Abnormal Ultrastructural Changes in Sodium Periodate-stimulated Lymphocytes from Patients with Chronic Lymphocytic Leukemia

Manley McGill,2 Thomas M. Monahan,3 Roger A. Novak, and Creed W. Abell

ABSTRACT

Lymphocytoid and plasmacytoid blasts have been identified in both normal and chronic lymphocytic leukemia lymphocyte cultures exposed to NaIO₄. However, the appearance of ultrastructurally abnormal blasts in chronic lymphocytic leukemia cultures suggests that NaIO₄ also stimulates the transformation of an abnormal subpopulation of lymphocytes. Development of invaginated nuclei produced morphological features similar to nuclear blebs, a cellular abnormality described in blood cells from other cancers. Furthermore, the consistent localization of nuclear invagination only to portions of nuclei adjacent to developing cytoplasmic microtubular complexes suggests a role for microtubules in the transformation process. The absence of these unusual blasts in cultures stimulated with other mitogens may indicate that these NaIO₄-sensitive cells are not responsive to the more commonly used plant lectins.

INTRODUCTION

Recent ultrastructural studies have demonstrated that functionally different lymphocyte populations can be identified by their distinctive morphological features. According to the reports of Greaves and Janossy (16) and Janossy et al. (17), ultrastructural morphology can be used to distinguish mouse T- and B-lymphocytes stimulated with plant lectins by determining the absence or presence of distended RER.4 The presence of distended RER in stimulated B-cells (plasmacytoid blasts) is a morphological visualization of increased protein synthesis and it is this morphological feature that distinguishes plasmacytoid blasts from lymphocytoid blasts (stimulated T-cells) in mouse lymphocyte populations. Douglas (11) and Biberfeld and Mellstedt (6) have described distended RER as a possible ultrastructural feature of human plasmacytoid cells. However, results obtained with mouse lymphocytes may not be analogous to the same studies performed with human lymphocytes (11), and ultrastructural morphology alone may not accurately identify human lymphoblasts as T- or B-cells. Yet, morphological studies of human leukemic lymphocytes have identified abnormal ultrastructural features indicative of abnormal cell function and abnormal lymphocyte population development.

In disease states such as CLL, prominent ultrastructural abnormalities in many lymphoblasts suggest that the majority of peripheral blood lymphocytes are functionally, as well as structurally, abnormal (8, 12, 13, 18). Biochemical characterization of CLL cultures supports morphological suggestions of abnormality by demonstrating a variety of biochemical anomalies in CLL lymphocytes after their stimulation with plant lectins (2, 4, 7, 11, 12, 20, 22). Furthermore, McGill and Brinkley (18) and McGill et al. (this report) have demonstrated unmistakable abnormalities in blast cell development and mitogen-induced blastogenesis in lymphocyte populations from leukemic patients. Thus, electron microscopic analysis may not identify human lymphocyte origins, but they do reveal abnormal lymphocyte morphologies that have proven to be ultrastructural visualizations of abnormal cell function.

In our investigations of the mitogenic effects of NaIO₄ on lymphocytes, ultrastructural examinations of treated cultures revealed the development of normal plasmacytoid and lymphocytoid blasts in cultures from both normal and CLL patients. In CLL cultures we also observed the formation of lymphoblasts with structural features unlike normal lympho- or plasmacytoid blasts. These blasts, which were observed in cultures from 4 different CLL patients, were not seen in NaIO₄-stimulated cultures from normal individuals and were not observed in previous studies of stimulated CLL lymphocytes. The unusual ultrastructural features that characterize these NaIO₄-responsive blasts and a comparison of these features with ultrastructural changes reported in blood cells from patients with other cancers are presented in this study.

MATERIALS AND METHODS

Populations of small lymphocytes were collected and cultured as previously described (20). Whole blood from normal donors was collected in citrate-phosphate-dextrose anticoagulant, clotted by the addition of 0.2 M CaCl₂, and clots were removed with sterile applicator sticks. The defibrinated blood was centrifuged at 500 × g for 30 min and
separated as serum and cell fractions into sterile containers. The cell fractions were resuspended in McCoy's 5A culture medium and layered onto Ficoll-Hypaque gradients to separate white cells from red cells. The white cell fractions, which contained 90% small lymphocytes, were washed in culture medium and resuspended at 5 × 10⁶ cells/ml in McCoy's 5A medium containing 10% autologous serum. CLL lymphocytes were treated in the same manner, except that blood samples from CLL patients were collected in heparin and cultured in McCoy's 5A media containing 10% fetal calf serum. The use of heparin and fetal calf serum facilitated the collection of CLL cells in smaller volumes and has not altered the growth characteristics of these cultures as demonstrated in previous studies (1, 20). Both methods of collection and culture have been used with normal and leukemic lymphocytes with no effect on ultrastructural morphology before or after stimulation with plant lectins. All 4 CLL patients included in this study had no history of treatment for leukemia prior to cell culture. Their initial white cell counts ranged from a low of 39,000/cu mm (86% lymphocytes) to a high of 58,700/cu mm (96% lymphocytes).

Lymphocyte cultures for NaIO₄ stimulation were washed in phosphate-buffered saline (0.15 M NaCl, 8.5 g Na₂HPO₄, and 1.07 g NaCl H₂O in 1 liter H₂O) and exposed to 5 × 10⁻⁷ M NaIO₄ in phosphate-buffered saline at 4° for 10 min. Normal cultures were washed in McCoy's 5A media and placed in McCoy's 5A media (supplemented with 10% (v/v) autologous serum and penicillin-streptomycin) at 2 × 10⁶ cells/ml. CLL cells were treated identically, except that culture media contained 10% fetal calf serum as discussed above. All cultures were incubated at 37° in a 95% air-5% CO₂ atmosphere. Lymphocytes were also stimulated with PHA-P (40 μg/10⁶ cells; Difco Laboratories, Detroit, Mich.) and cultured in complete McCoy's 5A media at concentrations of 2 × 10⁶ cells/ml.

DNA synthesis was determined by thymidine incorporation on all samples that were to be examined by electron microscopy. Lymphocyte cultures were pulse-labeled for 2 hr with [³H]thymidine (specific activity, 2.0 Ci/m mole; New England Nuclear, Boston, Mass.) at 0.5 μCi/2 × 10⁶ cells. Label was added to cultures at 24-hr intervals up to 144 hr after addition of NaIO₄ or PHA-P. At each 24-hr interval, replicate samples were processed for ultrastructural analysis by routine methods of fixation and embedding, and cell viability was determined by trypan blue exclusion.

RESULTS

In this study 7 normal and 4 CLL lymphocyte cultures stimulated with NaIO₄ produced peak levels of DNA synthesis and response profiles equivalent to those reported previously (20). Table 1 presents the response of 1 normal and 1 CLL lymphocyte culture to NaIO₄. These profiles are representative of all cell cultures used in this study. Thymidine incorporation into DNA in normal lymphocytesstimulated with NaIO₄ reached maximal values at 48 hr. This response was 70 to 90% of that obtained with PHA-stimulated cultures that reached peak activities 72 hr after PHA addition. However, thymidine incorporation into DNA of CLL lymphocytes stimulated with NaIO₄ was consistently greater than thymidine incorporation values obtained with PHA stimulation of the same donor. CLL lymphocytes reached maximal activities 96 hr after NaIO₄ exposure, while PHA-stimulated cultures demonstrated maximal activity at 120 hr. Both normal and CLL cell cultures stimulated with NaIO₄ were more than 85% viable throughout the 144 hr in culture.

The stimulation of normal and CLL lymphocyte cultures with PHA for various periods of time up to 144 hr induced the development of lymphocytoid blasts with ultrastructural characteristics of PHA-stimulated cells reported in previous studies (5, 12, 17). Fig. 1 shows a section of a normal lymphocytoid blast 72 hr after PHA (12) stimulation. Typically, this cell contains a prominent nucleus, a lobulated nucleus, swollen mitochondria, and decreased amounts of endoplasmic reticulum. Lymphocytoid blasts from a CLL culture stimulated with PHA for 120 hr are shown in Fig. 2. Their appearances are typical of CLL cells in culture 120 hr after PHA exposure and similar to those that have been described by others (12, 17). The extent of differences in blast ultrastructure between normal and CLL cultures stimulated with PHA increased with the length of time cells were maintained in culture and varied from one CLL donor to another. Yet, comparisons of normal and CLL lymphoblasts from cultures containing PHA for 72 and 120 hr, respectively (times of peak DNA synthesis), produced blast cells with the general appearances of those shown in Figs. 1 and 2. Two structural characteristics of CLL blasts that differed from normal blast morphology, and that were common to all leukemic cultures, included reduced blast cell diameters and smaller mitochondria containing larger numbers of cristae (Figs. 1 and 2). Plasmacytoid blasts were not observed in either normal or CLL cultures at times of peak DNA synthesis, although exposure of normal and CLL lymphocytes with PHA for longer time intervals did result in plasmacytoid development.

Both lymphocytoid and plasmacytoid blasts were observed in normal and CLL lymphocyte cultures stimulated with NaIO₄. Fig. 3 (electron micrograph) illustrates the effect of NaIO₄ in normal cultures 48 hr after stimulation. This blast cell has the general appearance of lymphocytoid development, with the exception that it is smaller and does not contain swollen mitochondria characteristic of PHA-stimulated lymphocytes (see Fig. 1). Fig. 4 is a section of a plasmacytoid blast from a normal lymphocyte culture 120 hr after NaIO₄ exposure. The increased amount of endoplasmic reticulum and the presence of dilated RER indicates that this cell was actively producing protein. More plasmacytoid blasts were observed in random sections of normal

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<th>Table 1</th>
<th>Response of normal and CLL lymphocyte cultures to NaIO₄ stimulation</th>
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<td>Normal</td>
<td>[³H]Thymidine incorporation into DNA (dpm/10⁶ lymphocytes × 10⁻⁶) at following Hr in culture after NaIO₄ treatment</td>
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<td>Normal</td>
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cultures 120 hr after NaIO₄ exposure than in samples examined during peak activities at 48 hr.

The ultrastructural analysis of CLL cultures stimulated with NaIO₄ revealed lymphocytoid and plasmacytoid blasts, but a number of blasts possessed very unusual ultrastructural morphologies (Figs. 5 to 10). Figs. 5 and 6 illustrate the tremendous increase in surface area of nuclei in cells from cultures 96 hr after stimulation. The nucleus sectioned in Fig. 5 displays elaborate invaginations of the nucleus, presumably due to an increase in nuclear membrane production. The shape of the nucleus in Fig. 6 also shows narrow projections of nuclear material surrounding small amounts of cytoplasm. However, contiguous thin sections of these nuclei indicate that the nuclear projections are narrow walls of nuclear material formed by concave invaginations of nuclear surfaces. Sections of CLL cells stimulated with NaIO₄ for only 24 and 48 hr did not reveal such elaborate changes in nuclear morphology but did show cells producing increased quantities of nuclear membrane (Fig. 7). Not shown here are many sections of other cells displaying whorls of membrane in the cytoplasm that were not associated with nuclear chromation. These observations indicate that increased nuclear membrane production precedes nuclear invagination. This conclusion is supported by the observation that cells displaying nuclear invagination contain nuclei with diameters equal to or greater than nuclei with normal blast morphologies. Thus, increased surface areas and increased quantities of nuclear membrane are characteristic features of invaginated nuclei.

Another interesting feature of the nuclear invagination process in CLL cells was observed by serial sectioning. Fig. 8 and the insert are noncontiguous sections of the same cell illustrating the localization of nuclear invagination to only a part of the nuclear surface. The cytoplasmic organelles of this cell are also organized near the site of nuclear invagination (Fig. 8), and few organelles are located in other areas of the cytoplasm (Fig. 8, inset). Extensive random sectioning of NaIO₄-treated CLL lymphocytes indicated that invagination of nuclei and organization of cytoplasmic organelles occurred in areas of cells containing centrioles and cytoplasmic microtubular complexes. Fig. 9 illustrates the close proximity of microtubules to the outer surface of invaginating nuclei.

The lymphocytes sectioned in Fig. 10 were from a CLL culture stimulated for 96 hr, but [³H]thymidine incorporation indicated that these cells exhibited less DNA synthesis than usually observed. Despite the low response, an increased number of cytoplasmic projections markedly increased cell surface areas and cell to cell contact. Increases in cell contact (Fig. 10) may be associated with increased phagocytosis observed in NaIO₄-stimulated cultures (1). However, Figs. 10 and 11 can only imply phagocytic activity until appropriate uptake experiments with latex spheres can be performed. Increases in plasma membrane surface area were observed in rapidly dividing CLL cultures, but these ultrastructural features were much more obvious in CLL cultures that had a lower response to NaIO₄.

A quantitation of the number of CLL blasts exhibiting extreme nuclear invagination can only be estimated because of the localization of this morphological feature to specific areas of cells. In random sections, 10 to 15% of all cells showing the morphological characteristics of stimulated cells exhibited nuclear invagination and organization of cytoplasmic organelles at cell centrosomes. The electron microscopic analysis of normal cultures did not reveal any blasts with the unusual morphological features demonstrated in all 4 CLL cultures and shown in Figs. 5 to 8. Furthermore, our investigations of ultrastructural changes in normal lymphocytes stimulated with NaIO₄ or CLL lymphocytes stimulated with PHA or pokeweed mitogen have not revealed the formation of a structured feature resembling those illustrated in Figs. 5 to 8 (unpublished data).

DISCUSSION

Stimulation of normal and CLL lymphocyte cultures with NaIO₄ produced blast cells with ultrastructural features of both lymphocytoid and plasmacytoid cells. Thus, our morphological data support other studies (1, 7, 20) that have identified NaIO₄ as a mitogen of both T- and B-lymphocytes. We have also observed blasts in NaIO₄-stimulated CLL cultures that possess ultrastructural morphologies unlike blasts we have seen in normal or CLL lymphocyte cultures stimulated with plant lectins. These NaIO₄-responsive blasts are characterized by increased amounts of nuclear membrane and extreme nuclear invagination. Since short-term lymphocyte cultures from 4 different CLL patients did not exhibit dilated RER or other ultrastructural characteristics of plasmacytoid development in blasts exhibiting nuclear invagination, it is uncertain whether the unusual NaIO₄-responsive cells we have observed are lymphocytoid or plasmacytoid blasts.

Our observations also show that areas of nuclear invagination in CLL blasts are adjacent to centrosomes of cells containing centrioles and cytoplasmic microtubular complexes (15, 23). In addition, the ultrastructural morphology of blasts with nuclear invaginations was further enhanced by localization of cytoplasmic organelles near sites of nuclear invagination. In other cell types, it has been demonstrated that the movement and localization of organelles is controlled by the assembly and disassembly of microtubules (19, 21, 24), but further experiments are necessary before we can relate location of organelles with the development of cytoplasmic microtubules in NaIO₄-stimulated lymphocytes. The consistent occurrence of nuclear invagination at centrosomes of NaIO₄-stimulated CLL lymphocytes and the presence of numerous microtubules adjacent to areas of nuclear invagination may offer a morphological method for the investigations of the possible interaction of microtubules between plasma and nuclear membrane during lymphocyte blastogenesis. In studies of lymphocyte transformation by plant lectins, Yahara and Edelman (25) have presented evidence that suggests that cytoplasmic microtubules interact with lectin receptors on plasma membranes and serve as mediators of lymphocyte stimulation. If microtubules are necessary for lymphocyte stimulation and subsequent nuclear invagination, inhibition of microtubule formation in these CLL cells may prevent nuclear membrane proliferation and nuclear invagination.
Finally, the ultrastructural features we have observed in some CLL lymphocytes have been compared with the ultrastructural changes described in blood cells from patients with other cancers (3, 9, 10, 14). Clausen and von Haam (9) demonstrated nuclear projections or blebs in neutrophils of patients with bronchogenic carcinoma. What they described as blebs or nuclear projections of heterochromatin is similar to areas of nuclear invagination in NaIO4-stimulated lymphoblasts. However, the blebs of nuclear material in their abnormal neutrophils were much less extensive and randomly oriented on nuclei surfaces. Ahearn et al. (3) have described nuclear bleb formation in human bone marrow granulocytes of patients treated with cytoxan arabinoside, and nuclear abnormalities similar to those described by Clausen and von Haam have been observed in lymphoblasts from Burkitt lymphoma by Dorfman (10) and Epstein and Achong (14). In each of these studies, the demonstration of nuclear abnormalities was performed by direct observation of cells as they existed in primary culture or in situ. Nuclear blebing in granulocytes and lymphoblasts from individuals with leukemia, bronchogenic carcinoma, and Burkett’s lymphoma is considered to be a morphological expression of cellular abnormality occurring as a result of disease processes.

The ultrastructural similarities between nuclear blebing and nuclear invagination implies that these 2 processes share similar pathogenic transformations. In the case of CLL small lymphocytes, this abnormal cellular event is not expressed until their transformation occurs via NaIO4 stimulation. Increased DNA synthesis activities and viabilities of greater than 85% in NaIO4-treated CLL cultures argue against the possibility that nuclear invagination is a toxic reaction to stimulation rather than a morphological expression of cellular abnormality. Furthermore, we have not observed nuclear invagination in normal lymphocytes stimulated with NaIO4 or in CLL lymphocytes stimulated with PHA or pokeweed mitogen. Thus, NaIO4 may stimulate a subpopulation of abnormal CLL lymphocytes that are not stimulated with plant lectins, or a subpopulation of CLL lymphocytes may develop into morphologically abnormal blasts in response to a particular mechanism of NaIO4-induced blastogenesis which is, as yet, unknown.

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REFERENCES

Leukemic Lymphocytes Stimulated with Sodium Periodate

Fig. 1. Normal lymphocytoid blast 72 hr after PHA stimulation. × 8,090.
Fig. 2. Typical lymphocytoid blasts from a CLL culture stimulated with PHA for 120 hr. × 5,620.
Fig. 3. Lymphocytoid blast produced by NaIO₄ stimulation of a normal culture for 48 hr. This section reveals a portion of the cell centrosome containing a centriole (C) with associated cytoplasmic microtubules, a Golgi apparatus (G), mitochondria (M), multivesicular bodies (MV), and lysosome-like bodies (arrows). × 15,800.
Fig. 4. Plasmacytoid blast from a normal lymphocyte culture 120 hr after NaIO₄ treatment. Dilated RER (arrows) is abundant in this thin section containing cytoplasm and a small portion of the nucleus. × 9,570.
Figs. 5 to 11. Sections of CLL lymphocytes from cultures stimulated with NaIO₄.
Fig. 5 and 6. Blasts exhibiting nuclear invagination 96 hr after stimulation. The projections of nuclear material that appear to surround areas of cytoplasm (arrows) are interpreted as cross-sections of walls of concaved pockets on the nuclear surfaces. Nuclear invagination occurs only on discrete areas of nuclei adjacent to areas of cytoplasm containing increased numbers of cytoplasmic organelles. Fig. 5, × 15,170; Fig. 6, × 17,580.
Fig. 7. CLL lymphocyte 24 hr after NaIO₄ exposure showing loops of nuclear membrane (arrows) extending into the cytoplasm. Microtubules (M) and microfilaments (MF) are closely associated with the outer surface of the nucleus. × 34,290.
Fig. 8. Two sections of a CLL blast illustrating the localization of nuclear invagination at the centrosome. A centriole in cross-section is at the arrow. Another section of the same cell (inset) did not reveal nuclear abnormalities and was devoid of cytoplasmic organelles. × 11,900; inset, × 3,870.
Fig. 9. Higher magnification of another NaIO₄-induced blast illustrating the close proximity of cytoplasmic microtubules to portions of the nucleus showing invagination. The microtubules at arrows are seen in longitudinal and cross-section and a centriole is at C. × 38,750.
Fig. 10. CLL lymphocytes from a 96-hr culture. Low levels of thymidine incorporation and the absence of ultrastructural changes characteristic of blastogenesis indicated this culture was only slightly responsive to NaIO₄ stimulation. Note the increased plasma membrane surface area and cell to cell contact of these cells. × 4,390.
Fig. 11. Lymphocyte with phagocytic vacuole from a culture 96 hr after NaIO₄ exposure. × 7,470.
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