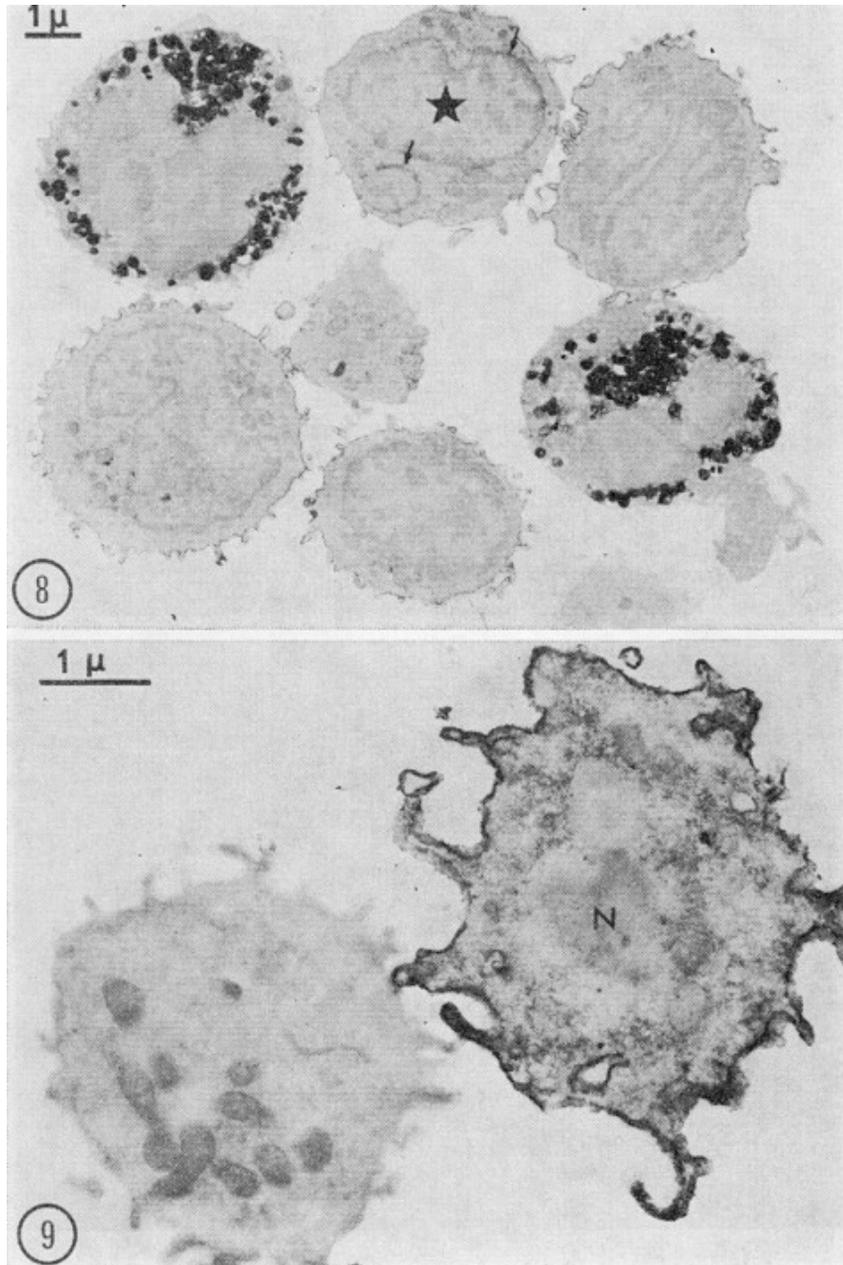
Fig. 8: Blood leukocytes from Waldenström macroglobulinemia. This micrograph shows two polymorphs with endogenous peroxidase in granules; their membrane is not labeled. Three villous B lymphocytes as revealed by anti-μ can be seen. In addition a smooth lymphoid cell (✱) with weak surface labeling also exhibits specific staining in perinuclear space and endoplasmic reticulum (→). x 7250.
Fig. 9: Tangential section of two villous lymphocytes in another sample from macroglobulinemia. One cell with long microvilli is heavily labeled by anti-μ reagent. N is the nucleus. x 14850.

anti-immunoglobulin reagents although segments of their membrane could exhibit numerous microvilli, the remaining surface being smooth (Fig. 10, 11).

3. Hairy cell leukemia

Blood buffy coat was obtained in four cases diagnosed by current clinical and morphological criteria (8, 9). In our experimental conditions these abnormal nononuclear cells exhibited three main characteristics (Fig. 12): 1) they had a very
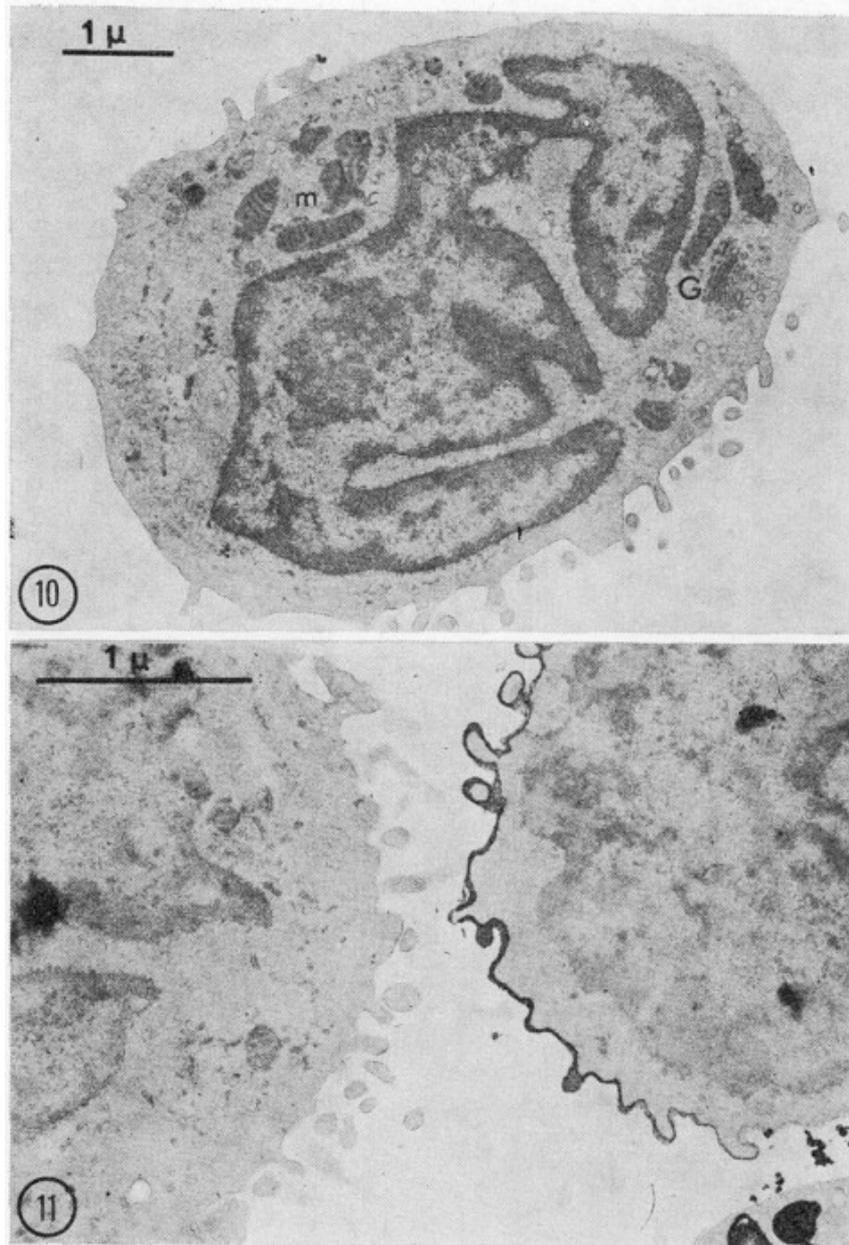
Fig. 10: Sezary syndrom. A typical abnormal cell with convoluted nucleus is seen. Cytoplasm contains lysosomes and numerous lucent vesicles; G is the Golgi apparatus, m are mitochondria. This cell has segments of villous and smooth membrane, without any detectable labeling by anti-Fab. x 15000.

Fig. 11: Sezary syndrom. Part of facing Sezary cell and B lymphocytes. Note that microvilli of Sezary cell are not labeled. Lower right: part of a polymorph with peroxidase containing granules. x 30000.

irregular surface, covered by numerous long microvilli and finger-like projections; 2) they had a high density of associated s.Ig., as revealed by anti-Fab reagent; 3) they lacked detectable endogeneous peroxidase in either endoplasmic reticulum or granules.

However, these cells failed to exhibit a monoclonal pattern of s.Ig. since both $\varkappa$ and λ light chains were detected in every sample. Moreover cells strongly reacted with anti-γ antibodies but not with anti-μ, anti-δ, or anti-α antibodies.
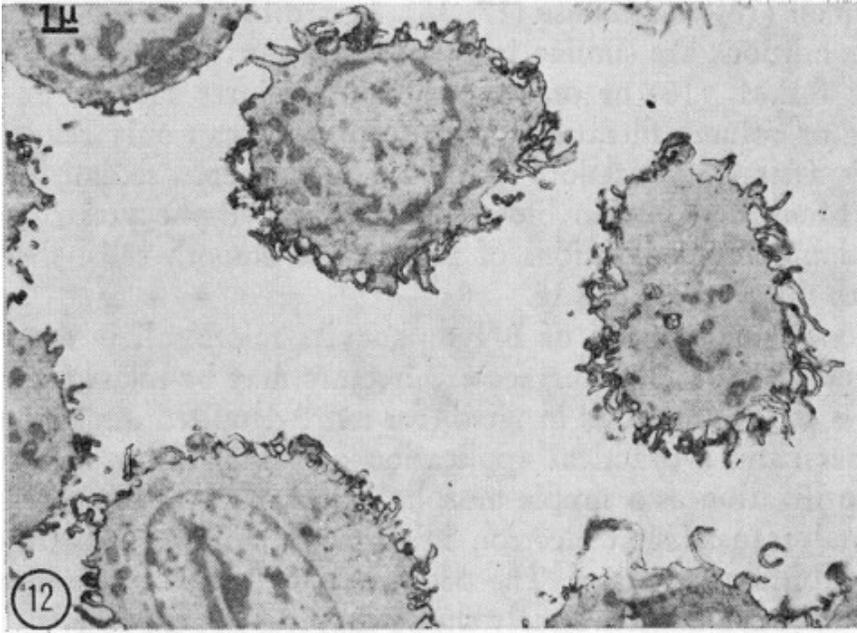
Fig. 12: Blood sample from hairy cell leukemia. These large mononuclear cells have a very irregular surface, covered by long villous processes. There is a high density of surface immunoglobulins as revealed by the diffuse black reaction product outlining the cell surface. No endogeneous peroxidase is detectable. These cells have been labeled with anti-γ reagent. x 5450.

Discussion

The present findings establish a clear duality based on the surface morphology of normal human blood lymphocytes, when first glutaraldehyde-fixed and then identified by peroxidase-conjugated anti-immunoglobin reagents. The distinction between normal villous B cells and smooth (presumed) T cells is not absolute since a minor population of intermediate forms can be found, most of them being however recognized as B cells (2, 3). Thus previous S.E.M. observations by Polliack and co-workers (4) are confirmed by I.E.M.

It is worth noting that until recently no major morphological distinction had emerged from previous I.E.M. studies using various markers; the reasons for this have been discussed already (2). We will only emphasize that when incubation with anti-immunoglobulin reagents is carried out on cells in a living state, B lymphocytes are rendered smooth as a result of a redistribution phenomenon which involves an invagination of surface microvilli (10). Similar observations of smoothing of the surface by antibody-induced redistribution have been made on cells from hairy cell leukemia (unpublished observations).

Such data illustrate the possible induction of morphological cell alterations by factors related to experimental procedures. Another example is the surface alteration of lymphoid cells when involved in rosetting with erythrocytes (11). Therefore the appreciation of the surface morphology of B and T cells after identification by rosetting procedures seems questionable and may explain conflicting reports (12, 13, 14).

However the villous nature of B lymphocytes as found in normal human blood – and recently in mouse lymphoid organs (15) – is still controverted by recent

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reports in human (16) and mouse (17, 18). It is difficult to know to what extent preparative conditions are similar in published reports, either at the level of cell collection for S.E.M. (16) or of separation procedures such as density gradient centrifugation or column filtration. At this point we can only reiterate that when observed after a simple procedure involving spontaneous sedimentation, washing and fixation of normal human blood buffy coat, lymphocytes can be separated into two predominant populations of villous and smooth cells, the former being associated with the presence of s.Ig.

The functional implications of B lymphocytes microvilli is not clearly determined however. This peculiar surface architecture may be related to some physical properties of B cells as revealed in vitro (for more detailled discussion see (2)).

On the other hand a practical application of this peculiar surface architecture would be its utilization as a simple morphological marker, detectable by scanning or conventional transmission electron microscopy, for the classification of cells from lymphoproliferative states. The present study which was undertaken to test the reliability of such a marker clearly shows that it is not the case.

In C.L.L. and macroglobulinemia, known as monoclonal B cell disorders (1) the villous nature of lymphocytes was confirmed. However samples also contained some smooth cells belonging to the same proliferative process as revealed by simultaneous immunologic identification; they were larger cells with a distinct nucleo-cytoplasmic features. The presence of smooth large B cells was further confirmed by examinating prolymphocytic leukemia samples in which this cell type was predominant over villous small lymphocytes, although they were part of the same proliferative process. A smooth surface also characterized those cells present in blood specimens from macroglobulinemia which were termed "intermediate" because of their developing endoplasmic reticulum; they were also shown to be of B deviration by the simultaneous detection of immunoglobulins both at the surface and in secretory apparatus.

These observations therefore demonstrate that B cells involved in the same proliferative process may have a different surface morphology. Similar conclusions were recently suggested by S.E.M. examination of C.L.L. samples (19, 20). These villous and smooth patterns may represent different stages in the maturation process of B cells. This view suggested by the present findings, is further supported by the surface pattern of B immunoblasts, as found in some lymphomas (to be published). It is also corroborated by the finding of smooth precursor B cells, as recently reported in chicken bursal cells (15).

Examination of abnormal Sezary cells also disclosed an exception to the general relationship between microvilli and s.Ig, as found in normal blood lymphocytes. Sezary cells have been shown to have membrane properties common to T lymphocytes (21, 22). In this study they were found to lack detectable s.Ig including at the level of their segment of villous membrane.

Taken all together our observations of B and T cell disorders emphasize that surface morphology alone is not a suitable criterion for classifying cells of lymphoproliferative disorders. Immunologic identification is in fact essential for that purpose.

The lymphocytic or monocytic nature of abnormal cells found in hairy cell leukemia keeps on being the subject of controversial reports (for review see (23,

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24, 25)), despite the use of various sophisticated methods. In this study cells lack detectable endogeneous peroxidase, an essential cytochemical feature of normal marrow and blood monocytes (26). Their peculiar surface morphology can be compared to that of some very villous, "hairy", B lymphocytes, but also to the ruffled membrane of monocytes (25). Despite the presence of great amounts of s.Ig. their lymphocytic nature is not ascertained. In the present experiment we failed to demonstrate surface IgM or IgD; such B cell markers were recently reported to be present in hairy cell leukemia (27). On the other hand surface polyclonal IgG were regularly detected in our study, a result suggesting that s.Ig were adsorbed cytophilic immunoglobulins rather than an actual cell product; the presence of receptors for the Fc portion of IgG has been previously demonstrated in hairy cell leukemia, favoring its monocytic origin (24). Culture experiments are actually in progress in order to clarify this problem. However, as recently pointed out by King and his collegues, the identification of these malignant cells is "plagued by the universal problem of categorizing abnormal cells on the basis of assays usually reserved for identifying normal cells". (28).

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