Endoplasmic Reticulum-associated Structures in Lymphocytes From Patients With Chronic Lymphocytic Leukemia

By S. Stefani, S. Chandra, R. Schrek, H. Tonaki, and W. H. Knospe

Fresh peripheral blood lymphocytes of 61 patients with chronic lymphocytic leukemia (CLL), 28 patients with other lymphoproliferative neoplasms, and 64 noncancerous human subjects were examined by electron microscopy. Lymphocytes from 13 (21%) CLL patients contained three endoplasmic reticulum (ER)-associated structures. Six of these patients had some drug treatment either for CLL or for some other malignancy and 7 had no cancer chemotherapy prior to the first blood sample collection. These structures were either fibrillar, crystalline, or granulofilamentous. Except for the crystalline structure which occurred in 80%-90% of lymphocytes of one CLL patient, the other ER-associated structures were infrequently observed. Lymphocytes from the noncancerous subjects and from the patients with other lymphoproliferative neoplasms did not contain these structures. The occurrence of these structures in untreated patients and their persistence in the lymphocytes of 7 patients who had provided two or more samples during a period of 9 yr suggested a physiologic defect in the lymphocytes of these patients and supported the hypothesis of a clonal origin of leukemic lymphocytes. The presence of the ER-associated structures in the lymphocytes of a small percentage of CLL patients was not considered pathognomonic of the disease. The structures were related to neither the chemotherapy administered to the patients nor to their lymphocyte count during the course of the disease.

THREE TYPES of “inclusion bodies” associated with endoplasmic reticulum (ER) have been described in lymphocytes from patients with chronic lymphocytic leukemia (CLL): (1) globular1 with a homogeneous mass within them, (2) crystalline,2, 6 and (3) cylindrical, made up of a complex array of granules and filaments.7,8 The third type of inclusion body has also been described in hairy cell leukemia.9,10 Since each observation has been made on only one blood sample from each patient and describes only one type of inclusion body, no inference can be drawn regarding a relationship of these bodies to the disease. The present communication results from a retrospective electron microscopic study of the peripheral blood lymphocytes of patients with CLL. It not only describes the existence of the latter two bodies in the leukemic lymphocytes from about 15% of the patients, but also reports a new type of ER-associated structure in the lymphocytes from about 8% of the patients. The electron microscopic observations have been in conjunction with and on the experimental controls of the studies relating to the effect of ionizing radiation11 and of drugs and reagents12,14 on peripheral blood lymphocytes from patients with...
CLL. Attempts have been made to determine if the presence of these bodies has a statistically significant relationship to the disease.

MATERIALS AND METHODS

Electron microscopic studies were made on the peripheral blood lymphocytes of 61 patients with CLL and of 28 patients with other types of malignant lymphoproliferative disease (Hodgkin disease, lymphosarcoma, and multiple myeloma). The studies were controlled by examination of blood cells from 48 normal persons and from 16 patients with nonmalignant disease (emphysema, ulcerative colitis, and multiple sclerosis). Six leukemic patients provided 2-3 blood samples during the 9-yr study period and one provided 6 samples in the same period.

Case Histories

Of the 61 patients with CLL, 13 had the ER-associated structures described in this study. Six of the latter patients had received a variety of therapeutic agents either for CLL or for some other malignancy, while the remaining 7 had received no cancer chemotherapy prior to the first blood sample collection. The ER-associated structures occurred in both treated and untreated patients, some of whom had provided more than two samples during the 9-yr study period. The clinical records of the 13 patients revealed that no attempt had been made to subdivide lymphocytes into large or small cells for any patient during the course of the disease, even for patient B (mentioned below), now living with CLL for the past 16 yr. Except for patient C, serum immunoglobulins were not determined. Case histories of four patients are summarized below.

Patient A. This patient was 68 yr old. A diagnosis of CLL was made during a routine physical examination on April 12, 1961, when the peripheral blood leukocyte count was 21,000/μl with 52% lymphocytes. The patient was followed in the outpatient clinic until February 1, 1971, without any treatment. During this period the proportion of lymphocytes in the peripheral blood remained at about 60%. Four blood samples were collected between 1968 and 1971. The lymphocytes in each blood sample contained the fibrillar matter within ER. The patient expired on April 16, 1971, and autopsy indicated that death was due to acute pulmonary edema and arteriosclerosis.

Patient B. This patient, age 54, was admitted on August 2, 1960 for dumping syndrome following a subtotal gastrectomy for peptic ulcer. The leukocyte count was 30,000 cells/μl with 94% lymphocytes. He had generalized lymphadenopathy and hepatomegaly 3 cm below the costal margin. A diagnosis of CLL was made and the patient has since been followed for both diseases periodically. The patient is alive and his peripheral blood lymphocytes have remained at the 85%-90% level without any antileukemic treatment. Since 1967 the patient has provided six blood samples, the latest one on January 14, 1976. Fibrillar structures and granulofilamentous bodies were always observed in about 4% of random sections of lymphocytes.

Patient C. This patient, age 68, had a diagnosis of carcinoma of the prostate in 1967 and received diethylstilbestrol for the following 5 yr. CLL was diagnosed in September 1971, for which the patient was given chlorambucil. Immunoelectrophoresis of serum done at that time showed reduced immunoglobulins with 5.2 mg/ml IgG (normal 8-14), 0.36 mg/ml IgA (normal 1-3), and 0.2 mg/ml IgM (normal 0.9-1.7). The first blood sample for electron microscopic observation was obtained in December 1972. Since then and until January 20, 1975, when another blood sample was received, the patient had received further treatment with cytoxan, prednisone, and radiotherapy. Immunoglobulins were not determined in 1975. In both blood samples, ER-associated crystals and granulofilamentous bodies occurred in approximately the same frequency. The patient died on March 16, 1975, and autopsy showed CLL involving lymph nodes, spleen, liver, and other organs and adenocarcinoma of the prostate with local metastases.

Patient D. The fourth patient was 69 yr old. CLL was first diagnosed in 1965, for which the patient was treated with steroids and chlorambucil. He was admitted on July 9, 1973, with the complaint of weakness for the preceding 4 wk. The leukocyte count was 240,000 cells/μl with 96% lymphocytes. The patient was hospitalized for 8 wk and was given chlorambucil, 2 mg daily. On the day of discharge the leukocyte count was 91,000 cells/μl with 87% lymphocytes. He expired.
on April 14, 1974, and no autopsy was performed. Only one blood sample was obtained from this patient in January 1970 and the lymphocytes contained an amorphous matter within ER.

Purification of Lymphocytes

Peripheral blood (15 ml) was drawn from healthy donors and from leukemic patients into sterile heparinized glass tubes and the lymphocytes were purified as described earlier. In this procedure monocytes and granulocytes adhered to the glass surface, leaving other blood cells in suspension. It is not known if the procedure alters the differential distribution of lymphocyte subpopulations.

Electron Microscopy

Lymphocytes were processed for electron microscopic examination as described earlier. Sections stained with uranyl nitrate and lead citrate were examined in an RCA EMU I11G electron microscope. From each sample of most patients, more than 100 lymphocytes were photographed for evaluation of any unusual ultrastructural characteristics.

RESULTS

The general ultrastructural morphology of peripheral blood leukocytes from normal subjects and from patients with CLL has been described in detail both qualitatively and quantitatively. Our observations will be confined to three structures associated with ER. These are fibrillar matter within the cisternae of ER, rhomboid crystals bound by ER, and granulofilamentous cylinders.

Fibrillar Matter Within the Cisternae of ER

The granular ER was infrequent in the peripheral blood lymphocytes of both normal individuals and leukemic patients. It was randomly disposed throughout the cytoplasm in short lamellar profiles. In some lymphocytes from CLL patients, the ER appeared polarized with respect to the cell, and at low magnifications its cisterna contained some dense homogeneous material (Fig. 1, arrow). At higher magnifications this material contained closely packed fibers, approximately 11 nm in diameter (Figs. 2 and 3). The arrow in Fig. 2 points to the transverse profiles of the fibers. In the longitudinal planes of sectioning, the individual fibers appeared to have a periodicity along the length (Fig. 3, arrow).

In certain planes of sectioning, lamellae of endoplasmic reticulum, whose cisterna contained fibrillar matter, were continuous with the outer nuclear membrane, with the fibers extending into the perinuclear space (Fig. 4, arrow heads). The amount of fibrillar matter seen in this space varied considerably (Figs. 4–6) depending on the plane of sectioning, and perhaps on the physiologic state of the cell. Lymphocytes of patient B, who had provided six blood samples over a 9-yr follow-up, during which the percent distribution of lymphocytes in the blood remained essentially the same, always contained fibrillar matter within their ER. The lymphocytes of CLL patient D contained some dense matter within the dilated cisternae of ER (Fig. 7) which never revealed a long fibrillar structure as described above.

The Golgi apparatus in lymphocytes from CLL patients exhibited its normal
Fig. 1. Electron micrograph of purified peripheral blood lymphocytes from patient with CLL (as are Figs. 2–14). Portion of a lymphocyte from patient B exhibiting the presence of some electron opaque material in the cisternae of ER. Nucleus (N), mitochondria (M). Bar, 1 μm.

Fig. 2. Electron-opaque material in the cisternae of ER of lymphocytes from patient B appears fibrillar in nature. Arrow points to transverse profiles of fibrils within ER. Nucleus (N); bar, 1 μm.

Fig. 3. High-magnification electron micrograph of fibrils in the cisterna of ER (patient B). Each fibril appears to have some striations along its length (arrow). Bar, 0.5 μm.
ultrastructural characteristics (Fig. 8). In the upper left portion of this figure, elements of the ER contain fibrillar matter. The unlabeled arrow points to a continuity between the lamella of ER and Golgi apparatus. Although various stages in the formation of large vesicles with electron-lucent material are observed in the Golgi apparatus in Fig. 8, neither in this nor in any other electron micrograph was fibrillar matter seen within the vesicles. Large spherical bodies, bound by a smooth membrane and containing material of the same electron opacity as the packed fibrils, were also not observed in lymphocytes of CLL patients. Such an observation would suggest storage of the fibrillar matter synthesized within ER prior to excretion by the cell.

**Crystalline Bodies**

The cytoplasm of lymphocytes from patient C contained long rhomboid crystals having a square or rectangular profile (Figs. 9–12). Their dimensions varied, one crystal being more than 3 μm long (Fig. 10). The greatest observed width was 0.8 μm. The crystals were enclosed by a lamella of rough ER all along their lengths (Figs. 9–12), and it is not known if they were open at the ends (Fig. 10, arrows). At higher magnification, the crystals revealed a laminated lattice structure with a periodicity of about 10 nm (Fig. 12). Each lamina measured 4.5 nm in width and was oriented parallel to the longitudinal axis of the crystal.

Approximately 20\% of the random sections of the lymphocytes of this patient had one or more crystalline structures in their cytoplasm. The crystals averaged 0.40 sq μm in area and the cytoplasm of the lymphocytes averaged 10.2 sq μm.
Fig. 5. Fibrillar matter in the perinuclear space of lymphocytes (arrows) from patient B. ER contains a fibril. Nucleus (N); bar, 1 μm.

Fig. 6. Another micrograph of fibrillar matter in the perinuclear space of lymphocytes (arrows) from patient B. Bar, 1 μm.
Fig. 7. Dense matter, probably fibrillar, within the ER of lymphocyte of CLL patient D. Nucleus (N); bar, 1 μm.

Fig. 8. Portion of a lymphocyte from patient B containing ER with fibrillar material in its cisternae, Golgi apparatus (GA) and a centriole (C). Arrow in the center points to the formation of a vesicle from a lamella which appears continuous with ER. Nucleus (N), mitochondria (M); bar, 1 μm.
From these data we estimated\textsuperscript{17} that the crystalline bodies were present in $88\%$ of the lymphocytes, i.e., practically all lymphocytes had these inclusions. In a second study 2 yr later, the crystalline bodies were again found in the lymphocytes in approximately the same frequency.

Elements of granular ER (Figs. 9 and 10, arrow heads) in the lymphocytes from this patient did not contain any electron-dense or fibrillar material which would suggest synthesis and stages in formation of these crystals within the reticulum. The only structure, other than the normal cell constituents, seen in
lymphocytes from this patient was the granulofilamentous cylindrical body (Figs. 9 and 11, unlabeled arrow) described in detail below.

**Granulofilamentous Structures**

In lymphocytes of a few patients (Table 1) with CLL, various profiles of a complex hollow cylindrical body were observed (Figs. 9, 13, and 14, unlabeled...
Table 1. Frequency of ER-associated Structures in Lymphocytes of Cancerous and Noncancerous Human Subjects

<table>
<thead>
<tr>
<th>ER-associated Structures</th>
<th>Chronic Lymphocytic Leukemia</th>
<th>Other Lymphoproliferative Neoplasms*</th>
<th>Control†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillar</td>
<td>5/61</td>
<td>0/28</td>
<td>0/64</td>
</tr>
<tr>
<td>Crystalline</td>
<td>1/61</td>
<td>0/28</td>
<td>0/64</td>
</tr>
<tr>
<td>Granulofilamentous</td>
<td>9/61</td>
<td>0/28</td>
<td>0/64</td>
</tr>
</tbody>
</table>

The numerator indicates the number of patients with the ER-associated structures and the denominator the total number of patients studied in each group.

*Other types of leukemia (n = 8), lymphosarcoma (n = 12), multiple myeloma (n = 1), Hodgkin disease (n = 7).

†Healthy individuals and patients with no history of any malignancy.

arrows). A close examination of many such bodies in different planes of sectioning suggested that they were enveloped by ER only in their central region. In longitudinal sections, the wall of the cylinders was observed to contain four or five layers of thin straight filaments with a uniform separation of about 47 nm (Fig. 11, unlabeled arrows). Rows of small granules similar in size and morphology to the cytoplasmic ribonucleoprotein granules occurred between the filaments but were not attached to them (Fig. 11). The cylinders were open at both ends and cytoplasmic material was always found in their core. At low magnifications, transverse sections of the cylinders produced images consisting of 4-5 layers of concentric “membranes” with granules between them (Fig. 11, double arrow). These membranes revealed a dotted structure at higher magnifications (Figs. 13 and 14, arrows), suggesting that they were composed of straight filaments arranged consecutively and parallel to each other along their longitudinal axes. Furthermore, the filaments exhibited a spiral formation (Fig. 14) similar to that of a loosely rolled bamboo curtain. Since filaments and granules were the main constituents of these cylindrical bodies, the latter have been designated as granulofilamentous structures.

Table 1 gives the incidence of the three ER-associated structures in the peripheral blood lymphocytes of CLL patients, patients with other lymphoproliferative neoplasms, and noncancerous human subjects. Only CLL patients, whether treated with chemotherapy or not, had these structures in their lymphocytes. Of the 13 CLL patients, the lymphocytes of 1, patient B, contained fibrillar and granulofilamentous bodies, while those of another, patient C, had the crystalline and granulofilamentous bodies. The lymphocytes of no one patient contained all three ER-associated structures. Except for the crystalline structures in a large proportion of lymphocytes of patient C, the frequency of fibrillar matter and the granulofilamentous structure was too low to provide an estimate of the percentage of lymphocytes which contained these structures.

DISCUSSION

In cells responsible for secreting globular proteinaceous material, the granular ER is involved in the synthesis of protein micromolecules. Its cisterna is often dilated and filled with electron-dense material. The synthesized matter is transported to the Golgi apparatus for storage, where maturation of the final product takes place before it is secreted from the cells. Similar phenomena...
Fig. 13. Transverse profiles of two adjacent granulofilamentous structures partially bound by ER in the lymphocyte of patient B. Bar, 0.5 μm.

Fig. 14. In the lymphocyte of another CLL patient, four granulofilamentous structures whose transverse profiles suggest that the straight filaments (arrows) constituting these structures are lined consecutively parallel to each other along their longitudinal axes and arranged to form a spiral, similar to a rolled bamboo curtain. Elements of ER appear randomly oriented with respect to these structures. Bar, 0.5 μm.

have been shown to occur in certain cells of the hemopoietic system but not in lymphocytes.

The fibrillar matter seen in the cisternae of rough ER of peripheral blood lymphocytes of five patients with CLL has not been described before. It is apparently synthesized by the rough ER of lymphocytes, and although the Golgi
apparatus in these lymphocytes is well developed and its lamellae are continuous with those of ER (Fig. 8), the fibrillar matter does not appear to be transported into the Golgi apparatus. Its eventual fate is thus unknown.

The rhomboid crystals seen in the peripheral blood lymphocytes of one patient with CLL are morphologically similar to those described by other investigators. In one report, the crystals have exhibited periodicity in an oblique direction and not along the two axes of the rhombus, as in the present study. Their crystalline nature and the association with rough ER suggest some protein as their main constituent. Hurez et al. and Cawley et al. believe that the crystals contain IgM and IgA, respectively.

In the lymphocytes from a patient with chronic lymphocytosis, Nardo and Norton described crystalloid inclusions filled with bundles of tubular structures, 12-15 nm in diameter. The crystals exhibited striations along the longitudinal axis. Similar tubular structures were also found in the lymphocytes of a CLL patient. In both these cases, the inclusions were different from the fibrillar and crystalline structures described in this study.

The granulofilamentous hollow cylindrical structure has been reported in the peripheral blood lymphocytes of patients with CLL, hairy-cell leukemia, monoblastic leukemia, and macroglobulinemia, in spleen and lymph nodes of patients with myelophthisis secondary to lymphosarcoma, and with macroglobulinemia (personal unpublished observations of one of the authors, S.C.). This structure has been variously described: either as a complex of membranes and granules, as a "granule-lamella complex," as a "granulolamellar structure," or as a "ribosome-lamella complex." In all these studies the membrane or lamella refers to the straight long profiles seen in the longitudinal sections through the core of these bodies.

In a three-dimensional reconstruction of these structures, Daniel and Flandrin have shown that the "lamellae component could be formed by a row of fibrils" which are demonstrable in transverse sections (Fig. 14). These fibrils are attached to a thin membrane whose spiral configuration forms the wall of these bodies. It is thus apparent that the fibrils and not the membrane or the lamella are one of the main structural components of these bodies; the other component being the ribosome-like granules. Since in longitudinal sections the fibrils always appear straight and rigid, we prefer to describe them as filaments, because of the connotation of greater rigidity attached to them. Krishan and Hsu and Krishan have described a polyribosome-filament complex structure in cultured cells treated with vincristine. In these cells, and also in adenoma cells, the bodies differ from those described in the above-mentioned studies in that the filaments are tightly packed without any granules between them and polysomes form complexes on the inner and outer surfaces of the hollow cylinders. Although we realize that calling a certain structure by different names can make the literature confusing, we hope the readers will accept our designation of these bodies as granulofilamentous, based on the observations of Daniel and Flandrin, Krishan and Hsu, and those described herein.

The association of the ER with the granulofilamentous bodies appears to be only partial, as evidenced from different planes of sectioning. The various images seen in the electron microscope suggest that the hollow cylinders are
enclosed by ER only near its central region. The extent of this envelopment could, however, depend on the physiopathologic state of the cell. It has been suggested that these structures originate from the rough ER.23

The three ER-associated structures generally occurred singly in the lymphocytes of only a small number of CLL patients irrespective of any chemotherapeutic treatment. No one particular structure was found in the lymphocytes of all these patients and the lymphocytes of not one patient contained all the three structures. Since the peripheral blood lymphocytes of CLL patients contain mononuclear cells with different buoyant densities27 and two subpopulations of B lymphocytes have been described in the blood of such patients,28 it is possible that we missed a particular cell type in the blood sample(s) of some patients, even though in our purification procedure both small and large lymphocytes could be assumed to be present in samples processed for electron microscopic examination.

To understand the etiology and nature of the ER-associated structures in the peripheral blood lymphocytes of CLL patients, we reviewed all the cases for the following factors: (1) chemotherapy prior to electron microscopic examination, (2) stage of the disease, (3) absolute lymphocyte count at the time of examination, (4) serum immunoglobulin profile, and (5) survival of patients. We found no correlation between the presence of the cytoplasmic structures and therapy. In fact, 7 of the 13 patients with one or two cytoplasmic bodies had no prior antileukemic treatment. Furthermore, in repeated ultrastructural studies on two patients, the therapy had no observable effect on the type or incidence of the ER-associated structures. Similarly, during the course of the disease, the type and incidence of these bodies did not change, irrespective of the variation in the absolute lymphocyte count. Based on their association with ER, the bodies were presumed to be proteinaceous. They were neither transported to the Golgi apparatus nor excreted by the cells. Since the serum immunoglobulins were determined for only one patient, no speculation could be made correlating the existence of these bodies with the level of immunoglobulin in the sera of the CLL patients.

The life span of our patients with CLL varied considerably. The shortest survival period was 3 yr and the longest (the patient still doing well) was 16 yr. The presence of the ER-associated structures did not affect the survival rate of the patients. One patient had these structures in the first sample of blood drawn during the first year of the disease and also in another sample drawn 4 yr later. This patient died the following year. Another patient who had one of these structures in a sample of blood drawn 9 yr after the diagnosis of the disease died within 4 mo of examination. A third patient, who incidently did not receive any cancer chemotherapy during the 16-yr period he had the disease, had two ER-associated structures in the first sample obtained 7 yr after the diagnosis of CLL and also in his sixth sample 9 yr later. All other samples from this patient had these structures in approximately the same frequency.

The ER-associated bodies thus appeared to be the result of a physiologic defect in the leukemic lymphocytes. This hypothesis was supported by the finding of two types of inclusions in the cells of a few patients. The peripheral blood lymphocytes from healthy individuals, from noncancerous patients, and from patients with lymphoproliferative diseases other than CLL did not contain any
of these structures. It is possible that lymphocytes in hematopoietic tissues other than blood of patients with Hodgkin disease and lymphosarcoma contain one or more of the ER-associated bodies described herein. We therefore believe that the presence of these structures in the lymphocytes of a small percentage of CLL patients should not be considered pathognomonic of the disease.

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Endoplasmic reticulum--associated structures in lymphocytes from patients with chronic lymphocytic leukemia

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