The Relative Osmotic Resistance of Chronic Lymphocytic Leukemia Lymphocytes

By David W. Westring and Suzanne B. Brittin

The hemolytic response of erythrocytes to hypotonic stress is well known. Complex enzyme systems are required by the cell to maintain membrane integrity during exposure to hypotonic solutions. When certain of these enzymes are defective, erythrocyte osmotic fragility may be altered. On the other hand, the response of leukocytes to osmotic stress has received very little attention. The activities of many leukocyte enzymes have been studied, however, and several deficiencies have been demonstrated in leukemic lymphocytes. It seems possible, therefore, that an altered leukocyte osmotic fragility might be one result of these metabolic dysfunctions. Using recently developed technics for obtaining pure cell populations, the experiments described below indicate significant differences in response to osmotic stress between lymphocytes from normal subjects and patients with chronic lymphocytic leukemia (CLL).

Materials and Methods

The diagnosis of CLL was established in all cases by characteristic clinical history, physical findings and laboratory data, including examination of peripheral blood films and bone marrow specimens. Lymph node biopsies were available from some patients. Donors of normal cells were allegedly healthy laboratory and hospital personnel.

Venous blood samples were drawn in glass syringes containing heparin and allowed to sediment at room temperature, in most cases without additives; in three normal subjects, dextran was required to enhance erythrocyte sedimentation. Ninety-five to 100 per cent pure suspensions of lymphocytes were isolated by the glass bead technic of Rabinowitz.

Osmotic Fragility. Isolated lymphocytes were washed twice in warm, balanced salt solution and aliquots of 5 x 10^6 cells were incubated 30 minutes in 2 ml. solutions containing 0.0 to 0.85 Cm. NaCl per 100 ml. double-distilled water at 37 C. The cells were then centrifuged 10 minutes at 170 g at 4 C. and the cell-free supernates were examined within 3 hours for ultraviolet light transmission on a Beckman DU Spectrophotometer at wavelengths of 260 and 280 mµ.

Cell Volume Studies. Fresh column-separated lymphocytes were examined by a Coulter counter with attached particle size distribution plotter and mean cell volume computer. The aperture diameter was 100 mµ. Sample purity, monitored from Wright-stained smears of the column effluents, was always greater than 97 per cent lymphocytes. The simultaneous measurement of several cells ("coincidence") was less than 3 per cent.

Nucleoprotein Extraction. Five million washed lymphocytes were subjected to total nucleo-protein extraction by the perchloric acid method of Ogur and Rosen. Duplicate determinations were performed when cell yield permitted. Optical density at 260 and 280 mµ was computed per ml. of extraction reagent.

From the Department of Medicine, University of Wisconsin School of Medicine, Madison, Wisconsin.

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David W. Westring, M.D.: Fellow in Hematology and Medical Genetics, University of Wisconsin School of Medicine; Present Address: Queens Hospital Center, Jamaica, N. Y.

Suzanne B. Brittin, M.T., A.S.C.P.: Research Assistant, University of Wisconsin School of Medicine.
RESULTS

Lymphocytes from 6 CLL patients were more resistant to hypotonic stress than lymphocytes of 7 normal donors in all concentrations of NaCl and in distilled water (Fig. 1). Optical density values became significantly different in solutions with salt concentration of less than 0.50 Gm. per cent. Regression analysis of values at all concentrations of NaCl—but not including distilled water values, which are known to be elevated by protein denaturation—revealed that the slopes are not parallel (Fig. 2).

Cell volume measurements (Fig. 3) indicated that isolated lymphocytes from 7 healthy and 7 CLL subjects were not significantly different. Size distribution analysis revealed a wider volume range of leukemic than normal lymphocytes, but similar mean cell volumes.

Sequential analysis of lipid, nucleotide, RNA, DNA and alkali-soluble fractions for total extractable nucleoproteins also revealed no difference between equal numbers of normal and leukemic lymphocytes (Fig. 4). Values of the ratio OD260/OD280 were the same for both groups at each step of the procedure.

An additional observation was evident from the data. Denaturation of nucleoproteins, especially DNA, is known to occur in salt solutions of less than 10^{-4} M, and this phenomenon may be measured as an increase in optical density of approximately 35 per cent at 260 m. When nucleoproteins from the lymphocytes of healthy subjects were exposed to distilled water, O.D. readings were nearly double the value obtained when the slope is extrapolated back to zero (Fig. 2, upper graph). Nucleoproteins from the leukemic cells, however, failed to undergo any increase in optical density under identical conditions (Fig. 2, lower graph).
Storti and co-workers\textsuperscript{11} were the first to describe the resistance of CLL leukocytes to hypotonic stress. Thomson et al.\textsuperscript{12} recently reported increased resistance of column-separated CLL lymphocytes when exposed briefly to distilled water. The studies reported here confirm, by another method of assay, the relative osmotic resistance of leukemic lymphocytes. To test the assay technic, cell volume and total nucleoprotein values were measured. These experiments showed that column-separated lymphocytes from healthy and CLL donors are not different with respect to mean cell volume and nucleoprotein content.

The relationship of osmotic forces to membrane permeability and enzyme participation has been described in considerable detail for the lysis of erythrocytes.\textsuperscript{8} Virtually no information exists about the character of lymphocyte membranes, and studies of enzyme content and activity have only produced reliable data since a technic for separation of leukocytes has become available.\textsuperscript{8} However, the very obvious dissimilarities preclude any reasonable attempt to apply erythrocyte physiology to lymphocytes.
A possible contribution to the differences reported here might be the mean cell age of the normal and leukemic samples. Despite the descriptive terminology still based upon it, lymphocyte age is difficult to determine. There is evidence, however, that CLL lymphocytes have a greater lifespan than normal lymphocytes, both in vivo\textsuperscript{2,6,13} and in vitro\textsuperscript{10} Thus an aliquot of leukemic cells has a greater mean cell age than its healthy counterpart. That a more efficient mechanism of cell preservation exists in old cells than in young seems contrary to the expected physiology of cell aging, but it cannot be excluded as a part of the altered genome following leukemic transformation.

A currently prevalent hypothesis that circulating lymphocytes are a func-
A tionally heterogeneous group must be considered. It has been suggested that a proliferative disorder in one cell line might account for the clinical and laboratory abnormalities observed in CLL patients. Such a clonal theory implies genetic disparity between populations of morphologically indistinguishable cells. Thompson and co-workers\textsuperscript{12} have described experiments in which many CLL lymphocytes were not recovered from columns of polysterene beads, while most lymphocytes from healthy donors passed through the beads. The recovered CLL lymphocytes more nearly resembled healthy lymphocytes than precolumn leukemic cells when cell respiration, responses to osmotic stress, and response to phytohemagglutinin in culture were measured. If such lymphocyte heterogeneity indeed exists, challenging CLL lymphocytes with hypotonic solutions, as described in this report, may be a feasible means of isolating the leukemic clone.

The effect of low ionic concentration on the denaturing of DNA is well known. It is likely that the unusually high values obtained from healthy nucleoproteins exposed to distilled water (Fig. 2) is due to the presence of nucleases and other cellular enzymes. That this increase in optical density was not detected in the samples from leukemic leukocytes is an intriguing observation which requires further study.

**SUMMARY**

Lymphocytes from healthy subjects and patients with chronic lymphocytic leukemia were exposed to graded hypotonic salt solutions and to distilled water. Nucleoproteins released into the media from $5 \times 10^6$ cells were measured spectrophotometrically at 260 nm as an estimate of cell fragility.

Leukemic lymphocytes were relatively more resistant to hypotonic stress than normal cells. Cell volume and extractable nucleoproteins were shown to be the same for both groups of cells. While nucleoproteins released from the healthy cells underwent a greater optical density increase in distilled water than anticipated, the leukemic cell products exhibited no significant color change in distilled water environment for periods up to 3 hours.

**SUMMARIO IN INTERLINGUA**

Lymphocytos ab subjectos normal e ab patientes con chronic leucemia lymphocytic esseva exponite a graduate solutiones salin e etiam a aqua destillate. Le nucleoproteinas liberate ad in le medios ab $5 \times 10^6$ celularas esseva mesurate spectrophotometricamente a 260 m$\mu$ como indice de valor estimatori del fragilitate cellular.

Lymphocytos leucemic esseva relativamente plus resistente al stress de medios hypotonic que celularas normal. Esseva mostra que le volumine cellular e le quantitate de extrahibile nucleoproteina esseva le mesmes pro le duo gruppos de celularas. Durante que le nucleoproteinas liberate ab cellularas normal manifestava un plus marcate augmento del densitate optic in aqua destillate que lo que habeva essite expectate, le productos del cellularas leucemic exhibiva nulle significative alteration del color in un ambiente de aqua destillate durante un intervallo de tempore de usque ad 3 horas.

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