Increased ascorbic acid content in chronic lymphocytic leukemia
B lymphocytes

(T lymphocytes/high-performance liquid chromatography)

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ABSTRACT Human lymphocyte extracts analyzed by high-performance liquid chromatography reveal a major UV-absorbing peak that was shown to be ascorbic acid by spectral, chemical, and enzymatic criteria. Because this peak appeared very prominent in the elution profile of chronic lymphocytic leukemia (CLL) lymphocyte extracts, we measured the ascorbic acid content in lymphocytes from the blood of normal subjects and untreated patients with chronic lymphocytic leukemia. A significantly higher concentration of 111 ± 15.3 nmol per 10^8 cells (mean ± SEM) was found in CLL lymphocytes than in normal blood lymphocytes, which contained 42.2 ± 3.3 nmol per 10^8 cells. Selective enrichment with B and T cells showed that this difference was limited to the chronic lymphocytic leukemia B cell, which had a 5- to 15-fold higher content of ascorbic acid than normal B cells had. In contrast, the ascorbic acid level was similar in normal and CLL T cells. The very high ascorbic acid content provides the chronic lymphocytic leukemia B cell with a reducing substance that could react with oxidants or free radicals.

The activity of 5'-nucleotidase is frequently decreased in lymphocytes from patients with chronic lymphocytic leukemia (CLL) (1), a neoplastic disorder characterized by the monoclonal proliferation of B cells (2). During a study of the consequences of this deficiency, we noted that lymphocyte extracts analyzed by high-performance liquid chromatography (HPLC) contained a major UV-absorbing peak that did not cochromatograph with nucleotide or nucleoside markers. Because this peak appeared to be more prominent in CLL than in normal lymphocyte extracts, it was decided to isolate and characterize the unidentified compound. Experimental results are presented in this communication in which the unknown compound, shown to be ascorbic acid, is quantitated in blood lymphocyte subpopulations from normal subjects and patients with CLL.

MATERIALS AND METHODS

Purification of Lymphocytes. Heparinized blood, obtained from normal subjects and untreated patients with CLL, was centrifuged on Ficoll/Hypaque gradients (3). The diagnosis and staging of CLL was established by standard criteria. Informed consent was obtained in accordance with the Helsinki Conference. The mononuclear cells were depleted of monocytes by adherence to Falcon plastic culture dishes (4) or by centrifugation through Percoll gradients (5). The degree of cellular purity was assessed by Coulter Counter sizing. The lymphocyte preparations generally contained <2% monocytes and <10% platelets.

T and B Lymphocyte Subpopulations. T and B cells were assayed by standard rosetting techniques that used neuraminidase-treated and complement-coated sheep erythrocytes, respectively. Preparations were enriched in T or B lymphocytes by the combination of two methods. T cells were purified after forming neuraminidase-treated sheep erythrocyte rosettes by centrifugation through Ficoll/Hypaque gradients (6) and lysis of sheep erythrocytes as described (7). In addition, B cells were further purified by using a modification of a procedure based on the attachment of B cells to antibody-coated plastic dishes (8). For this method, anti-F(ab')2 was purified as described by Chess and Schlossman (9). The antibody (5 ml; 1 µg/ml) was incubated at 4°C in 10-mm Falcon tissue culture dishes overnight. After decanting, the plates were washed five times with phosphate-buffered saline prior to use for cell purification. After most of the T lymphocytes had been removed by formation of neuraminidase-treated sheep erythrocyte rosettes, 5 ml of the resulting B-enriched cell suspension was placed in the anti-F(ab')2-coated dishes. The cells (5 × 10^6 cells per ml) were incubated in RPMI 1640 medium containing 15% (vol/vol) fetal calf serum for 30 min at room temperature. The nonadherent T and null cells were decanted gently and washed three times with phosphate-buffered saline. The B cells were eluted by incubation with 10 ml of RPMI 1640 medium/fetal calf serum containing human gamma globulin (5 mg/ml) for 1 hr at 37°C with gentle swirling. After decanting, the plates were washed twice with RPMI 1640 medium/fetal calf serum. The eluted cells were washed three times with phosphate-buffered saline. This procedure yielded preparations with 58–89% B cells and 87–99% T cells in normal subjects and from 90–97% B cells and 52–83% T cells in CLL preparations.

HPLC Assay Procedure. Extracts were prepared from 2–30 × 10^6 cells by using 60% (vol/vol) methanol as described by Donofrio et al. (10). After the methanol was evaporated under N2, the residue dissolved in H2O was analyzed on a Partisil 10 SAX column (4.6 × 25 cm). A guard column filled with pellicular anion exchanger and a solvent-conditioning precolumn (4.6 × 25 cm) filled with 37- to 53-µm silica were also used. A Spectra Physics 8000A liquid chromatograph equipped with a two-channel data system was used. On-line UV detection was with Spectra Physics model 8310 and model 770 detectors. The model 770 detector contained a wavelength drive and memory module (Kratos, Westwood, NJ) to allow for UV wavelength scanning. The separation conditions were a modification of the method of McKeeag and Brown (11). The elution conditions were as follows: temperature, 40°C; flow, 1.5 ml/min from 0–40 min, 3.0 ml/min from 40–52 min, and 2 ml/min from 52–72 min; buffers, 0.007 M KH2PO4/0.007 M KCl, pH 4.0 (buffer A) and 0.25 M KH2PO4/0.5 M KCl, pH 5.0 (buffer B); mobile phase, isotonic buffer A (min 0–5), linear gradient of buffers A→B (min 5–40), isotonic buffer B (min 40–52), linear gradient of buffers

Abbreviations: CLL, chronic lymphocytic leukemia; HPLC, high-performance liquid chromatography.
B→A (min 52–67), and isocratic buffer A (min 67–72). Under these conditions, ascorbic acid elutes with a retention time of 7.3 min and was found to be reliably detected over a 0.03- to 30-nmol range (12).

The ascorbic acid was quantitated by calibration with a crystalline ascorbic acid standard. In selected experiments, the ascorbate peak was trapped, and its UV absorption spectrum was determined. Ascorbate oxidase (1000 units/mg of enzyme protein; Boehringer Mannheim GmbH) was used to further identify the compound. In addition to quantitation by HPLC, in some cases the ascorbate analysis was carried out by the method of Roe et al. (13).

RESULTS

The typical HPLC elution patterns of lymphocyte extracts showed a major peak with a retention time of 7.3 min that was shown to cochromatograph with a crystalline ascorbic acid standard (Fig. 1). The UV absorption spectrum of this material was identical in extracts of normal and CLL lymphocytes (Fig. 2), with $E_{\text{max}}$ at pH 4.0 of 250 nm corresponding to that found with ascorbic acid at that pH (14). After the addition of ascorbic acid oxidase to a lymphocyte extract, the peak was no longer detectable, supporting its identification as ascorbic acid (Fig. 3).

![Fig. 1. Comparison of HPLC elution pattern in extracts prepared from normal and CLL lymphocytes. (A) Extract from 2×10⁶ CLL lymphocytes. (B) Normal lymphocyte extract from 3×10⁶ cells. Ascorbic acid, which elutes at 7.3 min, is denoted by the arrow. The other major nucleotide peaks were identified by comparison with known standards and $A_{280}/A_{264}$ ratios. $A_{264}$ units full scale = 0.16.](image1)

![Fig. 2. The UV absorption spectrum obtained on the trapped ascorbic acid peaks from normal and CLL lymphocyte extracts. The maximum UV-absorbing region of the ascorbic acid peak was selectively trapped and scanned over a wavelength range of 215–350 nm. —, CLL ascorbic acid peak; ..., normal lymphocyte ascorbic acid peak.](image2)

The ascorbic acid content of lymphocytes from patients with CLL who were not taking vitamin C supplements was significantly higher than that of normal lymphocytes (Fig. 4). The

![Fig. 3. Chromatographic separation from a CLL lymphocyte extract showing the effect of ascorbate oxidase. (A) Control: An extract from 2×10⁶ CLL lymphocytes in 20 μl of H₂O was mixed with an equal volume of O₂-saturated buffer A and incubated for 30 min at 38°C prior to HPLC. (B) Effect of ascorbate oxidase. An aliquot of the extract used in A was incubated with 20 milliunits of ascorbate oxidase under identical conditions. $A_{264}$ units full scale = 0.16.](image3)
cells had noted in lymphocytes B of normal subjects enriched in B or T cells was compared with CLL samples of leukemia, the ascorbic acid lymphocytic (Fig. 6).

Because cellular ascorbate content can be increased by dietary supplementation with vitamin C, the possibility was considered that the high levels observed in CLL could reflect consumption of this vitamin by patients. However, no significant differences were found between the lymphocyte ascorbic acid levels of patients taking vitamin C and control patients who denied taking this vitamin for at least a year prior to the assay (Fig. 5).

In view of the preponderance of B lymphocytes in chronic lymphocytic leukemia, the ascorbic acid content of samples from normal subjects enriched in B or T cells was compared with CLL samples that had been processed in an identical manner (Fig. 6). A striking difference was found between the CLL B lymphocytes, which contained 97.4 ± 9.7 nmol per 10⁸ cells (nine subjects), a level 10 times higher than that of normal B-lymphocyte-enriched cell populations, which contained 9.4 ± 3.3 nmol per 10⁸ cells (six samples) (P < 0.001). The average of normal T lymphocytes (41.5 ± 6.7 nmol per 10⁸ cells; seven subjects) did not differ from that of CLL T lymphocytes (37.5 ± 8.7 nmol per 10⁸ cells; nine subjects) (P = 0.36). Normal T lymphocytes were found to have higher ascorbic acid levels than normal B lymphocytes (P < 0.001), whereas the converse was noted in CLL, where T lymphocytes had a lower level than B cells had (P < 0.001).

![DISCUSSION](image)

The results show that ascorbic acid can be detected as a major UV-absorbing peak in methanol extracts of human lymphocytes. A significantly higher level of this compound was found in the

![FIG. 4](image)

**FIG. 4.** Ascorbic acid content of lymphocytes from normal donors and untreated patients with CLL.

mean ± SEM level in CLL lymphocytes was 111 ± 15.3 nmol per 10⁸ cells as compared with 42.2 ± 3.3 for normal lymphocytes. The difference was highly significant (P < 0.001, Student's t test; P < 0.01, Wilcox-Mann U test). Similar results were obtained when the determinations were carried out by the method of Roe et al. (13) (data not shown).

![FIG. 5](image)

**FIG. 5.** Comparison of ascorbic acid levels in lymphocytes from CLL patients taking ascorbic acid supplements and those denying any supplementation. The supplementation ranged from 60 to 500 mg daily.

![FIG. 6](image)

**FIG. 6.** Ascorbic acid contents of purified T (Right) and B (Left) lymphocytes from normal and CLL donors. The results of patients taking vitamin C are shown by open circles.
lymphocytes from patients with CLL than in lymphocytes from normal subjects. In view of the widespread consumption of vitamins in the general population, it was first considered possible that this reflected ingestion of vitamin C tablets or multivitamin preparations by the patients. This interpretation was not supported by dietary history, nor were significant differences found in the ascorbate content from CLL patients known to take vitamin C and those denying its ingestion.

Because the percentage of B lymphocytes is generally increased in the blood of patients with CLL, it was thought that B lymphocytes might contain more ascorbic acid than T cells and that the higher levels observed in CLL merely reflected the higher ratio of B to T lymphocytes present in patients with this disorder. The results of experiments with selective enrichment of B and T cells did not support this interpretation, showing instead that CLL B lymphocytes had higher levels than normal B lymphocytes. B lymphocytes from CLL patients had higher levels than T lymphocytes had, whereas B lymphocytes from normal subjects had a lower ascorbate content than that observed in normal T lymphocytes. Therefore, it appears that the CLL B lymphocyte differs from the normal B lymphocyte in this respect whereas no difference is found in the T lymphocytes.

The present studies do not define the reason for the increased ascorbate found in the CLL B lymphocyte. Because man, monkey, and guinea pig are unable to synthesize ascorbic acid, the higher levels are not likely to reflect increased synthesis (16). Other possibilities include a higher cellular uptake or a difference in ascorbic acid metabolism by these cells. Information is needed for CLL lymphocytes concerning dehydroascorbate reductase, the enzyme mediating the reduction and cellular uptake of dehydroascorbic acid (17).

Patients with chronic myelocytic leukemia (18) and acute lymphoblastic leukemia (19) were found to have reduced levels of ascorbic acid in their leukocytes when compared with leukocytes from normal subjects. In contrast, CLL B lymphocytes have higher levels than their normal counterparts. Increased levels of ascorbic acid have been reported in malignant epithelial tumors when compared to surrounding normal tissues (20). Electron spin resonance changes due to the ascoryl radical have been observed as an early change during murine leukemia (21).

The data reported here may have biologic significance because ascorbic acid can participate in a number of redox reactions, which include the conversion of proline to hydroxyproline (22), the formation of hydroxylysylsine in collagen synthesis (23) and the synthesis of catecholamines by the adrenal gland (24). Ascorbic acid has been reported to protect against superoxide radicals in vivo (25), and free radicals formed on oxidation of ascorbate can damage proteins (26, 27). To date, no information is available on the possible occurrence of these reactions in lymphocytes. Ascorbic acid also may react with such oxidants as hydroxyl radicals in its conversion to dehydroascorbate (28). Normal lymphocytes contain a reductase that converts dehydroascorbate to ascorbate (29). The in vivo interactions of this potential free-radical-scavenging system with oxidants or free radicals require further study.

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