The Ultrastructure of Phytohemagglutinin (PHA) Stimulated Lymphocytes of Chronic Lymphatic Leukemia

By Kathryn P. Clausen and Bertha A. Bouroncle

PHYTOHEMAGGLUTININ (PHA) is a potent mitogen, capable of stimulating lymphocyte transformation to blastoid or mitotic cells in vitro. Lymphocytes from patients with chronic lymphatic leukemia (CLL) are hyporesponsive or have a delayed response to PHA stimulation under the conditions of the usual short-term culture. In a separate study the delayed response of PHA-stimulated lymphocytes from patients with CLL was demonstrated when observed daily in cultures of up to 11 days. In this study, observations on the fine structure of both normal and leukemic PHA-stimulated lymphocytes are presented.

This study was carried out to determine if there are any morphologic differences between the blastoid cells derived from both normal and CLL lymphocytes, since both types of lymphocytes seem to differ functionally. Blastoid cells obtained from CLL lymphocytes of early (3 day) and long-term (7 to 8 day) cultures from the same patients were also compared to determine if there are any morphologic differences which might suggest a second population of blastoid cells associated with the delayed response.

MATERIALS AND METHODS

Eight cultures of concentrated peripheral blood lymphocytes from four subjects were examined.
1. Normal control, 5 day cultures, with and without PHA stimulation.
2. CLL, 6 day cultures, with and without PHA stimulation.
3. CLL, 3 and 8 day cultures with PHA stimulation.
4. CLL, 3 and 7 day cultures with PHA stimulation.

Technics for the concentration and culture of lymphocytes and thymidine labelling are described elsewhere. A leukocyte differential count was performed on each culture at the time of harvest. From each culture, 8 ml of suspended cells were concentrated into a pellet by centrifugation at 3000 rpm. for 15 to 20 minutes. The pellet was fixed in 5 per cent cold glutaraldehyde for 30 minutes, post-fixed for 45 minutes in buffered 1 per cent OsO₄, dehydrated through graded alcohols and embedded in epoxy resin. Ultrathin sections (600Å) for electron microscopy were stained for 15 minutes each with uranyl acetate and lead citrate. The sections were examined and photographed with an RCA EMU-3F electron microscope.

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RESULTS

Small Lymphocytes

Small lymphocytes from both stimulated and unstimulated control cultures (Fig. 1) were morphologically similar and were consistent with previously published descriptions of normal peripheral blood lymphocytes. The lymphocytes ranged from 6 to 8 μ in diameter. The nucleus was round or indented with abundant heterochromatin distributed throughout, but most concentrated at the inner nuclear membrane. In the appropriate sections, a large, centrally located nucleolus was apparent. The cytoplasm contained numerous free ribosomes, occasional strands of rough endoplasmic reticulum and small numbers of large oval mitochondria with well-preserved plate-like cristae. Occasionally, bundles of microfibrils were present in the cytoplasm.

Leukemic lymphocytes from cultures with and without PHA (Figs. 2 and 3) were 5 to 6 μ in diameter with a round dense nucleus and abundant heterochromatin. The cytoplasm was scant, representing only a thin rim containing many free ribosomes, occasional strands of rough endoplasmic reticulum and scattered mitochondria. Lamellar nuclear bodies, sometimes multiple, were prominent in the leukemic lymphocytes, but rare in the small lymphocytes from the control culture. Occasionally, lymphocytes with double or segmented nuclei were seen in the unstimulated leukemic culture.
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Fig. 2.—Chronic Lymphatic Leukemia. Small lymphocytes from 7-day culture with PHA. The cytoplasm is scant and the cells adherent. There is a prominent nuclear body (arrow, left). × 12,000.

Fig. 3.—Chronic Lymphatic Leukemia. Lymphocytes from 3-day culture containing PHA. There are multiple prominent nuclear bodies (arrows) and cellular cohesion. × 9,000.
Fig. 4.—Normal Control. Large blastoid cell from 5-day culture with PHA. There is a complex cytoplasm containing osmiophilic droplets (od), an elaborate Golgi apparatus (G), small dense mitochondria (m) and numerous vesicles (v). There are portions of two centrioles (c) with numerous microtubules, one of which joins the outer nuclear membrane (arrow). × 32,000.

Blastoid Cells

The blastoid cells were distinguished under the light microscope by increased size, a large vesicular nucleus with a prominent nucleolus and basophilic cytoplasm. Clear cytoplasmic droplets were common in the blastoid
Fig. 5.—Chronic Lymphatic Leukemia. Large blastoid cell from 3-day culture with PHA. There are numerous osmiophilic droplets (od) and a multivesicular body (mv). There is a small nuclear body (arrow). The small dense cytoplasmic bodies are probably lysosomes. X 16,400.

cells. We arbitrarily differentiated the blastoid cells into intermediate and large forms on the basis of size. Both cell types were capable of synthesizing DNA as demonstrated by the incorporation of tritiated thymidine. While both types of blastoid cells appeared in both the control and leukemic cultures, the intermediate blastoid cells frequently predominated in those cultures with a low total percentage of lymphocytic transformation.

Using the electron microscope, further distinction between large and intermediate blastoid cells was apparent, though occasional transitional forms were found.

Large blastoid cells (Figs. 4, 5 and 6) ranged from 8 μ to 20 μ in diameter. The nucleus was proportionately enlarged with an increased amount of euchromatin, a thin rim of heterochromatin adjacent to the inner nuclear membrane and occasional islands of heterochromatin distributed throughout the nucleus. The nucleolus, if included in the plane of section, was prominent. There was abundant cytoplasm characterized by numerous free ribosomes and polyribosomes, with occasional short strands of rough endoplasmic reticulum randomly oriented in the cytoplasm. Numerous cytoplasmic vesicles, as well as multivesicular bodies, were present in these cells. When included in the section, the Golgi apparatus was complex, consisting of abundant
Fig. 6.—Chronic Lymphatic Leukemia. Large blastoid cell from 7-day culture with PHA. The cytoplasm is complex with dilated Golgi vesicles and a prominent centriole with numerous microtubules. There are many prominent cytoplasmic polyribosomes. × 16,400.

lamellar profiles and vesicles. A centriole was often identified within the Golgi (Figs. 4 and 6) with prominent microtubules radiating from it. Mitochondria were numerous and usually large with well-developed cristae. These cells frequently contained round homogeneous osmiophilic droplets with distinct margins (Figs. 4 and 5). The droplets measured up to 0.8 μ in diameter. Lysosome-like structures were also occasionally identified in the cytoplasm of these cells (Fig. 5).

Intermediate blastoid cells (Figs. 7, 8 and 9) differed from the large forms essentially in having a less complex, less abundant cytoplasm, and large nucleus with very little heterochromatin. They were 7 to 11 μ in diameter and had a large nucleus consisting almost entirely of euchromatin with a very thin rim of heterochromatin adjacent to the inner nuclear membrane. When included in the section, the nucleolus (Figs. 8 and 9) was large and prominent. The narrow cytoplasmic rim was characterized by numerous polyribosomes, a few short strands of rough endoplasmic reticulum and sparse, small mitochondria with few cristae. Vesicles were rarely observed, and no osmiophilic droplets were seen. Occasional small lysosome-like structures were
identified (Fig. 9). Golgi vesicles, centrioles and microtubules were not seen in the intermediate cells.

There were no apparent differences between large blastoid cells of leukemic and control cultures. Intermediate blastoid cells from the leukemic cultures, in contrast to those derived from normal lymphocytes, commonly had double nucleoli (Figs. 8 and 9). Nucleoli were also frequently observed adjacent to the nuclear membrane in these cells. Lamellar nuclear bodies were prominent in the intermediate blasts from the leukemic cultures (Figs. 8 and 9), but were not found in the same cells from the control culture. No differences were noted between the blastoid cells, either large or intermediate, of the 3 and 7 to 8 day cultures of leukemic cells.

Intercellular cohesion was seen in all leukemic cultures, both with and without PHA, and was evident in clumped small lymphocytes (Figs. 2 and 3) and blastoid cells (Figs. 5, 8 and 9). Cohesion was occasionally observed among blastoid cells in the stimulated control culture but not in the culture of unstimulated normal lymphocytes (Fig. 1).

There were no plasma cells or proplasmacytes identified in any of the cultures examined. Occasional macrophages containing phagocytized debris were identified.

Fig. 7.—Normal Control. Intermediate blastoid cell from 5-day culture with PHA. There is a large vesicular nucleus, and narrow cytoplasmic rim containing numerous polyribosomes. × 12,000.
Fig. 8.—Chronic Lymphatic Leukemia. Intermediate blastoid cell from 6-day culture with PHA. There is a prominent lamellar nuclear body (arrow) and an eccentrically located nucleolus. × 12,000.

DISCUSSION

One objective of this study was to determine if there were distinguishing features of unstimulated and PHA-stimulated lymphocytes of chronic lymphatic leukemia compared to similar cultures of normal lymphocytes.

We have observed that the lymphocytes of CLL tended to be slightly smaller than the lymphocytes of the control culture with an increased nuclear-cytoplasmic ratio. Leukemic lymphocytes demonstrated more nuclear bodies and a greater tendency to cellular cohesion than normal lymphocytes in culture. Nuclear bodies have been described in many normal and abnormal mammalian cells and are of unknown significance in the leukemic lymphocytes.14-16 Cellular cohesion in lymphocyte cultures has also been observed previously2 and may be a nonspecific feature of the cultured cells.

The large blastoid cells of CLL were similar in every respect to the large blastoid cells of the control culture. The presence of osmiophilic droplets, dilated Golgi vesicles, polysomes, multivesicular bodies and prominent centrioles with an elaborate microtubular system have been previously reported as distinguishing features of PHA stimulated blastoid cells.1,2,17-20 The aggregation of cytoplasmic ribosomes into polysomes was a very characteristic feature of the blastoid cells in this study, compared to the free ribosomes
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Fig. 9.—Chronic Lymphatic Leukemia. Intermediate blastoid cell from 7-day culture with PHA. There is a prominent nuclear body (arrow) and two nucleoli. The cytoplasm contains numerous polyribosomes. The small dense bodies to the left of the nuclear membrane are probably lysosomes. × 9,000.

of small lymphocytes. Occasional membrane-bound cytoplasmic densities were identified which probably represent lysosomes. This is consistent with previous studies showing lysosomes in PHA-stimulated lymphocytes. Thus, no significant differences could be demonstrated by electron microscopy between large blastoid cells transformed from normal and leukemic lymphocytes in culture.

The most interesting finding in this study was a morphologically distinct intermediate blastoid cell in PHA-stimulated cultures of both normal and leukemic lymphocytes. Tanaka et al.\(^2\) mentioned the presence of a blastoid cell of intermediate size in PHA-stimulated cultures which seemed to contain more mitotic figures than the large cells, but they did not describe the structural characteristics of these cells. Caron\(^2\) studied thymidine-labelled, PHA-stimulated lymphocytes and has shown that the blastoid cells can produce daughter cells morphologically indistinguishable from small lymphocytes. No mention was made of an intermediate blastoid form in this process, either before or after mitosis. Douglas et al.\(^2\) described a unique "intermediate-sized PWM cell" in lymphocyte cultures stimulated with pokeweed mitogen (PWM). This cell appeared in cultures after 60 hours incubation and was approximately the same size as the intermediate blastoid cell described here. It was characterized by an eccentric nucleus with condensed clumped chromatin, numerous ribosomes, well-developed rough endoplasmic reticulum and a prominent Golgi apparatus with many vesicles. The similarity between this PWM cell and a plasmablast was noted by these authors and they
suggested that these PWM-stimulated cells might be engaged in immunoglobulin synthesis. They did not find a similar cell type in PHA-stimulated cultures. In contrast, the intermediate blastoid cell observed in the present study differed from the intermediate-sized PWM cell in having a centrally located nucleus with an abundance of euchromatin and virtual absence of heterochromatin which could not be explained as the result of plane of section through a larger cell. The cytoplasm was not abundant and was largely devoid of organelles except for numerous polyribosomes. It would be difficult to infer any special function of the intermediate blastoid cells in this study on the basis of morphology alone.

In the present study, the intermediate blastoid cells were not limited to leukemic cultures, but frequently predominated in those cultures with a low total percentage of lymphocytic transformation, all of which were leukemic. Pulse labelling with tritiated thymidine showed DNA synthesis as demonstrated by thymidine uptake in both the intermediate and large blastoid cells. This evidence of DNA synthesis and frequent intermediate blastoid cell predominance in hyporesponsive cultures suggest that the intermediate blastoid cells precede mitosis, and are perhaps precursors of large blastoid cells or are capable themselves of mitosis at this stage. Alternatively, it may be suggested that the intermediate blastoid cells, though synthesizing DNA, do not undergo mitosis at all, but are arrested in the premitotic phase.

We believe that the intermediate blastoid cells probably precede mitosis and are increased as a function of the hyporesponsiveness of the leukemic cultures. Whether they are an end-stage cell, capable of enlarging and synthesizing DNA but incapable of division, will have to be determined by further investigation.

**Summary**

Cultured lymphocytes of chronic lymphatic leukemia have been shown to have a delayed and diminished response to phytohemagglutinin (PHA) stimulation.

By electron microscopic examination, a morphologically distinct intermediate blastoid cell was identified in the stimulated cultures which showed minimal differences between those derived from normal and leukemic lymphocytes. It was distinguished by a large vesicular nucleus and relatively scant cytoplasm with few organelles. This intermediate blastoid cell frequently predominated in cultures with a low total percentage of blastogenesis. It is suggested that this intermediate blastoid cell represents a premitotic phase in lymphocytic transformation, though the possibility of it's being a non-mitotic blastoid cell cannot be excluded.

No morphologic differences were noted between the large blastoid cells transformed from normal and leukemic lymphocytes.

**SUMMARIO IN INTERLINGUA**

Esseva demonstrate que culturare lymphocytos de chronic leucemia lymphocytic responde retardatamente e minus fortemente al stimulation per phytohemagglutinin.

Studios a microscopia electronic ha permittite le identification in le stimulate culturas de
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un morphologicamente distinte cellula blastoide intermediari, ben que le culturas revelava solo minimas differentias seundo que illos essee derivate ab lymphocytos normal o ab lymphocytos leucemic. Le cellula blastoide essee distinguite per un grande nucleo vesicular e relativamente pauc cytoplasma con non numerose organellas. Illo predominava frequente mente in culturas con un basse procentage total de blastogenesis. Es suggestionate que iste cellula blastoide intermediari representa un phase premitotic in le transformation lymphocytic, ben que le possibilitate que illo es un cellula blastoide nonmitotic non pote esser escludite.

Nulle differentias morphologic esseva notate inter le grande cellulas blastoide transformate ab lymphocytos normal e ab lymphocytos leucemic.

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