Incorporation of Uridine-\(^{3}\)H and Sodium Acetate-\(^{14}\)C in Lymphocytes Derived from Normal and Leukemic Individuals

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SUMMARY

Patterns of isotope incorporation indicative of histone acetylation and RNA synthesis were studied in lymphocytes derived from patients with chronic and acute lymphoblastic leukemia and were compared to normal controls. Cultures with and without phytohemagglutinin stimulation were investigated. Statistically significant differences were observed between cultures of the leukemic patients and normal individuals but not between the two types of leukemia. Cells derived from leukemic patients incorporated both sodium acetate-\(^{14}\)C and uridine-\(^{3}\)H without phytohemagglutinin stimulation ("spontaneous"), while normal lymphocytes required the addition of phytohemagglutinin. This technique may prove useful in predicting the hematological relapse of leukemic patients currently in remission.

INTRODUCTION

Sequential activation and repression of genetic information may be the mechanism by which cellular and tissue differentiation is achieved (6), and the acetylation of nuclear histones may be one of the earliest indications of gene activation (1, 2, 4, 10—15, 19). Since it has been suggested that neoplasia may be a disease of differentiation (8, 16), investigations concerning gene activation in normal and neoplastic cells may prove to be highly informative. The incorporation of tritiated uridine into cells has long been used as an indicator of cellular RNA synthesis in a wide variety of systems, and this method is currently accepted as a routine procedure for such analyses. Additionally, the elegant biochemical investigations of Allfrey et al. (1) and Pogo et al. (12, 13) have shown conclusively that short pulses of sodium acetate-\(^{14}\)C taken up by lymphocytes are incorporated directly and almost solely into nuclear histones. Previously, we demonstrated cytological evidence of histone acetylation by autoradiography in normal human lymphocytes stimulated with PHA\(^{3}\) (9). The present investigations were designed to examine and compare the patterns of histone acetylation and RNA synthesis, measured by isotope incorporation, in cells derived from leukemic patients and normal individuals.

MATERIALS AND METHODS

A total of 28 individuals, 17 with CLL, 4 with acute lymphoblastic leukemia, and 7 controls (laboratory personnel), was studied. Initial diagnosis of the patients was made at the Roswell Park Memorial Institute, Buffalo, N. Y., and cells from all patients were studied prior to the initiation of chemotherapy regimens. Heparinized peripheral blood samples were allowed to settle by gravity for 2 hr at 37°. Following separation of the leukocytes, cultures (6 \(\times\) \(10^{6}\) leukocytes/ml) were initiated in Eagle's minimal essential medium (Spinner) with 20% fetal calf serum, pencillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml). Half of the cultures from each individual were stimulated with PHA (0.1 mg/ml; Burroughs-Wellcome, Inc., Tuckahoe, N. Y.), while the remaining half did not receive the mitogen.

Cultures were incubated at 37° for various periods of time ranging from 15 min to 60 hr. Each culture received a pulse of a radioactive isotope for the last 15 min of culture: either uridine-\(^{3}\)H, 2 \(\mu\)Ci/ml (Schwarz/Mann, Orangeburg, N. Y.; specific activity, 2000 mCi/mM) or sodium acetate-\(^{14}\)C, 5 \(\mu\)Ci/ml (New England Nuclear, Boston, Mass.). Three replicate cultures were initiated for each treatment per individual. After incubation the cells were centrifuged at 1000 rpm, and the supernatant was withdrawn, and the cell pellet was resuspended in single strength Earle's balanced salt solution. The cells were then washed 4 times in single strength Earle's balanced salt solution followed by fixation in 2 changes of 70% ethanol for 10 min each. Ethanol was used since it has been shown that more commonly used 3:1 methanol:acetic acid fixative removes 40 to 50% of the nuclear histones as opposed to a negligible amount removed with ethanol (7). Two to 4 drops of cell suspension were placed on a microscope slide (predipped in 70% ethanol) and passed through a flame for ignition.

 Autoradiography was performed by dipping the slides into prewarmed (40°) Kodak type NTB\(_3\) nuclear track emulsion and then allowing them to dry thoroughly. After drying, the slides were exposed for 14 days at 4° in light-tight boxes with silica gel as a drying agent. The autoradiographs were developed with Kodak D-19 developer and Rapid Fix at 18°.
Slides were stained with a buffered Giemsa stain, as described by Schmid (18), and dehydrated. Coverslips were mounted with Permount.

All slides were coded and scored "blindly" for the incorporation of isotope, visible as exposed silver grains. Only cells with 10 or more grains were considered labeled. At least 1000 cells/culture were scored, yielding a total of 3000 cells/treatment/patient. Statistical evaluation of the data was accomplished by a nested analysis of variance technique and a 3-way classification. The main effects of disease, PHA stimulation, and incubation times, as well as all possible interactions among these main effects, were tested for significance.

RESULTS

A striking difference in the rates of both sodium acetate-$^{14}$C and uridine-$^{3}$H incorporation was found between the cells derived from normal individuals and those from the leukemic patients. These differences between the normals and leukemias were statistically significant at $p = 0.01$ (uridine, $F = 24.06$; acetate, $F = 21.23$, d.f. = 2; 450). However, the differences in labeling patterns among the individuals within any disease group (e.g., acute lymphoblastic leukemia, CLL, or normals) or among the replicate cultures of any given individual were not statistically significant. Therefore, the data presented in Charts 1 and 2 represent the mean of each experimental group. Additionally, the differences in isotope incorporation between cultures with and without PHA stimulation for each group of subjects were also statistically significant (uridine, $F = 7.52$; acetate, $F = 3.13$, d.f. = 1; 450; $p < 0.01$).

Both isotopes were incorporated almost solely into the nuclei of the cells. The time course of isotope uptake during the 60-hr incubation period is depicted in Charts 1 and 2. PHA stimulation of normal lymphocytes resulted in a marked increase in the uptake of both sodium acetate-$^{14}$C and uridine-$^{3}$H (Charts 1a and 2a), while without PHA stimulation normal cells did not incorporate either isotope to any appreciable amount (Charts 1b and 2b). However, following PHA stimulation of normal cells, acetate incorporation was observed within 15 min, rising to a peak at 24 hr and then followed by a decline from 24 to 60 hr (Chart 1a). On the other hand, pronounced uridine incorporation in PHA-stimulated normal lymphocytes was not detected until 3 to 6 hr of culture and reached a peak at 48 hr with a subsequent decline to 60 hr (Chart 2a).

In contrast, the lymphocytes derived from both types of leukemic patients incorporated either isotope without PHA stimulation (Charts 1b and 2b). The greatest "spontaneous" isotopic incorporation into the leukemic cells occurred during early culture periods. At 15 min of culture, without the addition of PHA, approximately 66% of the cells from CLL patients and 74% of the cells from acute lymphoblastic leukemia patients were labeled by acetate in contrast of 4% of labeled cells in non-PHA-stimulated normal cells. Similar findings were observed for uridine incorporation. Subsequently, the rate of isotope incorporation in the cells of leukemic patients declined with lengthening time in culture. Although not statistically significant, this decline was more rapid in the cells derived from patients with CLL than in acute lymphoblastic leukemia cells (Chart 1). In the PHA-stimulated cultures of cells derived from the same patients, acetate incorporation declined from an initial level of 63% of labeled cells in CLL and 70% of labeled cells in acute lymphoblastic leukemia to 54% and 58%, respectively, by 12 hr in culture. Following this initial decline, the rates of incorporation began to increase, reaching a peak at 24 hr. This pattern was similar to that observed in normal PHA-stimulated cells (Chart 1a).

However, isotopic incorporation was not as great as the initial spontaneous rate in leukemic cells, i.e., without PHA stimulation (Chart 1b). This difference, measured by the "disease X PHA" interaction term in the analysis of variance, was significant at $p = 0.01$ ($F = 5.18$; d.f. = 2; 450).

The pattern of uridine incorporation was similar in the cells

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Chart 1. The rate of sodium acetate-$^{14}$C incorporation into lymphocytes derived from CLL patients and acute lymphoblastic leukemia (ALL) patients and normal individuals over a 60-hr incubation period. $a$, PHA added; $b$, no PHA.
of both types of leukemia (Chart 2) and differed significantly from that of the normal cells. A steady high rate of uridine incorporation was observed in leukemic cells without PHA stimulation for 36 hr of culture followed by a decline to 60 hr (Chart 2b). After PHA stimulation, however, uridine incorporation declined in the initial 12 hr of culture and then increased, reaching its peak at 24 to 36 hr, followed by a 2nd decline to 60 hr (Chart 2a).

DISCUSSION

Previous biochemical experiments have clearly demonstrated that sodium acetate-$^{14}$C in PHA-stimulated human lymphocytes is incorporated almost solely into the nuclear histone fraction of these cells (1, 12). Cytological and autoradiographic evidence of histone acetylation following sodium acetate-$^{14}$C incorporation has also been presented (2, 9). The addition of acetate during periods of active protein synthesis raises the possibility of its incorporation into molecules other than histones. For prevention of such incorporation, short “pulses” of isotope for the final 15 min of incubation were used. Previous experimentation with puromycin has shown that, following a 15-min pulse, sodium acetate-$^{14}$C is indeed incorporated into the histones and not into newly synthesized proteins (1, 9, 12).

PHA stimulation of normal lymphocytes significantly increases both histone acetylation and RNA synthesis during early periods of culture (4, 9, 12). In contrast, cells derived from leukemic patients showed a high rate of “spontaneous” sodium acetate-$^{14}$C incorporation without PHA stimulation, suggesting an active in vivo metabolic state for leukemic cells, which may represent a phase of dedifferentiation. Allfrey et al. (1) have suggested that histone acetylation and/or methylation may be one of the methods by which genes are regulated in the performance of their metabolic function and that therefore a defective acetylating system may lead to a loss of regulation. The incorporation of viruses into the genome of normal cells may induce such a defect which then allows “spontaneous” histone acetylation. Such an observation has already been described for scrapie virus infection of mouse brain, which increases the rate of histone acetylation in these cells (3). Furthermore, histone deacetylase, an enzyme of calf thymus, has recently been reported to prevent acetylation (5). In an abnormal metabolic system, such as exists in the neoplastic state, this enzyme may be affected in such a way that it may no longer regulate histone acetylation.

The decrease in incorporation of sodium acetate-$^{14}$C in leukemic cells following PHA stimulation is not clearly understood. However, it may be related to an abnormality in the immune mechanism of these cells, perhaps at the cell surface. The peak of sodium acetate-$^{14}$C incorporation of 24 hr may be expected, since it is conceivable that there is a small population of normal cells in leukemic patients which is stimulated by PHA and begins acetylation at this time, whereas the abnormal cells (spontaneously acetylating) maintain a high background. The decline in histone acetylation of leukemic cells between 24 and 60 hr may be due to death of such cells in culture. Since histone acetylation is one of the earlier indicators of gene activation and since a difference in the histone acetylation patterns of leukemic and normal cells has been observed, this procedure might prove useful in predicting hematological relapse of leukemic patients currently in remission.

ACKNOWLEDGMENTS

We are indebted to Dr. E. Z. Ezdinli for referring the patients, Dr. S. Addelman for statistical advice, and Dr. R. G. Davidson for critical reading of the manuscript.

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