SCANNING ELECTRON MICROSCOPY/1977 Vol. II
Proceedings of the Workshop on Biomedical Applications--
SEM as an Aid in Diagnosis
IIT Research Institute
Chicago, Illinois 60616, U.S.A.
March 1977

LACK OF CORRELATION BETWEEN IMMUNOLOGIC MARKERS AND CELL SURFACE ULTRASTRUCTURE IN THE LEUKEMIC PHASE OF LYMPHOPROLIFERATIVE DISEASES

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Abstract

In a prospective study of malignant cells from 13 patients with the leukemic phase of lymphoproliferative diseases, we wished to determine whether any correlation existed between the immunologic markers and the cell surface ultrastructure. Five patients had chronic lymphocytic leukemia, four had malignant lymphomas, poorly differentiated lymphocytic type, two had the Sézary syndrome, and one each had acute prolymphocytic leukemia and acute lymphocytic leukemia. Cell separation and isolation was done at room temperature for all specimens. Immunologic markers tested for were surface immunoglobins, a B-cell property, and E-rosettes, a T-cell property. Three patients had T-cell diseases, 6 had B-cell diseases, and 4 were classified as "null." All but one patient had moderate to large numbers of microvilli on their malignant cells. The single exception had a typical B-cell form of chronic lymphocytic leukemia. There appears to be no correlation between immunologic markers and cell surface ultrastructure; therefore, SEM appears not to be valuable in the diagnosis or classification of immunologic sub-types of certain lymphoproliferative diseases.

Key Words: Cell Surface Morphology, Surface Markers, Immunologic Markers, Leukemia, Lymphoma

Introduction

The identification of human B and T lymphocytes by scanning electron microscopy (SEM) was first reported by Polliack et al. in 1973.[1] By 1975, Polliack and DeHarven recognized that SEM allowed distinction between B- and T-derived lymphocytes in many, but not all cases. They stated that accurate identification of lymphocyte populations with overlapping surface features was difficult by SEM alone without parallel immunologic identification.[2] Our laboratory had previously investigated the surface ultrastructure and marker characteristics of leukemic cells, with emphasis on the types of projections present on cells in different types of leukemias.[3] The study reported here was a prospective one, designed to determine whether or not there was any correlation between surface ultrastructure and immunologic markers in patients with the leukemic phase of various lymphoproliferative diseases. Patients with hairy cell leukemia were excluded from this group, as it had previously been shown that their malignant cells have a distinctive surface ultrastructure characterized by large, undulating, broad-based ruffles with an occasional area of short microvilli.[4]

Methods and Materials

Thirteen patients with the leukemic phase of a lymphoproliferative disease were studied from September 1975 through September 1976 (Table 1). Forty to sixty cubic centimeters of peripheral blood were drawn into a plastic syringe and transferred into sterile vacutainers in which EDTA served as an anticoagulant. The lymphocytes were then separated out by the Ficoll-Hypaque density gradient method. The cells were washed 2 times with Hanks' Balanced Salt Solution (HBSS), counted in a hemocytometer, and the cell suspension was adjusted to 10 x 10^6 cells/ml with HBSS.

An aliquot of 0.5 ml was mixed with 2.5% glutaraldehyde in Millonig's buffer and slowly drawn down with gentle vacuum onto a 0.2μ silver filter membrane. The aspiration was stopped before the liquid level dropped to that of the

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membrane; the membrane was quickly removed while still wet, placed in 2.5% glutaraldehyde, post-fixed in OsO_4, taken through graded sets of alcohol and alcohol:amyl acetate, critical-point dried in a Bomar SPC-900EX, and coated with gold on a Seevac Conductavac I coater. The specimens were viewed in either a Coates and Welter Model 50 or a Hitachi HFS-2 SEM. The quantity of microvilli on the surface was judged and classified as few, moderate, or many.

A second aliquot of 5×10^6 cells was taken for surface immunoglobulin analysis with fluorescein-conjugated polyvalent (PV) antiserum (Meloy Laboratories, Springfield, Va.). The antiserum was diluted 1:4 with phosphate-buffered saline, filtered to remove any aggregates, and 100λ were added to the cell sample. This specimen was allowed to incubate for 30 minutes at ambient temperature, then was rinsed 3 times with HBSS. The cells were viewed on an American Optical fluorescent microscope and the percentage of fluorescing cells was calculated.

Another aliquot was taken for a test of E-rosette formation. A pellet of 5×10^6 cells was suspended in 0.4 ml adsorbed fetal calf serum, and 0.4 ml of a 0.5% solution of washed sheep red blood cells in phosphate-buffered saline was added. The mixture was allowed to incubate at 37°C for 30 minutes, then spun at 200 x g for 5 minutes and placed on ice for 2 hours. The mixture was resuspended by gentle rolling of the tube, and 100 cells were counted in a hemocytometer. Only those cells with 3 or more adhering erythrocytes were counted as E-rosettes.

Results

Results for the 13 patients are presented in Table 1. Seven normal adults were studied during the period in which the patients included in the table were under investigation. In these controls, the percentage of cells positive for SIg ranged from 10 to 24, with a median of 18; the percentage of E-rosettes ranged from 53 to 74, with a median of 55.

Table 1. Patient Characteristics, Cell Surface Markers, and Ultrastructural Projections

Patient No.	Sex	Age	Histological Diagnosis	WBC/mm^3	Abn. Cells (%)	P.V. (%)	E-Ros. (%)	Marker Characteristics	Surface Microvilli Few	Mod.	Many
1	M	53	CLL	7,400	85	2	60	T		X	
2	M	48	ML-PDL	40,000	50	0	33	T*			X
3	F	75	SS	19,100	95	0	55	T		X	
4	M	63	CLL	35,800	70	95	21	B		X	
5	M	68	CLL	69,000	95	87	0	B	X		
6	M	53	CLL	10,600	60	91	28	B			X
7	M	64	CLL	48,000	55	98	1	B		X	
8	M	54	ML-PDL	500,000	90	77	10	B		X	
9	M	57	ML-PDL	20,800	11	100	10	B			X
10	M	63	APL	380,000	82	2	12	Null			X
11	F	32	SS	40,000	71	18	12	Null			X
12	M	57	ML-PDL	25,400	70	6	4	Null			X
13	M	29	ALL	60,000	96	1	4	Null		X	

*The E-Ros. value could reflect normal T-lymphocytes and thus this could also be classified as "null."

Abbreviations:

Sex: M = Male; F = Female
WBC = White Blood Cell Count
Abn. Cells = Abnormal cells which are histologically diagnostic of disease
P.V. = Goat anti-human antisera
E-Ros. = Sheep erythrocyte rosettes

Histological Diagnoses:

CLL = Chronic lymphocytic leukemia
ALL = Acute lymphocytic leukemia
ML-PDL = Malignant lymphoma, poorly differentiated lymphocytic
SS = Sézary syndrome
APL = Acute prolymphocytic leukemia

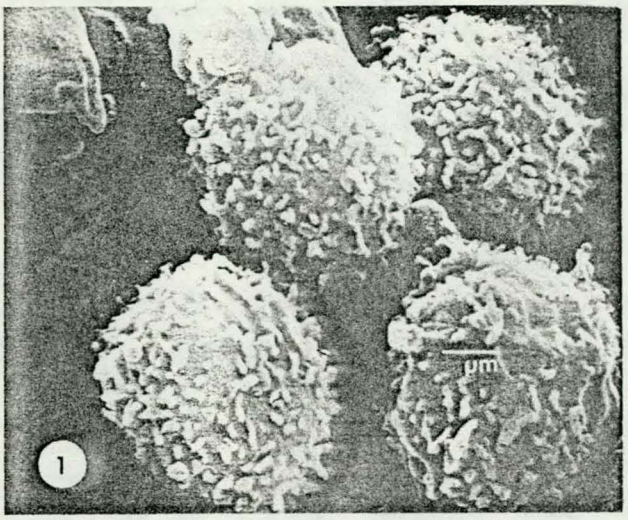

Figure 1. Cells from patient 1 (T-cell CLL), with moderate numbers of microvilli.

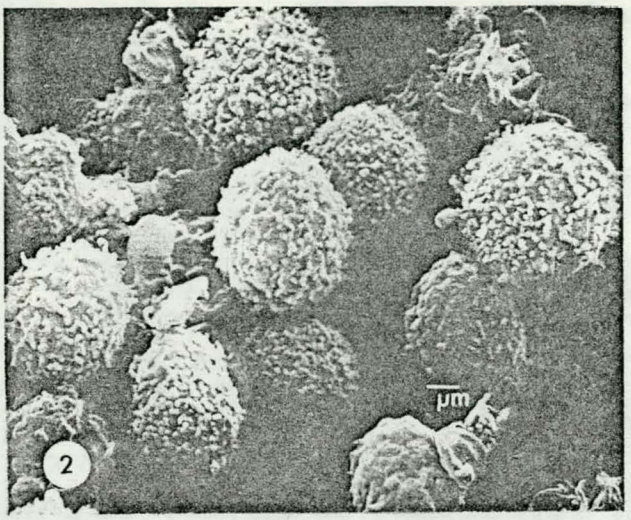

Figure 2. Cells from patient 3 (SS).

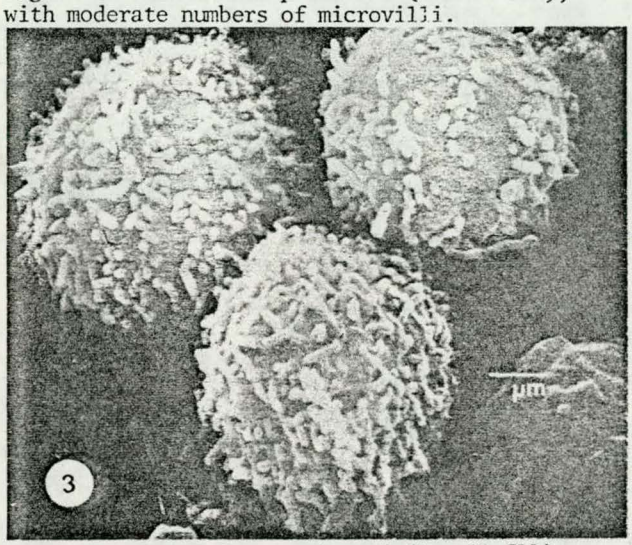

Figure 3. Cells from patient 4 (B-cell CLL).

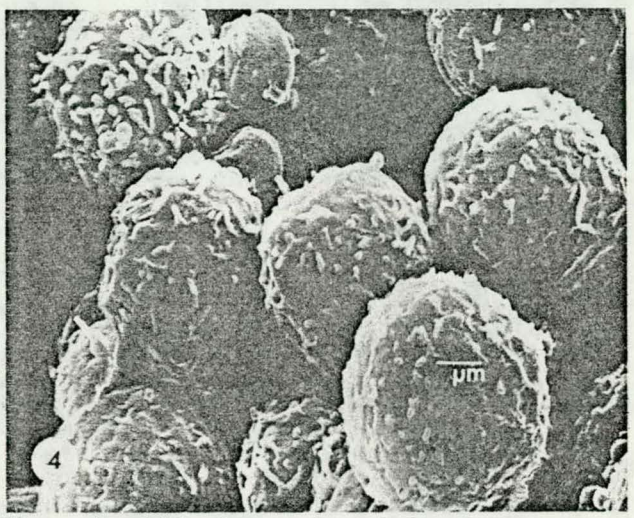

Figure 4. Cells from patient 5 (B-cell CLL), showing moderate to sparse numbers of microvilli.

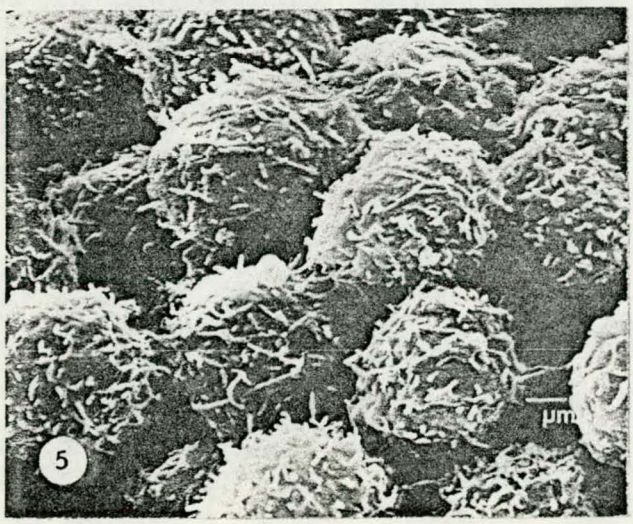

Figure 5. Cells from patient 8 (ML-PDL).

Representative examples of the surface ultrastructure for 2 patients with T-cell lymphoproliferative disease (patients 1 and 3) are shown in Figures 1 and 2. It is easily seen that these cells are moderately villous. Examples of the surface ultrastructure of cells from 3 patients (patients 4,5,8) with B-cell diseases are shown in Figures 3-5. The cells in Figure 4 are from the only patient who was judged to have few microvilli (patient 5), as was also evident in a second sample from this patient. Figures 3 and 5 demonstrate cells with moderate numbers of microvilli. Examples of the surface ultrastructure of cells from 2 patients (patients 10,13) with no surface markers, or "null" cells, are seen in Figures 6 and 7. Cells in Figure 6 have many microvilli, whereas cells in Figure 7 have only a moderate

number. It is obvious that, even though these "null" patients have different diseases and no evident markers, the cell surfaces are essentially indistinguishable from those of patients with B- or T-cell diseases.

Discussion

The lack of correlation between immunologic markers and surface ultrastructure on cells from patients with the leukemic phase of lymphoproliferative diseases that is documented in our study has been suggested previously. A study of Sézary syndrome cells in 1975 included a patient whose cells had no immunologic markers, but whose cells were smooth at one time and villous at two later times.[5] These changes were seen by both SEM and TEM, thus neutralizing the argument that they were a fixation artifact of filtration aspiration for SEM[6]; they certainly suggested, however, that cell surface ultrastructure might be less easily correlated with immunologic markers than first reported.[1] A study by Mann et al.[7] on a T-cell lymphoma demonstrated a villous surface structure of the abnormal cells. An earlier study in our laboratory showed several patients with non-B or "null" cell lymphoproliferative diseases who had abnormal cells, most of which were villous.[3]

Sanders et al.[8] stressed the importance of preparing cells fixed in suspension and demonstrated human mononuclear cells that had either multiple microvilli or ruffles. Alexander and Wetzel[9] viewed human lymphocytes fixed in suspension and found them to be uniformly covered with short microvilli. They also concluded that lymphocytes from subpopulations selectively enriched for T cells are villous and indistinguishable from B lymphocytes. Subsequently, Alexander et al.[6] showed that the purported difference between human T- and B-cell surface morphology was an artifact. They demonstrated that the technique of aspiration filtration smooths the surfaces of living cells in direct contact with the filters, and that portions of some of the cells are aspirated into the filter crevices. They studied one patient with T-cell Sézary syndrome whose percentage of smooth cells, when fixed in suspension, was 1.6; when they were fixed after aspiration filtration, their percentage was 81.2. Approximately the same percentages were found for a patient with B-cell chronic lymphocytic leukemia. Catovsky et al.[10] studied B, T, and "null" cell leukemias with SEM and found that some cells in each group had a smooth surface. Therefore they felt that leukemic B lymphocytes include a spectrum of cells possessing relatively sparse to very dense surface projections.

In order to prevent the preparation artifacts described,[6,9] we fixed our cells in suspension prior to membrane aspiration and only viewed groups of cells which were not directly adjacent to the membrane. Using the suggested methodology in a prospective study of the leukemic phase of lymphoproliferative diseases, we found no correlations between the immunologic markers and cell surface ultrastructure. Our observations support the suggestion made by Catovsky et al.[10] and are certainly consistent with the explanations advanced by both Alexander and Wetzel[9] and by Alexander et al.[6] We conclude that the original report[1] of an association cannot be substantiated, and that SEM analysis has little value in the diagnosis or classification of immunologic subtypes of lymphoproliferative diseases.

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This research was supported in part by the Leukemia Research Foundation, Inc., the Thomas Moore Fund, and the Hematology Research Foundation. The Franklin McLean Memorial Research Institute is operated by The University of Chicago for the United States Energy Research and Development Administration under Contract No. EY-76-C-02-0069.

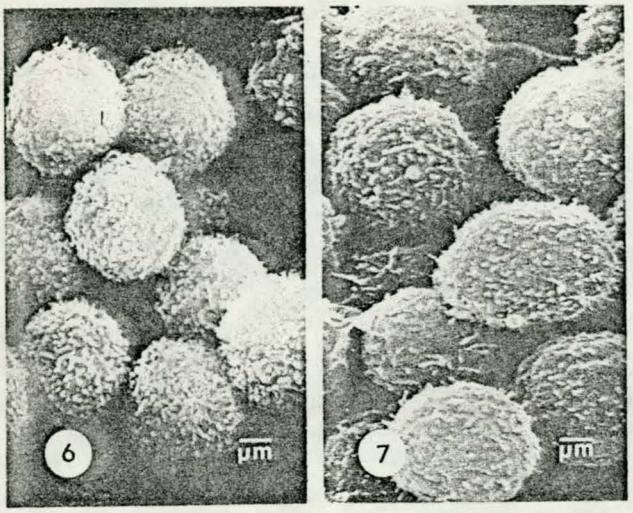

Figure 6. Cells from patient 10 (APL).

Figure 7. Cells from patient 13 (ALL).

The authors wish to acknowledge the scanning electron microscopy user laboratory at the University of Chicago in which these results were obtained. The laboratory is operated on a resource grant from the National Institutes of Health.

DISCUSSION WITH REVIEWERS

Reviewer I: In leukemic circulating cells, it seems that surface structure and immunological markers do not correlate. But to conclude from that, that "SEM appears not to be valuable in the diagnosis or classification..." is an unwarranted pessimistic statement. Please comment.
Authors: SEM is helpful in distinguishing differences between various lymphoproliferative diseases such as hairy cell leukemia and CLL as demonstrated in text reference 4. However, in the diagnosis of immunologic sub-types of certain lymphoproliferative diseases such as CLL or ML-PDL, SEM does not appear to differentiate cells classified functionally as B or T.

Reviewer V: Reference is made to patients who have neoplastic cells, some of which have smooth surfaces and some with villous surfaces. Reference is also made to a patient with Sézary syndrome whose cells were smooth at one time and villous at 2 later times. Has any correlation been attempted with degree of anaplasia or with any other parameter which might give insight as to the mechanisms of this feature of surface ultrastructure of the neoplastic cell?
Authors: The histological diagnosis is a reflection of the degree of anaplasia as ALL is the least differentiated, ML-PDL is of intermediate differentiation, and CLL is the most differentiated. Thus, cell surface microvilli do not seem to correlate with degree of anaplasia.

Reviewer III: 2.5% glutaraldehyde was used as a fixative. Seeing that osmolality affects surface architecture, could the authors state what the osmolality of the fixative used was? How long were the cells fixed for prior to aspiration on to the filters?
Authors: The osmolality was approximately 320 mOs and the cells were fixed for between 1 and 5 minutes.

Reviewer I: Could the authors indicate on what ground they apparently assume that they are looking at the entire population of cells and not at a "selected" sample?
Authors: Only patients with the leukemic phase of a lymphoproliferative disease were studied. In all patients, except No. 9, the great majority of cells were abnormal. Thus, cells harvested on the membrane should be representative of the population, regardless of cell loss.

Reviewer III: Could the authors give details of how many cells were counted per sample? How was the "quantity of microvilli judged and classified" as few, moderate, and many?
Authors: Many cells were examined directly by SEM and approximately 200 cells per sample were evaluated for microvilli from micrographs. The quantity of microvilli was judged on a qualitative basis as is reflected by the figures. Since the study was undertaken to determine trends in associations between SEM and immunologic markers, and since there was not even a suggestion of a correlation, no extra effort was made to do cell-by-cell microvilli counts.

Reviewer II: Have you compared lymphoid cells in these patients fixed directly from venous blood in glutaraldehyde and those prepared in the present method?
Authors: We have not fixed lymphoid cells in these patients directly from venous blood in glutaraldehyde. However, we have fixed spleen tissue from patients with hairy cell leukemia directly into glutaraldehyde and compared it to spleen cells after separation in a Ficoll-Hypaque gradient. There was little difference between the projections on the cells from either specimen.

Reviewer III: It looks from the authors scanty tabulated data that in CLL only one of the 5 cases has many microvilli. Despite the fact that it would be difficult to draw conclusions relating to such small numbers of cases (9 in all), it appears that lymphoma cells more frequently have many microvilli. Do the authors feel that this may be of significance?
Authors: The importance of a qualitative analysis of just nine cases as to the amount of microvilli on CLL versus ML-PDL cannot be drawn from this study. The message of the study is that there is no apparent correlation between immunologic markers and cell surface projections in spite of earlier suggestions in the literature.

DISCUSSION WITH REVIEWERS CONT'D.

Reviewers II and IV: In Patient No. 1, where the count is 7,400/mm^3, how was the percentage of abnormal cells obtained? It may be that this was a case of lymphoma, well-differentiated, and the T cells obtained were normal peripheral lymphocytes. It is interesting that this is the only CLL with T cells.
Authors: The high percentage of lymphocytes results in an absolute count of greater than 5000/mm^3, which is consistent with CLL. In a patient with adenopathy and a lymph node biopsy also consistent with CLL, and with an E-rosette percentage of 60%, we have classified this patient as T-CLL. We agree that an alternative explanation could be considered. Time and follow-up should answer this question.

Reviewer III: Is it correct to assume from the table that cases 4 and 6 had from 16 - 19% of cells with both markers, seeing that the number of E-rosetting cells and those positive for PV add up to 116 and 119% respectively?
Authors: We doubt whether 16-19% of cells had both markers.

Reviewer I: The authors seem to mix bibliographical references related to normal lymphocytes to those concerning leukemic lymphocytes. Do they rule out the possibility that surface differences might exist between subpopulations of normal cells which do not persist in leukemic cells?
Authors: Text reference 9 has answered this question. The authors demonstrated "similarity of B and T cell surface morphology."

Reviewer III: Are not these findings in relation to CLL certainly similar to the conclusions recorded in much earlier publications in the literature?
Authors: Although Polliack (Scanning Electron Microscopy/1976/II, 31-40) reported that most cases of CLL had moderate to markedly villous surfaces, he made no correlation with immunologic markers.

Reviewers I and III: How do your findings relate to Reyes et al., 1975 (J. Exp. Med. 141, 392, 1975)?
Authors: They demonstrated a relationship between villous surface and surface immunoglobulin in normal cells. However, two cases of CLL had smooth surfaces and detectable surface immunoglobulin, while one case of Sézary syndrome had villous projections and lacked surface immunoglobulin.