Comparison of Histone Variant Synthesis in Human Lymphocytic Leukemia Cells and in Normal Lymphocytes

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Abstract

The synthesis of core histone variants and of histone H1 variants was determined in fresh leukemic cells of eight patients with leukemia [seven acute lymphoblastic (ALL) and one chronic lymphocytic (CLL)], in normal lymphocytes from healthy donors or from ALL patients in complete remission. Histone variant synthesis was evaluated by incubating cells with [3H]Lys and [14C]Arg in medium without Lys and Arg and then by two-dimensional polyacrylamide gel electrophoretic separations (acetic acid-urea-Triton x-100 acetic acid-urea-hexadecyltrimethylammonium bromide for core histone variants; sodium dodecyl sulfate/acetic acid-urea-hexadecyltrimethyl ammonium bromide for H1 variants). As previously reported, quiescent lymphocytes and lymphocytes stimulated with phytohaemagglutinin (PHA) showed clearcut changes in the proportions of synthesis of core histone variants and H1 variants. Leukemic lymphocytes freshly obtained from blood showed a pattern of core histone synthesis and H1 synthesis intermediate between that of quiescent and PHA-stimulated lymphocytes; this is probably due to the presence of a mixture of resting and growing cells. When leukemic cells were stimulated to grow by mitogens, the pattern of core histone and H1 variant synthesis was similar to that in mitogen-stimulated normal lymphocytes. Histone variants whose synthesis is associated with the S-phase were not synthesized in leukemic cells treated with the DNA synthesis inhibitors hydroxyurea and l-/3-D-arabinofuranosylcytosine (Ara-C). The pattern of acetylation of histone H4 was also apparently similar in leukemic cells and normal lymphocytes.

The radioactivity associated with the ubiquitinated forms of H2A increased in non-growing lymphocytes and in leukemic cells treated with DNA synthesis inhibitors whereas they decreased after mitogenic stimulation. Variability was wide in the synthesis of ubiquitinated H2A in different cases of leukemia.

The only clear-cut difference between leukemic cells and normal lymphocytes was that leukemic cells from ALL patients, but not lymphocytes from normal donors or from ALL patients in complete remission, synthesized appreciable amounts of H1*, increasing after hydroxyurea/Ara-C treatment and decreasing after PHA-stimulation. In leukemic cells from a CLL patient H1* synthesis was undetectable.

Introduction

Nucleosomes, the repetitive structural units of eukaryotic chromatin, contain about 200 base pairs of DNA tightly complexed with an octamer of core histone molecules and more loosely associated with the linker histone H1 (1, 2). Primary structure variants have been detected for core histones (H2A, H2B, H3, and H4) and for H1 by gel electrophoresis systems (3, 4). The separation of histone variants and their modified forms has been much improved by using 2D-PAGEgel electrophoresis (5–7).

The relationship between histone and DNA synthesis is now well established (7–12). Though most histone synthesis occurs in S-phase of the cell cycle (7), during the quiescent (G0) state and during the G1 phase a significant amount of histones is synthesized (8–11). In several cell type studies the synthesis pattern of core histone variants differs in S-phase, G1, or quiescent cells. Three H3 variants are synthesized in S-phase; in G0 and G1 cells H3.3 is the only H3 variant synthesized. It is therefore possible to distinguish between cells in G0/G1 and S-phases only on the basis of the H3 variant synthesis pattern (9). The synthesis pattern of H2A variants is similar in G0 and S-phases, with the expression of all H2A variants (H2A.1, H2A.2, H2A.x, and H2A.z). Cells in G1 have a lower H2A.1 + H2A.2/H2A.x + H2A.z ratio than G0 or S-phase cells (10).

The H1 synthesis patterns in quiescent and G1 cells show three H1 isoprotein species, H1.1, H1.2, and H1.3; in S-phase cells two other H1 variants, namely H1.4 and H1.5, are synthesized (12). H1* is an additional H1 subtype which is generally expressed more in better differentiated (13–15) or in low proliferating cells (16). Lymphocytes (T, B, and LGL) of many animal species studied (12, 17) do not synthesize any appreciable amount of H1*, which is instead present in all other non-proliferating cell types studied so far (12, 17, 18). Except for the absence of H1*, the histone synthesis pattern of unstimulated lymphocytes is as described for other quiescent cells (11, 12).

Limited attention has been paid so far to the study of possible differences in chromatin structure between normal and tumor cells. How DNA synthesis and histone variant synthesis are coordinated is still unknown, but some very precise mechanism of control must be postulated (19). As malignant transformation is essentially characterized by an abnormal control of proliferation, in neoplastic cells the mechanism governing the regulation of DNA and histone synthesis may well be altered. In addition it has been reported that neoplastic cells can synthesize embryonal histone variants (20), not present in the normal tissue they originate from. Not only the synthesis of histone variants but also their posttranslational modifications (e.g., acetylation) have been proposed as playing a role in the regulation of cell growth and differentiation (21).

In this study we compared the synthesis of core histone variants, H1 variants and H1* in freshly isolated leukemic cells and in normal lymphocytes (from healthy donors or leukemic patients in complete remission) and investigated how inhibition or stimulation of DNA synthesis affects histone variant synthesis in these cells. The acetylation of histone H4 and the ubiquitination of histone H2A was also investigated.

Materials and Methods

Patients. Table 1 summarizes the main clinical features of eight cases of human leukemia studied: five cases of T-ALL, one common ALL, one B-ALL and one T-CLL. Patients suffering from acute leukemia ranged in age between 3 and 17 years. The patient with T-CLL was 82
RESULTS

Fig. 1 shows the fluorographic picture of a 2D (AUT-AUC) gel electrophoresis of HCl cellular extract, as an example of the pattern of core histone synthesis in human normal lymphocytes and lymphoblasts taken from a patient suffering from ALL. The pattern of core histone synthesis in quiescent lymphocytes was typically the following (Fig. 1A): only the H3.3 variant was synthesized, without any appreciable synthesis of H3.1 and H3.2 variants. Only two forms of H4, b1 (monoacetylated) and b2 (diacetylated), were detectable in appreciable amount. Synthesis of H2A and H2B was low but detectable in quiescent lymphocytes.

Forty-eight h of PHA mitogenic activation of DNA synthesis produced an enhancement of histone synthesis, particularly a dramatic switch in the H3 synthesis pattern (Fig. 1B): all three variants of H3 were synthesized with H3.2 predominating. Four forms of H4 were expressed: the unmodified form b0, the monoacetylated b1, the diacetylated b2, the triacetylated form b3. An example of the core histone synthesis pattern in leukemic cells taken from a patient suffering from ALL (case 1) is illustrated in Fig. 1, C–E. A histone synthesis pattern between the G0- and S-phase pattern was found in fresh leukemic cells (Fig. 1C), consistent with the presence of a small proliferating cellular fraction; three variants of H3 were synthesized but H3.2 was not the most important, as in activated PBL (Fig. 1B). All forms of H4 were expressed, but b2 clearly much more than in PBL (Table 2) but their values were relatively low, between 1.9 and 15.4.

<table>
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<tr>
<th>Case No.</th>
<th>Sex</th>
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<th>Diagnosis</th>
<th>Chemotherapy</th>
<th>WBC (no./µl)</th>
<th>Blasts (%)</th>
<th>%G0/1</th>
<th>%S</th>
<th>%G2-M</th>
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<td>F</td>
<td>82</td>
<td>T-CLL</td>
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<td>90</td>
<td></td>
<td>1.5</td>
<td>3.9</td>
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Table 1 Clinical features of the eight cases of lymphocytic leukemia investigated

Table 2 Flow cytometric analysis

YEARS OLD. Cases 1, 4, 6, 7, and 8 were never previously treated with antineoplastic agents.

Cell Culture. PBL and leukemic cells were obtained from normal donors and leukemic patients by centrifugation on Ficoll-Hypaque gradient. Cells were incubated overnight at 37°C in culture medium RPMI 1640 containing 10% fetal calf serum. PBL and leukemic cells were stimulated to proliferate with 1 µg/ml of PHA (Wellcome Research Limited, Beckenham, England) 48 h before labeling.

Measurement of Histone Synthesis. To measure the synthesis of core histone and H1 variants PBL and leukemic cells were incubated, for a labeling time of 2 h at 37°C, in lysine- and arginine-free RPMI 1640 medium supplemented with 10% fetal calf serum, 5 µCi/ml L-[U-¹⁴C]lysine (348 mCi/mmol) and 10 µCi/ml L-[2,3,4,5⁻³H]arginine (52 Ci/mmol), (Amersham International, Bucks, England).

To inhibit DNA synthesis 1 mM HU and 10 µMArs-C were added 0.5 h before labeling, then left for 2 h.

Histone Extraction. Whole cells were extracted with 0.5 N HCl containing 1% β-mercaptoethanol and 1 mM phenylmethylsulphonyl fluoride (Sigma Chemical Company, St. Louis, MO) at 4°C overnight, in a volume of 200–300 µl for 10⁵ cells. After microcentrifugation (8000 × g) for 5 min at 4°C the supernatant was frozen and lyophilized.

2D-PAGE and Determination of Radioactivity. The 2D-PAGE system was prepared according to the method of Bonner et al. (22). The first dimension consisted of AUT gel for core histone variant separation and sodium dodecyl sulfate gel for the resolution of H1 isoforms. In both cases the second dimension was an AUC gel.

The 2D gels were prepared for fluorography by the methods of Bonner and Laskey (23) and Laskey and Mills (24); the gel was then dried and exposed to Kodak X-Omat AR film at –80°C for a period ranging from 24 h (for PBL stimulated with PHA) to 2 months (for quiescent PBL). A frame of radioactive ink (which comes out on the film) was drawn on the dried gel to ensure the precise placing of the gel on the film and made possible accurate excision of the spots corresponding to histone variants.

The excised spots from the gels were digested overnight at 37°C in 1 ml hydrogen peroxide-ammonium hydroxide mixture (95 parts 30% H₂O₂ and 5 parts of concentrated NH₃) in a scintillation vial. Ten ml acidified Aquassure scintillation liquid (NEN Research Products; Dreieich, West Germany) were added to each vial and each sample was counted for 10 min. Pieces of gels which were negative at fluorography in both cases the second dimension was an AUC gel.

Flow Cytometric Analysis. DNA index and the proportion of cells in different phases of the cell cycle; three variants of H3 were synthesized but H3.2 was not the most important, as in activated PBL (Fig. 1B). All forms of H4 were expressed, but b2 clearly much more than in PBL (Table 2) but their values were relatively low, between 1.9 and 15.4.

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In leukemic cells the percentage of cells synthesizing DNA was higher than in PBL (Table 2) but their values were relatively low, between 1.9 and 15.4.

RESULTS

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Forty-eight h of PHA mitogenic activation of DNA synthesis produced an enhancement of histone synthesis, particularly a dramatic switch in the H3 synthesis pattern (Fig. 1B): all three variants of H3 were synthesized with H3.2 predominating. Four forms of H4 were expressed: the unmodified form b0, the monoacetylated b1, the diacetylated b2, the triacetylated form b3. An example of the core histone synthesis pattern in leukemic cells taken from a patient suffering from ALL (case 1) is illustrated in Fig. 1, C–E. A histone synthesis pattern between the G0- and S-phase pattern was found in fresh leukemic cells (Fig. 1C), consistent with the presence of a small proliferating cellular fraction; three variants of H3 were synthesized but H3.2 was not the most important, as in activated PBL (Fig. 1B). All forms of H4 were expressed, but b2 clearly much more than the others.

When DNA synthesis was shut down, after 2.5 h of 1 mM HU and 10 µM Ara-C treatment, the only H3 variant synthesized in leukemic cells (Fig. 1D) or in normal lymphocytes was H3.3 (data not shown). After PHA activation of cell proliferation (Fig. 1E) leukemic cells showed H3 and H4 synthesis patterns similar to PHA-activated normal PBL.

The synthesis of histone H2A subtypes (H2A.1, H2A.2, H2A.x, and H2A.z) and histone H2B was invariably evident in untreated (C), HU/Ara-C treated (D) or in PHA-stimulated (E) leukemic cells. The synthesis of ubiquitin adducts of H2As was visible in normal PBL and leukemic cells. The pattern of core histone synthesis of PBL from patient 1 after complete remission (F) was the same as in normal donor PBL.

H3 variant synthesis ratios are reported in Table 3. The (H3.1 + H3.2)/H3.3 ratio was very variable (range, 0.6–3.9) in the different leukemic cells investigated. The lowest value was found in the case of T-ALL (case 8). When leukemic cells were exposed to DNA synthesis inhibitors (HU and Ara-C) the (H3.1 + H3.2)/H3.3 ratio dropped steeply, while it increased after 48
Fig. 1. Synthesis of core histone variants in normal lymphocytes and in lymphoblastic cells taken from ALL patient 1. PBL and leukemic cells were purified from peripheral blood as described in "Materials and Methods." Cells were labeled for 2 h with 10 μCi/ml [3H]arginine and 5 μCi/ml [14C]lysine in arginine- and lysine-free RPMI 1640 medium, supplemented with 10% fetal calf serum. PHA-activated PBL and leukemic cells were incubated with 1 μg/ml of PHA 48 h before labeling. Leukemic cells were treated with HU (1 mM) and Ara-C (10 μM) 0.5 h before labeling and during the labeling time. Histones were HCl extracted from whole cells and separated on AUT/AUC 2D gel system. Fluorograms are: A, normal quiescent PBL; B, PHA-activated normal PBL; C, leukemic cells; D, leukemic cells treated with 1 mM HU and 10 μM Ara-C; E, PHA-activated leukemic cells; F, PBL from patient 1 after 7 months of complete remission. Dotted lines, position of the missing variants; solid lines, variants which were visible on the fluorograms.

Table 3 H3 variant synthesis ratios (H3.1+H3.2/H3.3)

H3 synthesis patterns were studied in normal PBL and in leukemic cells from eight patients. Histone extraction and separations were performed as described in Fig. 1. To quantitate histone synthesis, individual spots corresponding to the H3 variants were cut from the dried gel and dissolved in a mixture of 95 parts of 30% H2O2 and 5 parts of concentrated NH3. Ratios were obtained from the amount of radioactivity incorporated in each spot (as [3H]arginine).

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<tr>
<th></th>
<th>+HU/Ara-C</th>
<th>+PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>0.6</td>
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</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>1*</td>
<td>0.2</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>4</td>
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<tr>
<td>4*</td>
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</tr>
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<td>0.6</td>
</tr>
<tr>
<td>8</td>
<td>0.6</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* PBL obtained after complete remission.

Table 4 Histone uH2A synthesis (% of total H2A synthesis)

The synthesis of uH2A and H2A were studied in normal PBL and in the 8 cases of leukemia. Core histones were separated on AUT/AUC 2D-PAGE. The spots corresponding to uH2As or to H2As were processed as described in Table 3.

<table>
<thead>
<tr>
<th></th>
<th>+HU/Ara-C</th>
<th>+PHA</th>
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<tbody>
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<td>37.2</td>
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</table>

* PBL obtained after complete remission.

Fig. 2 (A–C) reports a typical H1 synthesis pattern of normal PBL; the three H1.1, H1.2, and H1.3 isoprotein variants were expressed in resting PBL (A) and after HU and Ara-C treatment (B). Synthesis of two other variants H1.4 and H1.5 was evident after PHA-activation of DNA synthesis (C); H1° synthesis was not detectable in quiescent PBL, in quiescent PBL after HU and Ara-C treatment (B) or in PHA-activated PBL (C).

In leukemic cells of ALL patients at least three H1 subtypes plus H1° were synthesized (see as an example case 1, Fig. 2D). H1° synthesis, in contrast to normal PBL in which it was not detectable, increased relatively to other H1s after HU and Ara-
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In normal lymphocytes the patterns of H3 and H1 subtypes were similarly coupled to DNA synthesis: H3.1 and H3.2 are synthesized concomitantly with H1.4 and H1.5. A similar association was found in leukemic cells of six out of eight cases investigated.

Two cases presented some discrepancies: in case 1 no H1.4 or H1.5 was detectable whereas all the H3 subtypes were expressed (compare Fig. 2D and Fig. 1C). Instead, in case 4 H1.4 and H1.5, but not H3.1 and H3.2, were synthesized (Fig. 3, A and B).

DISCUSSION

Many recent studies, aimed at evaluating differences between normal and neoplastic cells, have focused on DNA alteration and on the altered expression of some genes (e.g., oncogenes). Not much attention has been given so far to changes of chromatin structure or, particularly, to possible differences in histone proteins in transformed cells. This possibility is worth investigating considering that the expression of different histone variants and their posttranscriptional modifications have been recently proposed to play a crucial role in regulation of the cell cycle (21, 26). In the present study we investigated whether there are differences in histone subtype synthesis between normal and neoplastic cells (i.e., lymphocytes and lymphocytic leukemic cells). The pattern of histone variant synthesis in unstimulated lymphocytes found in the present study was the same as that previously described (11, 12), typical of quiescent cells (where the H3.3 variant but not H3.1 and H3.2 was synthesized; of HI only the three isoproteins H1.1, H1.2, and H1.3 were expressed). After mitogenic stimulation the synthesis pattern of histone variants changed markedly (all core histone variants were synthesized, the H3.2 variant being the dominant H3 species, and all five H1 isoproteins being synthesized).

Leukemic cells synthesized the same core histone and H1 variants as normal lymphocytes with a pattern generally inter-

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Table 5 H1 variant synthesis ratios (H1 (3+4+5)/(1+2))

<table>
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<th>Variant</th>
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<td>8</td>
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* PBL obtained 7 months after complete remission.
+ PBL obtained 16 months after complete remission.
+ PBL obtained 10 months after complete remission.

C inhibition of DNA synthesis (E). The mitogenic activation of cell proliferation (F) produced H1.4 and H1.5 isoprotein synthesis like in normal lymphocytes in addition to the basal pattern.

After mitogenic stimulation H1** synthesis was no longer detectable in leukemic cells. In contrast to ALL cells, in the only CLL case investigated H1** synthesis was undetectable. H1** synthesis was undetectable in PBL from patient 1 after 7 and 16 months of complete remission, and in patient 4 after 10 months of complete remission (data not shown).

Table 5 shows H1 variant synthesis ratios (3 + 4 + 5/1 + 2): in normal PBL and cases 1, 2, and 8 the ratio was 0.4 or lower; higher values were due to increased synthesis of H1.4 and/or H1.5 variants rather than to a decrease in the synthesis of H1.1, H1.2, and H1.3. As for H3 ratios, inhibition of DNA synthesis reduced H1 ratios through the reduction or total disappearance of H1.4 and H1.5 isoprotein synthesis. After mitogenic stimulation of cell proliferation there was enhanced expression of variants H1.4 and H1.5 with consequently higher H1 ratios in normal PBL and leukemic cells.
mediate between quiescent and proliferating cells. This might reasonably be due to the fact that only a portion of leukemic cells was cycling. Generally the H3 and H1 patterns were in good agreement, probably giving an adequate image of the proliferation index, as previously documented for normal lymphocytes (11, 12).

In two cases, however, there were some discrepancies, the S-phase associated variants of core and H1 histones being differently expressed (see cases 1 and 4). This might be due to imperfect regulation of the coupling of DNA synthesis with the synthesis of S-phase core histone variants and with S-phase H1 variants.

When leukemic cells were exposed to HU and Ara-C the synthesis of H3.1 and H3.2 and of H1.4 and H1.5 was abolished, thus giving a picture similar to that found for normal quiescent lymphocytes. On the other hand when leukemic cells were stimulated to grow with PHA the core histone and H1 patterns were again similar to those in activated normal lymphocytes.

Therefore the present study did not reveal profound differences between normal lymphocytes and leukemic cells in core histone and H1 variants (the exception of H1* will be discussed later). In addition the changes in the relative expression of histone variants caused by induction or inhibition of DNA synthesis were very similar in lymphocytes and leukemic cells.

Although we did not analyze in detail all the posttranslational modifications of histone proteins, the gel electrophoresis methods used enabled us to separate the acetylated forms of H4 and the ubiquitinated forms of H2A with good resolution. It was of interest to assess both these modifications comparatively in leukemic and normal lymphocytes since several reports have related them to the regulation of gene expression (21, 27–30).

Acetylation in particular has been related to cell differentiation (e.g., sodium butyrate was proposed as inducing differentiation by inhibiting the deacetylation of histone H4) (28) and histone ubiquitination with chromatin condensation (26). In leukemic cells and normal lymphocytes the radioactivity associated with the dimodified form of H4 was clearly related to the proliferation status. The radioactivity associated with the dimodified form of H4 was proportionally much higher in nonproliferating and HU/Ara-C treated cells than in proliferating cells. This finding has been already reported in other cell types (8, 31) and reflects the pattern of H4 processing (31). H4 is synthesized in cytoplasm as the dimodified form, then undergoes further processing in the nucleus before and after being deposited in chromatin. Although S-phase cells synthesize more H4, they deacetylate it much faster than G0 or G1 cells (9); thus the relative increase of radioactivity associated with the dimodified H4 after HU/Ara-C treatment may mainly reflect this difference in the rate of deacetylation.

In some cases the relative synthesis of uH2A was similar to that in normal lymphocytes, in other cases it was clearly greater. On inhibiting or stimulating DNA synthesis there was, respectively, an increase and a decrease in the synthesis of radioactive molecules of uH2A. It is known that ubiquitinated H2A is present in interphase (32) and is lost when chromatin is condensed in mitosis (26). Therefore it is not surprising that PHA stimulation caused a reduction in uH2A forms because of a higher proportion of cells entering mitosis. The increase of uH2A over H2A after DNA synthesis inhibition by HU and Ara-C treatment may be due to a decrease in H2A synthesis. Since the synthesis of ubiquitin is reportedly not inhibited by DNA synthesis inhibitors (33), radioactivity found in uH2A complexes is probably due in large proportion to ubiquitin synthesis.

A clear-cut difference found between ALL cells and normal lymphocytes concerns the synthesis of H1*. We previously reported that normal lymphocytes do not synthesize this histone protein (12, 17) whereas fresh leukemic cells do (34). The present study confirmed this and showed that, as for other cells which synthesize H1* (16, 35), there is an apparent inverse relationship between DNA synthesis and H1* synthesis which in fact became undetectable after stimulation of DNA synthesis. As previously discussed (34) H1* synthesis may play a role in the regulation of gene expression and it would therefore be attractive to characterize its synthesis and its function in different human lymphocytic leukemias. Further studies are also in progress to assess whether this protein can be used as a marker for early detection of relapse of ALL patients in complete remission.

REFERENCES


SYNTHESIS OF HISTONE VARIANTS IN HUMAN LEUKEMIC CELLS


Comparison of Histone Variant Synthesis in Human Lymphocytic Leukemia Cells and in Normal Lymphocytes

Cecilia Mannironi, Vincenzo Rossi, Andrea Blondi, et al.