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Original Articles

Albumin Enhanced Morphometric Image Analysis in CLL

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Background: The heterogeneity of lymphocytes from patients with chronic lymphocytic leukemia (CLL) and blood film artifacts make morphologic subclassification of this disease difficult.

Methods: We reviewed paired blood films prepared from ethylene-diamine-tetraacetic acid (ETDA) samples with and without bovine serum albumin (BSA) from 82 CLL patients. Group 1 adhered to NCCLS specifications for the preparations of EDTA blood films. Group 2 consisted of blood films containing EDTA and a 1:12 dilution of 22% BSA. Eight patients were selected for digital photomicroscopy and statistical analysis. Approximately 100 lymphocytes from each slide were digitally captured.

Results: The mean cell area ± standard error was 127.8 μm^2 ± 1.42 for (n = 793) for group 1 versus 100.7 μm^2 ± 1.39 (n = 831) for group 2. The nuclear area was 88.9 μm^2 ± 0.85 for group 1 versus 76.4 μm^2 ± 0.83 for group 2. For the nuclear transmittance, the values were 97.6 ± 0.85 for group 1 and 104.1 ± 0.83 for group 2. The nuclear:cytoplasmic ratios were 0.71 ± 0.003 for group 1 and 0.78 ± 0.003 for group 2. All differences were statistically significant ($P < 0.001$).

Conclusions: BSA addition results in the reduction of atypical lymphocytes and a decrease in smudge cells. BSA also decreases the lymphocyte area and nuclear area, whereas nuclear transmittance and nuclear:cytoplasmic ratio are increased. A standardized method of slide preparation would allow accurate interlaboratory comparison. The use of BSA may permit better implementation of the blood film-based subclassification of CLL and lead to a better correlation of morphology with cytogenetics and immunophenotyping. Published 2003 Wiley-Liss, Inc.†

Key terms: chronic lymphocytic leukemia; morphology; atypical morphology; atypical chronic lymphocytic leukemia; morphometric analysis

The morphologic heterogeneity of chronic lymphocytic leukemia (CLL) lymphocytes on peripheral blood films is variable between patients and within patients. Attempts have been made to subclassify CLL, but the morphologic heterogeneity has limited the use of this approach. The French-American-British (FAB) cooperative group has published guidelines for the subclassification of CLL based on blood film lymphocyte morphology (1), and other investigators have used modified FAB (2–9). The subclassification of CLL is important because the survival of patients correlates with subclassification, and the latter appears to be predicted in part by CLL lymphocyte morphology. Although controversy exists over the subclassification of CLL, patients with large cells and prolymphocytes, morphologic subtypes of CLL, are believed to have more aggressive disease (10).

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In this investigation, we used morphometric analysis to document the morphologic heterogeneity of lymphocytes in the peripheral blood films from patients with CLL in the presence or absence of bovine serum albumin (BSA). Although there have been several reports on the use of morphometric analysis of CLL lymphocytes and other B-cell leukemias, none have addressed the role of BSA. Benattar and Flandrin (11) carried out a morphometric and color analysis of blood films from T- and B-lymphoid leukemias. They were able to resolve small cell CLL, large cell CLL, and atypical CLL (trisomy 12 positive, increased prolymphocytes [PLs]), in addition to B-prolymphocytic leukemia, hairy cell leukemia and its variant, splenic lymphoma with villous lymphocytes, and cells from follicular lymphoma and mantle cell lymphoma. They pointed out the need for methodologic standardization when using the red cell for size and color control. Malik et al. (12) applied spectral morphometric analysis to CLL lymphocytes. Although they noted distinct chromatin patterns in the nuclei of CLL and normal lymphocytes, the promise of this approach to spectrally map CLL nuclei remains to be fulfilled.

These reports (11,12) and an earlier study by Robinson et al. (13) suggested that the application of image analysis to CLL blood films made with BSA deserves further study. Because we were finding a larger number of CLL blood smears that contained atypical large lymphocytes and PLs than would be expected by cytogenetics and immunophenotyping, BSA addition was selected for its known ability to reduce smudge cells. We believe this report provides further evidence of the usefulness of this approach and suggests a novel methodologic basis for addressing the question of whether morphology, as provided in the clinical laboratory, can assist in subclassification and predicting prognosis.

MATERIALS AND METHODS

Slide Selection and Preparation

In this investigation, 164 peripheral blood films (82 ethylene-diamine-tetraacetic acid [EDTA] and 82 BSA) from patients with CLL were selected for microscopic analysis. Cases were selected from an archived library of slides of past and/or current CLL patients at the National Institutes of Health (NIH). Slides were obtained in paired sets including one slide with the addition of BSA and the other a normal (EDTA) peripheral blood smear. All patients gave written informed consent according to the National Cancer Institute internal review board for protocol evaluation.

The BSA-containing slides were prepared with a 3-ml transfer pipette (Falcon, Franklin Lakes, NJ) at a 1:12 drop ratio dilution of a 22% BSA (Immucor, Norcross, GA) solution to whole blood. Five minutes after mixing, the BSA-containing blood and the blood devoid of BSA were manually smeared onto 3-in. × 1-in. × 1-mm microscope slides (Columbia Diagnostics, Fredricksburg, VA) and allowed to air dry. A modified Wright's stain was applied to each slide by using an automatic stainer (EM Science, Cincinnati, OH). Each blood film was then coverslipped and archived. Three investigators (M.S.-S., M.E.R., and G.E.M.) performed microscopic analysis of the paired blood films. Individual lymphocyte morphology was examined at 100× oil immersion for both slides. Morphology was compared with and without BSA, and any changes in the lymphocyte morphology based on the slide preparation technique were documented. A *change* was defined as a reduction greater than 50% the number of "atypical lymphocytes" demonstrated by a rounding up of the lymphocytes in BSA and/or greater than 50% reduction in the presence of smudge cells. Eight pairs of slides were selected for further analysis on the basis of a high white blood cell count and noticeable differences between paired slides. A high absolute lymphocyte count reduces the time required for digitization.

Digital Microscopy

The eight patients' paired slides were photomicrographed with a Cool Snap Fx camera (PhotoMetrics, Huntington Beach, CA) attached to an Axiophot microscope (Carl Zeiss, Thornwood, NY) and were captured with IPLab 3.5 (Scanalytics, Fairfax, VA). Approximately 100 randomly chosen lymphocytes were photographed from optimal areas of the slide where red cells were just abutting.

Morphometric Analysis

For analysis of the entire lymphocyte area, nuclear area, and nuclear transmittance of the segregated lymphocytes, a macro program from IPLabs was provided (Jim Palidino, BioVision, Exton, PA). The macro allowed for the analysis of the lymphocytes directly from the composite gallery. A 10-μm micrometer was used to convert square pixels to square microns. For analysis, the whole lymphocyte was segmented by using color channel segmentation. Each sequential cell in the composite was numbered and correlated with the value for the area. This procedure was performed again to obtain nuclear area and nuclear transmittance. The nuclear:cytoplasmic (N:C) ratio was calculated by dividing the nuclear area by the entire cell area. Graphs were prepared with KaleidaGraph (Synergy Software, Reading, PA), and JMP (SAS Institute, Cary, NC) was used to test for statistical significance. Means, standard errors, and P values were calculated.

RESULTS

Observation

Group 1 cells (EDTA only) show typical and atypical morphologies, including the presence of smudge cells (basket cells, Gumprecht shadow). In addition, the morphology of group 1 preparations shows the presence of stellate or atypical lymphocytes (also referred to as virocytes, Downy type II cells, or "ballerina skirting with radial basophilia") rather than the usual more spherical shape (e.g., Fig. 1, top). Group 2 cells (BSA) display a significant reduction in smudge or basket cells, and the cells tend to round up, losing their stellate, atypical appearance. An increase in nuclear transmittance can be noted in some patients, and there is an increase in chro-

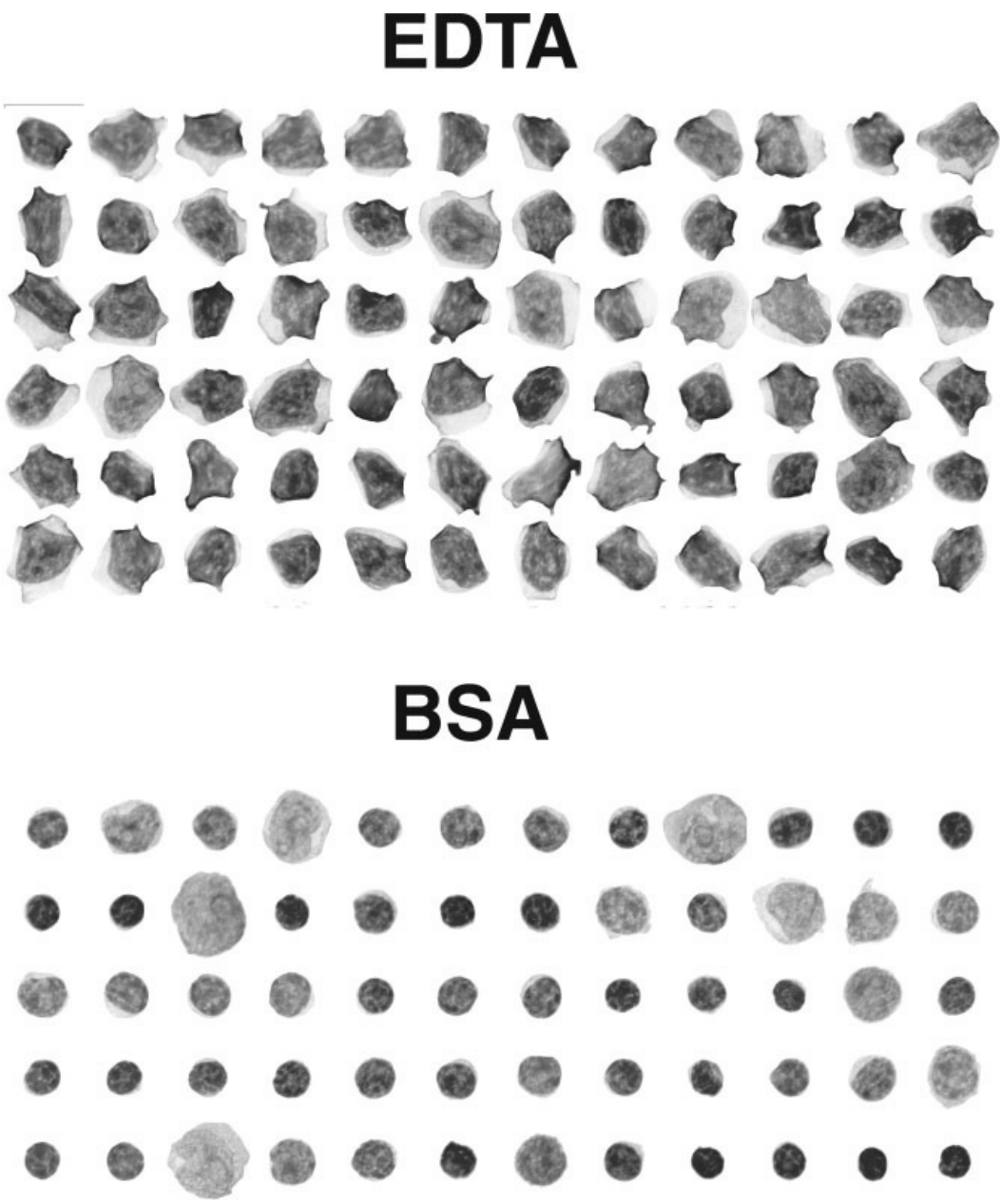

FIG. 1. Comparison of the lymphocyte morphology of patient 1 between group 1 (EDTA) and group 2 (BSA).

matin detail and the nuclear irregularities present are better appreciated. Intracytoplasmic detail and cytoplasmic projections appear to be better preserved in the slides prepared with BSA (e.g., Fig. 1, bottom).

Statistical Analysis

The computational analysis of each composite (Table 1) showed that the mean whole cell area ± the standard error was 127.8 μm^2 ± 1.42 (n = 793) for group 1 versus 100.7 μm^2 ± 1.39 (n = 831) for group 2, and the difference was statistically significant ($P < 0.001$). The area of the nucleus was also significantly different ($P < 0.001$): 88.9 μm^2 ± 0.84 for group 1 versus 76.4 μm^2 ± 0.83 for group 2. The differences in the nuclear transmission values were also significant ($P < 0.001$), with 97.6 ± 0.85 for group 1 and 104.1 ± 0.83 for group 2. The N:C ratio was found to be statistically different ($P < 0.001$) at 0.71 ± 0.003 and 0.78 ± 0.003 for groups 1 and 2, respectively. Therefore, the whole cell area and nuclear area decreased, whereas the nuclear transmittance and N:C ratio increased with the addition of BSA.

Table 1
*Morphometric Analysis Results Between Group 1 (EDTA) and Group 2 (BSA)**

Patient no.	n		Whole cell area (μm^2)		Nuclear area (μm^2)		Transmittance		N:C ratio	
	EDTA	BSA	EDTA	BSA	EDTA	BSA	EDTA	BSA	EDTA	BSA
1	82	92	112.3 ± 3.25	59.5 ± 3.10	85.3 ± 1.98	47.1 ± 1.90	91.5 ± 2.15	111.1 ± 3.91	0.77 ± 0.009	0.82 ± 0.007
2	100	102	99.2 ± 2.56	65.2 ± 1.71	73.9 ± 1.22	55.1 ± 1.09	82.2 ± 1.87	103.3 ± 2.30	0.77 ± 0.010	0.86 ± .008
3	107	107	115.9 ± 2.95	105.3 ± 1.93	85.0 ± 1.17	76.1 ± 0.92	106.6 ± 1.85	89.9 ± 1.35	0.75 ± 0.008	0.73 ± 0.007
4	104	106	128.4 ± 2.98	74.9 ± 1.98	86.1 ± 1.48	60.3 ± 1.26	111.6 ± 1.43	74.5 ± 1.50	0.69 ± 0.007	0.82 ± 0.010
5	87	107	139.9 ± 3.70	127.5 ± 2.68	91.8 ± 1.60	91.5 ± 1.22	105.9 ± 1.69	93.4 ± 1.31	0.67 ± 0.010	0.73 ± 0.009
6	105	108	134.6 ± 3.11	87.1 ± 2.09	89.5 ± 1.27	68.8 ± 1.30	102.0 ± 1.66	118.6 ± 2.45	0.68 ± 0.006	0.80 ± 0.009
7	108	108	121.3 ± 2.40	113.6 ± 2.45	81.0 ± 1.10	87.6 ± 1.23	85.3 ± 1.35	122.7 ± 1.96	0.68 ± 0.008	0.79 ± 0.007
8	100	102	170.1 ± 4.37	169.0 ± 3.88	119.6 ± 2.78	122.0 ± 2.46	95.1 ± 1.91	121.0 ± 1.91	0.71 ± 0.009	0.73 ± 0.009
Mean	793	831	127.8 ± 1.42	100.7 ± 1.39	88.9 ± 0.85	76.4 ± 0.83	97.6 ± 0.85	104.1 ± 0.83	0.71 ± 0.003	0.78 ± 0.003

*Data are presented as mean ± standard error. BSA, bovine serum albumin; EDTA, ethylene-diamine-tetraacetic acid; N:C, nuclear:cytoplasmic.

To depict and compare the results of all eight patients examined, the 1st, 25th, 50th, 75th, and 100th percentile lymphocytes for both treatments were selected based on whole cell area (Fig. 2). The changes from EDTA to EDTA plus BSA as described above were reproducible. Figure 2 allows the BSA-treated blood films to be easily compared for any pattern of changes in cell size and nuclear configuration within a patient and between patients. Although only one time point was examined, patients could be followed longitudinally for sequential changes or to document stability.

Figure 3 shows a plot of whole cell area, nuclear area, and N:C ratio of each patient plotted by rank order of the whole cell area across both groups. Figure 3 shows that the effects of EDTA and BSA may result in separate populations (pa-

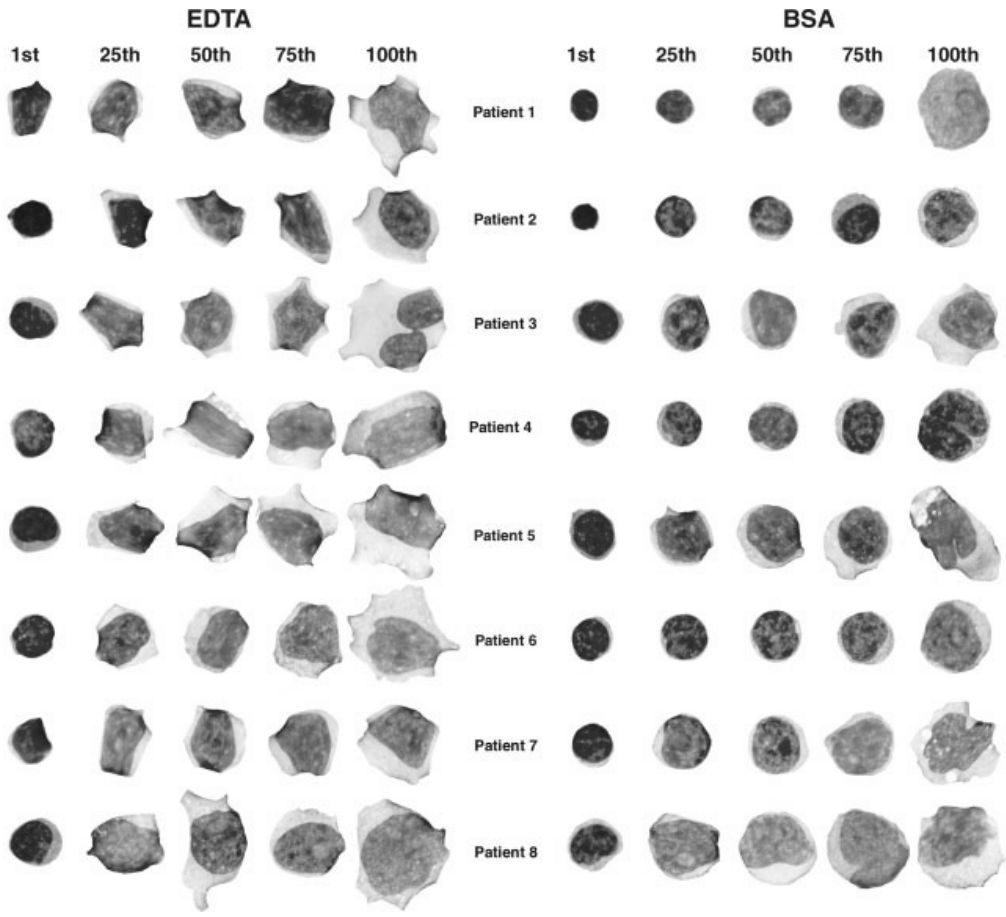

FIG. 2. Comparison of the 1st, 25th, 50th, 75th, and 100th percentile lymphocytes between group 1 (EDTA) and group 2 (BSA) for all eight patients studied.

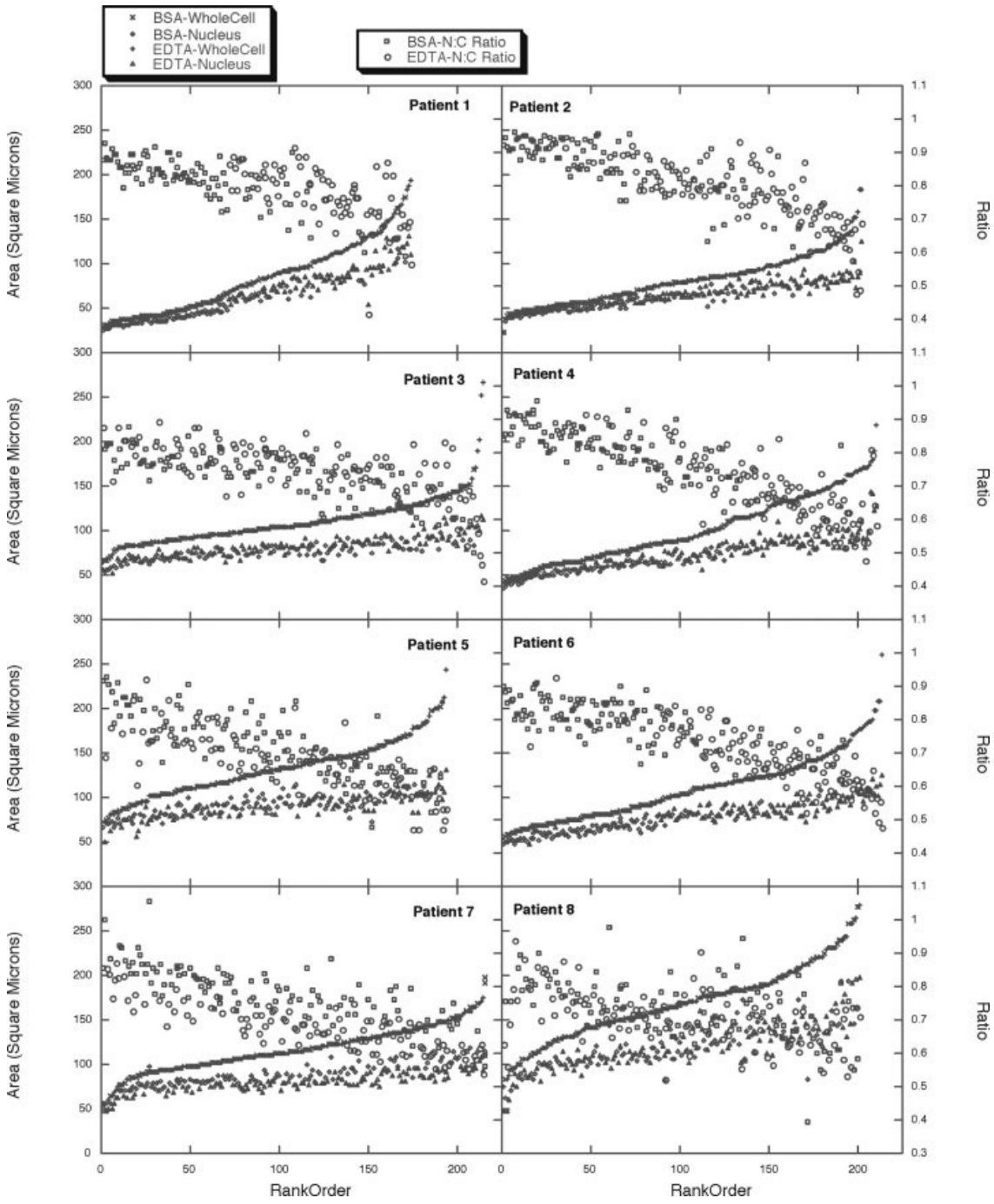

FIG. 3. Plot of whole cell are, nuclear area, and N:C ratio by rank order between each patient and group 1 (EDTA) and group 2 (BSA).

tients 1, 2, 4, and 6) or in populations that are parallel and overlapping (patients 3, 5, 7, and 8). Figure 4 displays nuclear transmittance versus nuclear area and shows different degrees of separate populations in all eight patients. These graphs suggest that the subjective visual changes noted on the addition of BSA to conventional EDTA blood films can be quantitated. Limited morphometric analysis of simple cellular parameters confirmed some of these observations. Addition of BSA appears to allow for easier, more uniform, morphologic subclassification of subpopulations within patients with CLL.

DISCUSSION

This study shows that the addition of BSA to the preparation of EDTA blood films reduces the number of smudge cells and "atypical" lymphocytes and aids in the

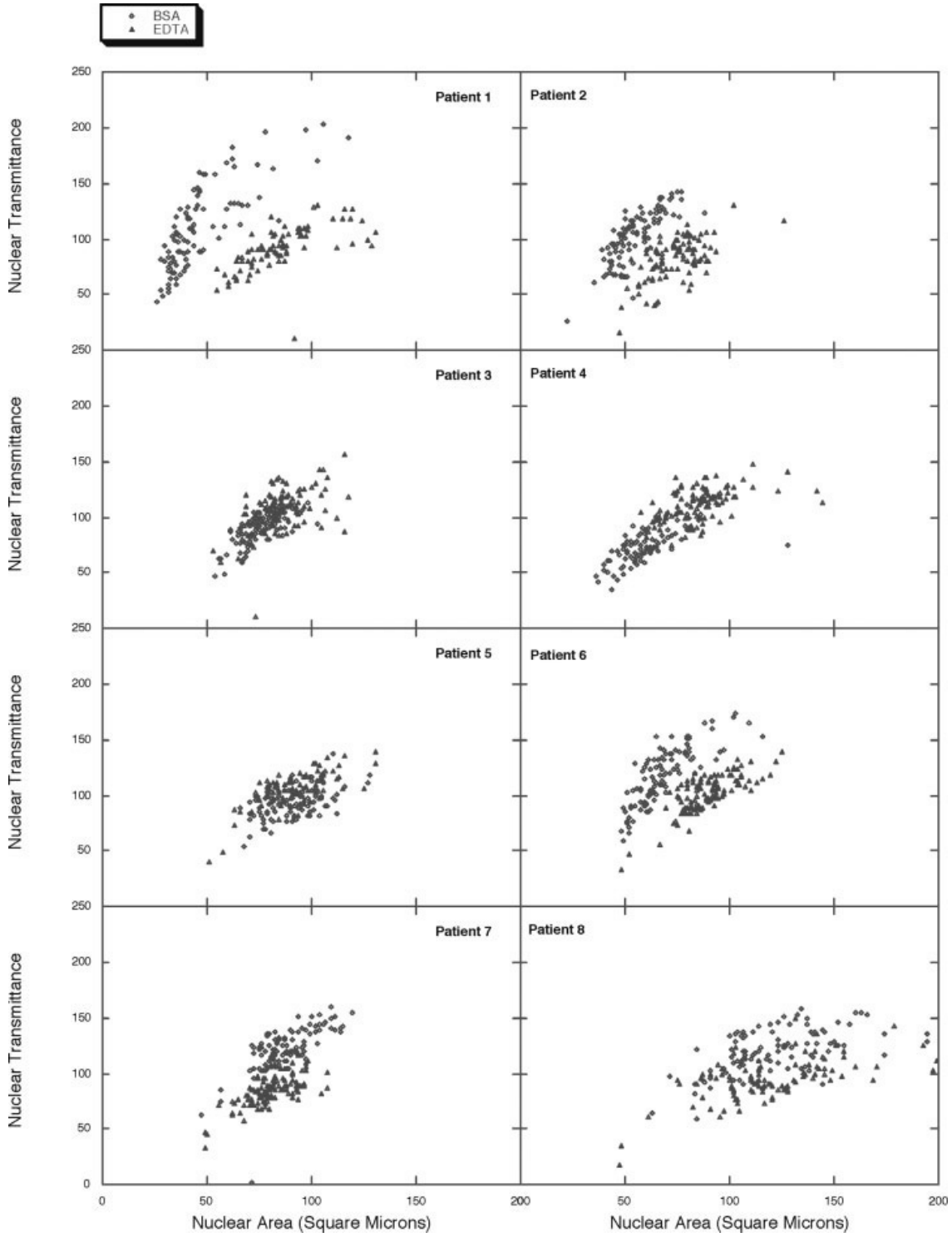

FIG. 4. Plot of nuclear transmittance versus nuclear area between group 1 (EDTA) and group 2 (BSA).

consistency of the morphologic evaluation of the peripheral blood film in CLL patients. There is increased preservation of nuclear and cytoplasmic details on visual inspection, and the morphometric analysis indicates that these subjective observations can be quantitated. Further morphometric analysis of CLL blood films may provide more

objective information for the subclassification of this disease. Morphologic features are important because they continue to be used in conjunction with other laboratory evaluations as a basis for subclassification and prognosis in CLL.

Guidelines initially published by the FAB for the diagnosis of CLL patient groups using morphologic features defined three groups: conventional CLL, CLL with PLs (CLL/PL), and CLL mixed (1). The Revised European and American Lymphoma (REAL) and World Health Organization classifications also recognize the CLL/PL subgroup, i.e., between 10% and 55% PLs (14,15). Since the initial description of prolymphocytic leukemia (16) and the definition of prolymphocytoid transformation in CLL (17), the significance of PLs has been more fully defined for subclassification and for clinical relevance (18–21). Many studies have indicated a worse prognosis and refractoriness to treatment in patients with these morphologic features (18–23).

A second morphologic variation, large lymphocytes, also has been correlated with prognosis. Large lymphocytes initially were correlated with longer survival (24); however, Binet and others have shown that CLL patients with larger lymphocytes are more likely to have advanced disease (25–27). Orfao et al. (28) found an intermediate survival for mixed CLL (small and large lymphocytes) and postulated that this is a transitional state in the formation of large cell CLL and does not lead to CLL/PL.

Nuclear notching, a third aspect of lymphocyte morphology, has been more ambiguous as a marker for prognosis in CLL patients. Ralfkiaer et al. (29) noted that patients with this morphology have an increased mortality, but others have found no survival differences (30,31).

The recognition of prolymphocytes, conspicuous nucleoli, large cells, plasmacytoid lymphocytes, clefting, notching, and other plasma membrane changes in CLL has been based primarily on the careful analysis of well-made peripheral blood films. Given the perceived value of the morphology of CLL lymphocytes in predicting advanced clinical stage, progression, and transformation, Matutes et al. studied the relation between morphology and specific cytogenetic abnormalities; they demonstrated a relation between CLL with atypical morphology (CLL/PL and CLL mixed) and trisomy 12 (32–34). However, the presence of trisomy 12 does not always correlate with atypical morphology because conventional CLL can be positive for trisomy 12 (5,35). In addition, the presence of trisomy 12 and atypical morphology do not always predict whether patients will have an unmutated germline or somatically mutated heavy chain V region immunoglobulin gene (36,37). Atypical morphology also has been correlated with cytogenetic abnormalities of chromosome 14 (38,39). Recently, Mauro et al. found no predictive relation between typical and atypical CLL in terms of response and duration of response to fludarabine and prednisone at the time of presentation or progression (40).

Despite the ambiguity about the role of morphology in subclassification and prognosis in CLL patients, morphology is readily available and continues to be used. However, the correlation between morphology, immunophenotype, and cytogenetics remains unresolved. This study suggests a way to improve the usefulness of morphologic examination by the relatively simple procedure of including albumin in the preparation of EDTA blood films. It has the potential to decrease the variation in morphology within and between laboratories so that clinical correlations can be studied more easily. It will be of interest to see whether this methodology and the associated morphologic observations can be confirmed and whether it will lead to decreased intraobserver and interlaboratory differences. This simple, standardized method to prepare blood films in CLL may further contribute to the morphologic subclassification of CLL and its association with the biological and clinical behaviors of CLL.

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