Acid Hydrolases as Markers of Maturation in B-Cell Chronic Lymphocytic Leukemia

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Malignant lymphocytes from 30 B-cell chronic lymphocytic leukemia (B-CLL) patients were studied for the cytochemical localization of two acid hydrolases, alpha-naphthyl acetate esterase (ANAE) and acid phosphatase (AP). The large majority of the cells stained for both ANAE and AP in 7 cases, for AP only in 18 cases, and were negative for both the enzymes in 5 cases. Ultrastructural analysis revealed that the cells that displayed more mature morphological features, such as well developed smooth and rough membrane compartments, were those positive for acid hydrolases. That ANAE and AP are expressed by B cells at late stages of maturation was confirmed by the finding that some lymphocytes and all of the plasmacytoid lymphocytes and plasma cells from Walderström's macroglobulinemia, from mixed cryoglobulinemia, and from multiple myeloma patients stained strongly for both ANAE and AP. Using the expression of acid hydrolases and certain ultrastructural features as markers of cell differentiation, it was possible to demonstrate a process of maturation within the single B-CLL clones with accumulation of the cells at stages that differed in the various cases.

CHRONIC LYMPHOcytic LEUKEMIA usually represents the proliferation of a single B-cell clone (B-CLL). Cells from the single patients display a substantial homogeneity as for their morphology and surface membrane phenotype. Recently, a correlation between the different morphological features and the surface membrane phenotype of malignant B cells has been reported. All of the above observations have originated the concept that the malignant B-CLL clones are "frozen" at certain stages of maturation. In other lymphoproliferative disorders, a morphological and surface marker homogeneity is not always observed. For example, this is the case in Waldenström's macroglobulinemia, multiple myeloma, and among the T-cell malignancies, in acute lymphoblastic leukemia and T-CLL. In some disorders (i.e., those derived from T cells), the reason for such heterogeneity is not completely understood, while in others (i.e., macroglobulinemia, myeloma, and B-CLL with serum monoclonal protein), the heterogeneity is consequent to a process of maturation occurring within the malignant clone. This is best demonstrated by the finding that in macroglobulinemia lymphocytes, plasmacytoid lymphocytes and plasma cells all express immunoglobulin molecules sharing the same light chain type and idiotype.

In addition to being used for the identification of distinct lymphocyte lineages (markers of lineage), enzyme markers have proved to be of value in determining the stage of maturation of the cells within lymphocyte subpopulations (markers of maturation). An example of this is offered by the analysis of two acid hydrolases—acid phosphatase (AP) and alpha-naphthyl acetate esterase (ANAE). These two enzymes are expressed by peripheral blood resting T cells but not by resting B cells, thus serving as markers of lineage. On the other hand, AP is expressed by the thymocytes, the majority of which do not express ANAE. Therefore, for the T-cell lineage, the presence or absence of ANAE represents a means for the identification of maturational stages. Interestingly, acid hydrolases have been employed to assess the maturational stage of the cells from certain T-cell malignancies.

The present study deals with the localization of ANAE and AP in B-CLL cells and shows that: (1) the enzyme phenotype of the cells varies in the different patients, (2) the large majority of the cells from any given patient display the same reaction pattern; and (3) cytochemical patterns suggestive for a maturational process, although limited, occurring within the malignant clone can be observed.

MATERIALS AND METHODS

Patients

Thirty patients with B-CLL, 8 with multiple myeloma, 4 with Waldenström's macroglobulinemia, and 7 with mixed, type II cryoglobulinemia were examined.

The white blood cell count of B-CLL patients ranged from 20,000 to 700,000/cu mm, with a minimum of 80% lymphocytes. In 22/30 patients, 80% or more of the malignant cells expressed surface immunoglobulin as assessed by immunofluorescence with anti-class and anti-type specific reagents. Surface immunoglobulin was of the lambda type in 14 of the patients and of the kappa type in the remaining 8. The cells expressed both IgM and IgD in 8 patients, IgM in 7 patients, IgD in 4 patients, and IgG in 3 patients. In the other 8/30 patients, the B-cell nature of the malignant cells was confirmed by the presence of receptors for mouse red cells and for the third complement component. One patient had a serum monoclonal component of the same class and type as the surface immunoglobulin of the...
lymphocytes (IgGk), and immunofluorescence for cytoplasmic immunoglobulin showed that 2%-3% of the peripheral blood monoclonal cells with the morphology of young plasma cells contained IgGk molecules. The mean percentage of cells forming rosettes with neuraminidase-treated sheep erythrocytes (Ew) of all but one patient was 13.6%-2.0% (see Fig. 1). In one patient, in spite of their B-cell nature confirmed by the presence of surface IgGk molecules and receptors for mouse erythrocytes and C3', 80%-90% of the lymphocytes were capable of forming Ew rosettes. This case will be the subject of a separate report. The clinical stage of all of the patients was determined at the onset of the disease according to the Rai classification. Of the 30 patients, 25 were examined while on regular chlorambucil treatment, whereas the remaining cases were studied at the onset of the disease prior to any therapy.

In 3/8 of the myeloma patients, the monoclonal protein was IgGk, IgAλ in 3 others, IgAλ in one, and κ in one. Bone marrow aspirates contained a minimum of 15%-20% plasma cells as determined by May-Grünwald-Giemsa staining. Immunofluorescence with specific reagents demonstrated cytoplasmic immunoglobulin of the same class and type as the serum monoclonal component in the large majority of these plasma cells (80%-100%). All but one of the patients were examined prior to any therapy, whereas one was studied during relapse following long-term treatment with Melphalan.

Two patients with macroglobulinemia had an IgMκ and two and IgMα monoclonal protein. Bone marrow aspirates contained a minimum of 15% cells with the morphology of plasmacytoid lymphocytes or young plasma cells and variable numbers of lymphocytes. Cytoplasmic immunofluorescence revealed that the majority of these cells (70%-90%) contained molecules of the same class and type as the monoclonal component.

Six mixed cryoglobulinemia patients had an IgMκ and one an IgMα monoclonal component, all of which reacted in the cold with polyclonal IgG.

The amount of cryoprecipitate detectable, relative to the total serum, ranged between 5% and 30% as measured in a hematocrit tube. The pattern of bone marrow aspirates did not vary significantly from that of macroglobulinemia, although a lower percentage of lymphoplasmacytic cells was generally detected (8%-15%). Again, immunofluorescence studies showed that the majority (60%-70%) of these cells contained molecules of the same class and type as the monoclonal component. All of the macroglobulinemia and cryoglobulinemia patients were studied prior to any therapy.

**Isolation of Peripheral Blood Mononuclear Cells and Bone Marrow Cells**

Mononuclear cells were obtained from heparinized peripheral blood of patients by Ficoll-Hypaque density gradient centrifugation and subdivided into aliquots for immunologic, cytochemical, and electron microscopy analyses.

Single cell suspensions from bone marrow aspirates of myeloma, macroglobulinemia, and cryoglobulinemia patients were prepared by repeated passaging through fine gauze needles and deprived of erythrocytes by centrifugation on Ficoll-Hypaque gradients. Cells collected from the interface were treated for AP or ANAE cytochemistry.

**Cytochemistry**

The methods for the cytochemical localization of ANAE and AP are detailed elsewhere. Briefly, cells were fixed in suspension with cold Baker's formol-calcium, pH 6.7, for 15 min and cytocentrifuged. Slides were incubated in the substrates at 37°C for 60 min. For the demonstration of ANAE activity, 1.3 mM alpha-naphthyl acetate with 6% hexazotized p-rosanilin was used as substrate in 6.7 mM phosphate buffer, pH 5.8. The substrate for AP was 1 mM naphthol-AS-Bi-acetate with 6% hexazotized p-rosanilin, buffered in 0.15 M barbital-acetate. Generally, the cytochemical preparations were examined under phase-contrast microscopy, which permitted the detection of smaller quantities of the reaction product than did conventional light microscopy.

**Electron Microscopy**

Cells were fixed in suspension with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, at room temperature for 30 min. Following extensive washing in cacodylate, cells were postfixed in 1% osmium tetroxide in the same buffer at room temperature for 30 min, dehydrated with ethanol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined under a Siemens Elmiskop 101 transmission electron microscope.

**RESULTS**

**Expression of ANAE and AP by B-CLL Cells**

Figure 1 shows the percentage of ANAE-positive cells in B-CLL. Of 30 patients, 23 had a low proportion...
of positive cells (Fig. 2A and C). In these patients, the percentage of ANAE-positive cells was similar to that of the $E_N$ rosettes and therefore compatible with the presence of residual normal T cells. In 7/30 patients, ANAE-positive cells were above 50%, and in some cases, almost represented the totality of the cells. Unlike T cells, which display a dot-like pattern of staining, ANAE-positive B-CLL cells showed a diffuse reactivity with the reaction product, mostly evident in a paranuclear area (Fig. 2E).

When stained for AP, only 5/30 patients had low numbers of positive cells (Figs. 1 and 2B). In the remaining patients, the majority of the cells were positive and, in the single cases, displayed either a uniform staining pattern consisting of a focal paranuclear accumulation of the reaction product or a diffuse staining of the whole cytoplasm in a rim-like fashion (Fig. 2D and F). The one patient with a monoclonal IgG serum protein had a minority of cells diffusely stained for ANAE or AP, with the appearance of typical plasma cells.

Positivity of 50% of the cells was set arbitrarily as the limit to classify any given B-CLL case as positive or negative for one of the two enzymes. Accordingly, when the results of the two cytochemical reactions were compared in the same patient, three groups could be distinguished, namely, ANAE-negative, AP-negative B-CLLs (5/30), ANAE-negative, AP-positive B-CLLs (18/30), and ANAE-positive, AP-positive B-CLLs (7/30) (Fig. 1). No significant correlation was found between the cytochemical phenotype of the cells...
Fig. 3. The main ultrastructural features of B-CLL cells. (A) A cell (type 1) with numerous ribosomes and very scarce membrane systems. (B) A cell (type 2) with a fairly well developed smooth membrane compartment. (C) A cell (type 3) with numerous strands of rough endoplasmic reticulum mainly located in the cytoplasmic periphery. See Table 1 for the relative frequency of these three cell types in the various B-CLL cases.
and the clinical stage of the patients. Furthermore, there was no correlation between the expression of surface immunoglobulin and that of acid hydrolases.

**Ultrastructure of B-CLL Cells**

When B-CLL cells were analyzed by electron microscopy, three types of cells were identified. One of them, named type 1 for convenience, was characterized by disperse nuclear chromatin and by the remarkable absence of cytoplasmic membrane compartments. The cytoplasm was predominantly occupied by monoribosomes or dispersed polyribosomal clusters beside rare and isolated mitochondria (Fig. 3A). Another cell type (type 2) was identifiable through the presence of nuclear heterochromatin clumps and a smooth membrane system in the cytoplasm. The smooth membranes were more often assembled in parallel arrays of curved cisternae in a paranuclear localization, as is characteristics of the Golgi apparatus (Fig. 3B). The rough endoplasmic reticulum was either absent or constituted by single isolated strands. In the third cell type (type 3), the nucleus contained heterochromatin clumps and the cytoplasm displayed numerous strands of rough endoplasmic reticulum more commonly located in the cytoplasmic periphery and around the Golgi apparatus. The latter was always well developed (Fig. 3C). Type 3 cells never showed clear plasmacytoid features in spite of the relative abundance of rough endoplasmic reticulum, because this was seldom arranged in parallel arrays of cisternae.

By contrast, 2%-3% of the cells with a well developed rough endoplasmic reticulum and the typical appearance of plasmacytoid lymphocytes and plasma cells were detected in the B-CLL patient with a monoclonal IgGx serum component.

In each of the patients, one of the above cell types was predominant over the others (Table 1). The comparison of the ultrastructural studies with the results of the cytochemical analysis revealed that cases with the predominant type 1 cells were ANAE-negative, AP-negative, cases with type 2 cells were ANAE-negative, AP-positive, and those with type 3 cells were ANAE-positive, AP-positive. This is also evident when the results of Table 1 are compared with those of Fig. 1.

**Expression of ANAE and AP in Secretory B-Cell Malignancies**

Plasma cells in bone marrow preparations from myeloma patients were always positive for both ANAE or AP. The cytochemical localization of the two enzyme activities had a diffuse pattern of staining distributed over the entire cytoplasm (Fig. 4A).

In the four cases of macroglobulinemia, typical plasma cells as those described above for myeloma were also observed. However, other more numerous cells resembling large lymphocytes and plasmacytoid lymphocytes were positive for AP or ANAE (Fig. 4, B and C). In all of these cells, the pattern of staining was diffuse and readily distinguishable from that of other cells. These included macrophages and maturing cells of the myeloid lineage; the former were positive for ANAE or AP with a granular pattern of staining, whereas the latter were negative for ANAE and positive for AP with a faint granular staining (see legend to Fig. 4).

The cytochemical pattern of cryoglobulinemia bone marrow cells resembled that of macroglobulinemia, since diffusely stained lymphoid cells at different maturation stages were also observed (Fig. 4D). In the single macroglobulinemia and cryoglobulinemia patients, the proportion of cells identified as lymphoid through cytochemical criteria was consistent with that of cells positive for cytoplasmic immunoglobulin as identified by immunofluorescence (data not shown).

**DISCUSSION**

Previous studies have shown that ANAE and AP are preferentially expressed by T lymphocytes, whereas B cells are negative for these markers.17-20,29 Plasma cells represent a remarkable exception, in that they stain strongly for both the enzymes, independent of whether they are obtained from normal subjects or from patients with plasma cell dyscrasias.29,32 These findings suggest that the expression of acid hydrolases by B cells could occur during (and represent a marker for) the process of maturation. The present studies further support this concept as they demonstrate that, in addition to the plasma cells, the plasmacytoid lymphocytes from macroglobulinemia and cryoglobulinemia also are ANAE and AP positive.

In the course of this work, the lymphocytes from a number of B-CLL patients were found to express ANAE and AP. These data were best evaluated fol-
allowing a number of changes that greatly improved the sensitivity of the techniques for the localization of hydrolytic enzymes on both B and other lymphoid cells. These included fixation of the cells in suspension (prior to cytocentrifugation and incubation with the substrate) and observation by phase-contrast microscopy. However, it should be stressed that all of these changes, although useful, are not essential, since some positivity can be observed in CLL cells with the commonly used techniques. This is also demonstrated by the findings of Pinkus et al., who also described ANAE positivity in some CLL cases.

Three groups of B-CLL could be distinguished. In the first, the cells were negative; in the second, the cells expressed AP only; and in the third, the cells were positive for both AP and ANAE. If the expression of acid hydrolases reflects the degree of maturation, then the first group would comprise B-CLLs with cells less mature than those positive for both the enzymes. Expression of AP but not of ANAE would identify clones at an intermediate stage of maturation. If this were the case, then the pattern of expression of acid hydrolases by maturing B cells would resemble that of maturing T cells. The hypothesis that the various B-CLL clones were at different maturational stages was confirmed by the ultrastructural observations, which showed that the ANAE-negative, AP-negative B-CLLs were characterized by the predominance of cells rich in free ribosomes and with scarce or absent membrane-bound organelles. ANAE-negative, AP-positive B-CLLs consisted predominantly of cells with well-developed smooth membranes, whereas numerous strands of rough endoplasmic reticulum were observed in the majority of the cells from ANAE-positive, AP-positive B-CLLs.

Quite unexpectedly, no correlation was found between the expression of surface immunoglobulin (or other membrane markers) and the degree of maturation of the cells as assessed by cytochemical or morphological criteria. This finding is not easily explained but could suggest that the expression of surface immunoglobulin is not merely dependent on the degree of differentiation reached by the single cells.

In the single B-CLL cases, besides the predominant cell type, cells falling into different morphological or cytochemical categories were detected, although in lower proportions (see Table 1). Owing to the high proportion of circulating malignant lymphocytes, these cells were clearly part of the neoplastic lymphocytes. These findings, therefore, suggest that a process of maturation was occurring in the single CLL cases. An arrest of this process at stages that varied in the different patients possibly resulted in the accumulation of cells displaying uniform phenotypes. Previously, the hypothesis that B-CLL lymphocytes could mature into cells capable of secretion (but not identifiable as plasma cells) was suggested by the finding that certain patients had serum immunoglobulin sharing the same idiotype as the surface immunoglobulin of the malignant cells. Furthermore, preliminary in vitro labeling studies with radioactive amino acids have shown that cells from certain B-CLLs synthesize two types of immunoglobulin molecules, one with the physicochemical properties of the secreted molecule and the other with those of the membrane molecule (Sitia et al. unpublished results). B-CLL, therefore, resembles a number of other chronic malignancies derived from different hemic cells, such as, for example, chronic myelogenous leukemia, where concomitant processes of proliferation and differentiation occur.

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such a process appears somewhat limited and seems to differ from that of myeloma or macroglobulinemia, where the malignant clones comprise cells from the stage of the small lymphocyte, or even of pre-B cells, to that of mature plasma cell.

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