Synthesis of a Peroxidase Activity by Cells of Hairy Cell Leukemia: A Study by Ultrastructural Cytochemistry

By F. Reyes, M. F. Gourdin, J. P. Facet, B. Dreyfus, and J. Breton-Gorius

The nature of cells present in the blood, marrow, and spleen of patients with hairy cell leukemia is largely debated. These cells have been tentatively categorized on the basis of either monocytic or lymphocytic markers, and the accumulating data points to the fact that they share some characteristics of both cell types. Although hairy cells are known to lack myeloperoxidase-positive granules, present in normal human monocytes, we investigated the possible presence of other peroxidase activities differing from the granule-bound myeloperoxidase. The study was carried out with several methods based on the incubation of fixed and unfixed cells in the presence of diaminobenzidine and hydrogen peroxide. A peroxidase activity was found in hairy cells, located always in the endoplasmic reticulum but not in the Golgi apparatus or in any granule. By its cytochemical characteristics it appears to be closely related to that of tissue macrophages, activated blood monocytes, and other nonlymphocytic hematopoietic cells. This peroxidase is not found in lymphocytes with B or T phenotypes.

Hairy cell leukemia (HCL) is a now well-defined clinical and cytologic entity. Since the pioneer work of Bouroncle et al., the origin of the proliferating mononuclear cells has remained uncertain; this is reflected by the varying terminology used in the literature, such as “leukemic reticuloendotheliosis” or “histiolymphtocytosis.” This has led to a morphologic compromise based on the “hairy” appearance of the surface of the proliferating cells.

Despite the recent accumulation of morphologic, cytochemical, functional, and immunological data, difficulties still exist in the categorization of hairy cells (HC): they have characteristics that may be found in either normal monocytes or lymphocytes. In this respect the detection of surface immunoglobulins (SIg) on HC has been of particular interest, the presence of SIg being essential to the definition of B lymphocytes but also possibly resulting from an exogenous Ig adsorption as in monocytes. Recent data, however, support the view that at least part of SIg is a genuine product of HC.

In this paper we report on the demonstration of a peroxidase activity in HC, detected by ultrastructural cytochemistry and distinct from myeloperoxidase (MPO). This peroxidase is a new cytochemical characteristic of the proliferating cells of HCL and is not found in lymphocytes having B or T phenotypes.

MATERIALS AND METHODS

Cell samples. Blood HC obtained from seven patients were typical of the disease clinically and morphologically by blood smear examination (kindly performed by Dr. C. Sultan from Hôpital Henri Mondor, Créteil and Dr. G. Flandrin from Hôpital St. Louis, Paris). Cytochemical studies were performed before and after culturing cells (see Table 1).
Table 1. Hairy Cell Peroxidase

<table>
<thead>
<tr>
<th>Case</th>
<th>Noncultured</th>
<th>Cultured</th>
<th>Controls</th>
<th>Alkaline DAB Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fre</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mad</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bla</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Boe</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Van</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bol</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mal</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

a When repeated in a given case, studies were done on samples obtained at intervals during the course of the disease.

b When repeated in a given case, studies were done at intervals varying from 24 hr to 4 wk of culture.

c Method (see text).

As a comparison for peroxidase reactivity various lymphocyte cell samples from normal individuals and patients were examined, some of them also being studied after culture (see Table 2). Raji cell line was kindly provided by Dr. J. L. Preud’Homme from Hôpital St. Louis, Paris. All lymphocyte samples were treated by method C (see below), which appeared to be the most sensitive for peroxidase detection.

Preparation of cell suspensions. Since HC were to be recognized by their morphology at the electron microscope, no attempt was made to separate them from other buffy coat cells. Heparinized venous blood was sedimented at room temperature and the buffy coat pipetted off. Plasma was centrifuged at 200 g for 10 min and discarded; the cell pellet (approximately 3 x 10^6 cells) was then reacted as indicated below in order to detect peroxidase activities by ultrastructural cytochemistry. Blood lymphocyte samples were prepared either in the same way or after Ficoll purification.

Cell cultures. Blood samples to be cultured were obtained by sterile venipuncture either asuffy coat by single sedimentation (HCL and CLL samples) or as Ficoll purified (normal transformed lymphocytes). They were washed in RPMI-1640 medium (Eurobio) supplemented with 2% fetal calf serum (FCS) (Gibco) and viability assessed by trypan blue exclusion. Cells were then concentrated at 1 x 10^6/ml in RPMI-1640 supplemented with 20% FCS and kept in a humidified atmosphere of 5% CO₂ and 95% air. When necessary, culture medium was replaced weekly (see Tables 1 and 2). Normal transformed lymphocytes were obtained by culturing in the presence of 10 μ/ml phytohemagglutinin (PHA) M (Bacto), 50 μ/ml concanavalin A (con A) (Calbiochem) and pokeweed mitogen (PWM) (Gibco) 1:100.

Table 2. Lymphocyte Samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Noncultured</th>
<th>Cultured</th>
<th>Time of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal blood buffy coat</td>
<td>2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ficoll-purified normal blood</td>
<td>5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sézary syndrome buffy coat</td>
<td>2</td>
<td>1</td>
<td>day 4</td>
</tr>
<tr>
<td>T Cell CLL buffy coat</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B Cell CLL buffy coat</td>
<td>6</td>
<td>1</td>
<td>day 1, day 25</td>
</tr>
<tr>
<td>Waldenström disease buffy coat</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Raji cell line</td>
<td>—</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>PHA-transformed normal blood lymphocytes</td>
<td>—</td>
<td>2</td>
<td>48 hr, 72 hr</td>
</tr>
<tr>
<td>Con A-transformed normal blood lymphocytes</td>
<td>—</td>
<td>3</td>
<td>48 hr, 72 hr</td>
</tr>
<tr>
<td>PWM-transformed normal blood lymphocytes</td>
<td>—</td>
<td>2</td>
<td>day 7</td>
</tr>
</tbody>
</table>

* T and B phenotypes defined by E rosettes and immunoperoxidase staining. Raji cell line has surface and cytoplasmic B characteristics (Preud’Homme JL, Gourdin MF, Reyes F, Fellous M: Human lymphoblasticoid cell lines with pre-B cell features. Manuscript in preparation).
Cytochemical procedures. Peroxidase activities were studied by incubating cells in the presence of 3,3'-diaminobenzidine (DAB) and H$_2$O$_2$ using three separate methods:

**Method A.** Cells were fixed by resuspending in 1.25% phosphate-buffered (0.1 M) glutaraldehyde (TAAB Lab) for 30 min at room temperature, washed in buffer, and reacted for 30 min at room temperature in the dark in Graham-Karnovsky medium$^{33}$ containing 10 mg DAB and 0.1 ml 1% H$_2$O$_2$ in 10 ml Tris-HCl buffer 0.05 M, pH 7.6. After washing in phosphate buffer, cells were post fixed by 1% OsO$_4$.

**Method B.** Cells were fixed by resuspending in a mixture of tannic acid and aldehydes at low concentration for 1 hr at 4°C as described by Anderson et al.$^{34}$ After washing they were kept overnight in phosphate buffer 0.1 M before being reacted for 1 hr with a medium containing 20 mg DAB 0.1 ml 1% H$_2$O$_2$ in 10 ml Tris-HCl buffer 0.05 M pH 7.6. They were then washed in phosphate buffer and postfixed by OsO$_4$.

**Method C.** Unfixed cells were directly resuspended for 1 hr in a medium containing 20 mg DAB 0.01 ml 3% H$_2$O$_2$ in 10 ml Ringer-Tris buffer 0.05 M pH 7.3 according to the method of Roels et al.$^{35}$ After washing in Ringer-Tris buffer cells were fixed either directly by OsO$_4$, or by glutaraldehyde followed by OsO$_4$.

Cytochemical controls and inhibitors. Controls consisted of cells from incubations in DAB-containing media without H$_2$O$_2$. Inhibitions were carried out by preincubating cells in 0.01 M KCN or 0.02 M aminotriazole followed by incubation in the DAB medium supplemented with the corresponding inhibitor. In another experiment glutaraldehyde-fixed cells were incubated in an alkaline DAB medium (pH 9.7), which detects catalase.$^{36}$

Processing for electron microscopy. After osmium post fixation cell pellets were dehydrated in alcohols and propylene oxide and embedded in Epon according to usual procedures. Ultrathin sections were cut on a Reichert ultramicrotome and examined in a Philips EM-300 electron microscope.

RESULTS

**HCL Samples**

**Method A.** HC lacked dense staining suggestive of peroxidase activity in any cell organelle (Fig. 1). Rare contaminating polymorphs could be recognized in blood samples by their myeloperoxidase (MPO)-containing granules, but no monocytes were found. Contaminating erythrocytes as well as occasional reticulocytes and normoblasts had a black diffuse cytoplasmic staining owing to the peroxidatic activity of hemoglobin.

**Method B.** HC were reactive in only two patients of six studied by this method. In one case (Fre) the cytochemical reactivity had a moderate intensity and was found only in about 60% of cells. In the other case (Mad), the study of which was repeated at a few months interval, all HC were strongly reactive. In both cases the cytochemical reactivity was located in the perinuclear envelope and cytoplasmic strands of endoplasmic reticulum (ER); no peroxidase-positive granules or Golgi saccules were found. Mitochondria were unreactive (Figs. 2 and 3). Identical results were obtained in case Mad after culturing HC that remained fully reactive in the same organelles. In other cases cells were negative and remained unreactive when cultured in several repeated studies (see Table 1). As in method A, occasional polymorphs were identified by their MPO-containing granules.

**Method C.** When unfixed HC were incubated in the DAB-containing medium a strong cytochemical reactivity was observed in six of seven cases. The reactivity was present in 100% of HC in every case but one; in the latter (case Bla) only half of the HC were moderately stained. The peroxidase activity was located in the perinuclear envelope and ER cisternae but never in Golgi saccules or any granule (Figs. 4 and 5).
Fig. 1. HCL blood sample (case Ma!) treated by method A. No dense staining found in perinuclear envelope, ER strands (arrow), or granules. Mitochondria (m) were unreactive. Surface of cells had typical villous projections. × 6800.

Fig. 2. HCL blood sample (case Mad) treated by method B. Black reaction product present in perinuclear space and ER strands (arrows) of all HC. × 6800.
Fig. 3. Same sample as Fig. 2, method B. Peroxidase activity strongly detected. Narrow ER strands seen as dots when cross-sectioned (single arrow) or as elongated profiles (double arrow). Mitochondria (m) were unreactive. × 13,800.

Fig. 4. HCL blood sample (case Van) treated by method C. This HC had a strong peroxidase activity within perinuclear space and ER. Other reactive structures were numerous mitochondria (m), whose membrane and cristae stained because of the cytochrome peroxidase activity. In this experiment cells were postfixed only by osmium after incubation in the DAB medium; for this reason their morphology was less well preserved than when postfixed by glutaraldehyde plus osmium (see next figures). × 11,800.
In case Mal all HC were negative; in this case, however, a strong peroxidase activity became detectable in 100% of HC after 24 hr of culture and was further confirmed by repeated studies of these cultured cells (Figs. 6 and 7). A somewhat similar observation was made in case Bla, in which only half of the HC were moderately reactive but where 100% of HC became strongly positive after culture.

Two other samples (cases Mad and Bol), the cells of which were 100% stained, also remained fully reactive when cultured.

Taken together, these results established that in every sample studied by method C before or after culture, HC showed a peroxidase activity located exclusively in the perinuclear space and ER.

In two cases (Fre and Bla) the cytoplasm of HC contained ribosome-lamellar complexes previously described by others; a peroxidase activity was detectable at the periphery and center of these cylindrical complexes, thus identifying such reactive structures as ER strands (Fig. 8).

As with the two methods described above, the peroxidase activities of other buffy coat cells were also obvious with method C. In addition, round cells with a typical nucleocytoplasmic lymphocyte morphology were easily recognized in sections and lacked any ER reactivity. It must be also noted that in virtually all cells of samples studied by method C mitochondria were reactive at the level of cristae and outer membrane as a result of the peroxidatic activity of cytochrome.
Fig. 6. HCL cultured sample (case Mal) treated by method C, postfixed by glutaraldehyde plus osmium. HC initially peroxidase negative were strongly positive after 24 hr of culture. Localization of peroxidase activity is unchanged. E, two erythrocytes whose hemoglobin reacted strongly in presence of DAB. \( \times 7600 \).

Fig. 7. Case Mal, sample examined at 4 wk of culture, method C and single osmium postfixation. Peroxidase activity persisted with identical distribution, as well as mitochondrial staining. L, lipid droplets appearing in cultured cells. E, reactive erythrocytes. \( \times 5600 \).
Case Fre, sample treated by method C, single osmium postfixation. Two cross-sectioned ribosome-lamellar complexes: these structures surrounded and centered by ER reactive for peroxidase (single arrows); three mitochondria (m) also stained. N, part of nucleus surrounded by strong peroxidase staining (double arrow). Perinuclear envelope and ER surrounding ribosome-lamellar complex connected by reactive ER strand (triple arrow). x 48,000.

Mitochondrial staining was found both in ER-positive HC and in ER-negative lymphocytes.

**Lymphocyte samples.** The detection of ER peroxidase activity was carried out by method C in various samples (see Table 2). All these experiments gave negative results, with the exception of the mitochondrial cytochrome reactivity (Figs. 9 and 10). The absence of ER reactivity in T and B lymphocyte cells was further confirmed after culturing cells of various origin for varying periods of time.

In normal blood buffy coat lymphocytes were easily recognized among cells with MPO-positive granules (polymorphs and monocytes). In Ficoll-purified blood samples a high proportion of monocytes (up to 40%) was identified by the presence of MPO-positive granules, monocytes having no ER reactivity; contaminating platelets were also present having some ER peroxidase activity; lymphocytes were clearly negative (Figs. 10 and 11). However, in three of five Ficoll-purified samples of normal blood, very rare round lymphocytic-looking cells were found with a faint unique perinuclear reactivity (not shown).

**Cytochemical controls.** They were carried out in cultured HC from three patients, the peroxidase activity being detected by methods B and C (see Table 2). The incubation of cells in DAB-containing media lacking H₂O₂ consistently abolished the cytochemical staining of perinuclear and cytoplasmic ER in all cells; that of mitochondria was inconsistently abolished when this control was carried out with method C.
The ER reactivity of HC was totally inhibited when aminotriazole was added before and during incubation in DAB, by either method B or method C; in the latter method aminotriazole did not inhibit mitochondrial staining. KCN inhibited both the ER peroxidase activity and mitochondrial staining. Finally, no ER staining was found when glutaraldehyde-fixed HC were reacted in the alkaline DAB medium that detect catalase.

**DISCUSSION**

The main result of this study is the positive reaction of HC in the presence of DAB and H₂O₂, i.e., the synthesis of a peroxidase activity located in the ER of these cells. This finding should be added to the list of characteristics already investigated in order to functionally categorize HC.

HC have been hitherto considered as peroxidase negative on the basis of optical and ultrastructural cytochemistry. However, in these studies the methods used can be considered as suitable only for the detection of MPO, which is highly resistant to fixatives; MPO is indeed regularly detected in the maturing granulocyte and monocyte series after glutaraldehyde fixation of cells (for review see ref. 39). Therefore HC can be considered as MPO-negative cells, thus differing from normal monocytes. They have, however, a peroxidase activity sensitive to aldehyde fixatives, as shown by comparing the results of the three methods used in the present study: no HC are found to be reactive under conditions of MPO detection (method A), while reactivity is detectable in cells of a few patients using a low-dose aldehyde fixative (method B), and all samples are fully reactive when cells

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**Fig. 9.** Blood B lymphocytes from patient with CLL; method C and single osmium postfixation. Six lymphocytes (L) shown, devoid of ER peroxidase activity; their only reactive structures were mitochondria. x 5800.
Fig. 10. Ficoll-purified normal blood mononuclear cells; method C and double postfixation. Three monocytes (Mo) shown, identified by their content in MPO-positive granules. Six peroxidase-negative lymphocytes also shown, some but not all having reactive mitochondria. × 6800.

(including cultured cells) are reacted with DAB without prior fixation (method C). HC peroxidase is also characteristic in that its localization is restricted to the perinuclear space and other ER strands. By this criterion also it differs from MPO, which in addition to ER always involves the Golgi apparatus and granules of the granulocytic and monocytic precursors.39

Although of unknown identity, the cytochemical reactivity found in HC can be considered on the basis of the present findings as a peroxidase activity. First, the possibility of an artefactual staining (resulting from a nonspecific fixation of DAB in cells followed by oxidization in the presence of OsO₄) is ruled out by controls in which cells remain unstained when incubated in DAB media lacking H₂O₂. On the other hand, DAB oxidization in the presence of H₂O₂ (giving rise to a dense visible reaction product) is not specific for peroxidases, since it can also be induced by other heme-containing molecules such as hemoglobin39 or enzymes such as cytochromes and catalase.37 The absence of ER staining in HC treated by method A, i.e., glutaraldehyde fixed (contrasting with the persistent hemoglobin reactivity as shown by erythrocyte staining), and its presence in HC treated by method C, i.e., unfixed, indicates that this peroxidase activity is enzymatic in nature.35 Aminotriazole- and KCN-induced inhibition of the HC cytochemical reactivity is similar to that of other known tissue peroxidases.34,37,11 Aminotriazole-induced inhibition also clearly discriminates between the ER peroxidase activity and that of mitochondrial cytochrome. Finally, the consistent finding of a peroxidase activity when HC were incubated in DAB as unfixed cells (method C) indicates that the
oxidation of DAB does not result from catalase. This is further confirmed by the absence of ER staining when fixed HC were incubated in the alkaline DAB.

On the basis of its sensitivity to fixatives and inhibitors and of its localization restricted to ER, HC peroxidase appears to be similar to peroxidases of other tissues as well as to the peroxidase activity of the megakaryocyte series. Also relevant to the problem of the functional characterization of HC is the detection of a peroxidase activity sharing some of the above characteristics of HC peroxidase in Kupffer cells and peritoneal macrophages of some animal species. Moreover, it has recently been shown that human blood monocytes (already equipped with MPO-containing granules) are able, after adhering in vitro for a short time, to develop a peroxidase activity that has an identical ER localization to that of Kupffer and peritoneal cells and, as shown here, to HC. It must be recalled that in two of our cases the peroxidase activity was lacking in some or all HC when studied before culture but becomes fully detectable in the ER of all HC after 24 hr of culture.

Strikingly, this study failed to detect any peroxidase activity in the various T and B cell samples obtained from patients with proliferative disorders (including cultured samples) as well as in immunoblasts obtained in vitro after culturing in the presence of PHA, con A, and PWM and in a continuous B cell line. The study of normal blood buffy coat or Ficoll-purified mononuclear cells gave a similar result: peroxidase-negative lymphocytes were clearly identified among MPO-containing leukocytes. Incidentally, this study confirms that 30-40% of Ficoll-purified cells are monocytes. Contaminating platelets were also recognized by
their ER peroxidase content. Thus on the basis of this single cytochemical marker HC appear to be related more to some tissue macrophages and blood monocytes (as well as to other myeloid cells) than to lymphocytes.

However, the study of Ficol purified cells deserves an additional comment. In three of five samples treated by method C a survey of numerous sections disclosed very rare round cells, small and lymphocytic looking, with a faint perinuclear peroxidase activity (unpublished results). The significance of this finding is at the present time unknown. This very minor population of peroxidase-positive lymphocytic-looking cells may correspond to some circulating nonlymphocytic committed percursors, but there is also the possibility that it corresponds to a normal lymphocyte subpopulation that needs further functional characterization. This problem is currently under investigation in this laboratory.

In conclusion, this study shows that the mononuclear cells of HCL have a peroxidase activity synthesized in ER. By contrast, this peroxidase is not detected in normal or malignant B and T cells at various maturation steps. In the current controversy on the nature of HC it would be tempting on this basis to classify these cells not as lymphocytic but rather as cells related to monocytes and tissue macrophages, a possibility already suggested by previous morphologic and functional studies. However, as pointed out by others, the identification of HC is "plagued by the universal problem of categorizing abnormal cells on the basis of assays usually reserved for identifying normal cells." Despite discrepancies in immunofluorescence studies, data are now accumulating that favors the B lymphocyte nature of HC by demonstrating synthesis of SIg and Ig production in vitro.

Therefore one may speculate on the possibility that HCL is composed of cells expressing a hybrid phenotype; such a possibility was recently shown independently in one case of HCL whose cells bore B and T surface properties.

Since HC endogenous peroxidase can be inhibited, work is now in progress to try to detect intracellular Ig synthesis in HC by means of immunoelectron microscopy using peroxidase-labeled reagents, a method that has proved to be useful for detecting Ig synthesis by lymphoma cells.

ACKNOWLEDGMENT

We are grateful to Dr. W. Vainchencker and Dr. S. Xeneumont-Moritz for their help in cell cultures, to Dr. G. Flandrin, Dr. B. Varet, and Dr. M. Tulliez, who provided some HCL samples, to Dr. C. Sultan, who performed some of the routine smear examinations, and to Dr. J. L. Preud'Homme, who provided the Raji cell line. We also acknowledge the collaboration of Ph. Reboul in photographic assistance and M. Segear in secretarial work.

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