METHYLATION OF TRANSFER RNA DURING TRANSFORMATION OF HUMAN LYMPHOCYTES BY PHYTOHEMAGGLUTININ*

Opendra K. Sharma and Lawrence A. Loeb†

Department of Microbiology, University of Colorado Medical Center, Denver, Colorado, and †The Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania

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SUMMARY: Methylation of transfer RNA during phytohemagglutinin induced transformation of human lymphocytes was studied by labeling the tRNA in vivo with (methyl-H^3)-methionine and measuring the distribution of tritium in the methylated nucleotides. An alteration in the pattern of methylation occurred within hours after PHA-stimulation and this change was maintained through several cell generations. There was a 50 to 94% increase in the relative amount of methylated N^2-methyl-guanine (1 to 3 hr) and a 40 to 59% decrease in the relative amount of 1-methyladenine (1 to 12 hr). Treatment of the stimulated cells with Actinomycin D prevented the subsequent methylation of tRNA. However, inhibition of protein synthesis by puromycin did not effect the early changes observed in the methylation of tRNA.

Human lymphocytes are specialized cells which seldom divide in vivo. However, they can be induced to divide in culture by the addition of phytohemagglutinin (PHA) and other mitogens as well as by appropriate antigenic stimuli. When stimulated, the small lymphocyte which contains a compact nucleus and very little cytoplasm, increases in size, replicates its DNA, and is "transformed" (over a period of 48 to 72 hr) into an actively dividing lymphoblast (1). Since these changes occur in culture, lymphocyte transformation has been studied as a model system for analyzing the nature of regulatory processes involved in the conversion of a resting cell into an actively dividing cell. An increase in RNA synthesis occurs immediately following stimulation by PHA (2). Protein synthesis increases at about 2 hr (3). DNA replication as measured by thymidine incorporation

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begins between 18 and 24 hr and reaches a maximum rate in about 2 to 3 days (4). We now report an alteration in the pattern of methylation of newly synthesized transfer RNA (tRNA) during the initial stages of PHA induced lymphocyte transformation.

Lymphocytes were isolated from human peripheral blood by the method outlined by Cooper (5). The final preparation was suspended at a concentration of 7.5×10^5 cells/ml in Eagles MEM (Spinner modification) (GIBCO, Grand Island, New York) with 20% foetal calf serum, 1% L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml). Five ml portions of the cell suspension were incubated in 30 ml Corex Tubes at 37°C in the absence or presence of 125 µl of PHA-M (General Biochemical Corp., Chagrin Falls, Ohio). At the times indicated, the cells were harvested by centrifugation at 1500 g for 15 min at 37°C. To each culture was added 0.2 mC of methyl-^{3}H methionine (Amersham/Searle, Sp. Act. 5.2 C/mmole) in 2 ml of balanced salt solution containing 0.02 M sodium formate (Cooper, H. L., personal communication). Incubation was for 2 or 3 hr at 37°C and the cells were isolated by centrifugation at 6,000 g for 10 min and stored at -70°C.

The time of exposure to methionine of 2 or 3 hr was selected so as to allow sufficient incorporation of the isotope into the tRNA. The radioactivity in tRNA represents methylation, per se, and not incorporation into the carbon rings of the bases via the 1-C pool since 5S RNA, which is known not to be methylated in other cells was found to be devoid of radioactivity. The distribution of the methylated constituents of tRNA after PHA stimulation is given in Table I. We find that within three hours after PHA stimulation there is an increase from 12.5% to 18.7% in the amount of label found in N^2 methylguanine and a decrease from 16.1% to 11.2% in 1-methyl adenine. This altered distribution is more pronounced 12 hr after PHA-stimulation and is found consistently in stimulated cells until the experiment terminated at 60 hr. The rate of incorporation of appropriate radio-

TABLE I

Percent Distribution of Methylated Constituents of tRNA Labeled with (Methyl-^{3}H) Methionine during Lymphocyte Transformation

Methylated Constituent	Control No PHA	Time after Stimulation by PHA 10 min	20 min	60 min	180 min	12 hr	40 hr	60 hr
Methylated Cytidylic Acids	26.0	24.9	23.1	24.7	25.3	26.7	27.2	26.2
Methylated Uridylic Acids	9.5	11.0	12.1	12.1	11.4	10.2	9.9	9.7
1 MeG + 7 MeG	20.4	19.4	18.1	20.4	18.2	15.5	15.2	16.2
N^2MeG	12.5	14.1	11.9	13.2	18.7	23.7	21.9	21.9
N_2^2 DiMeG	11.6	12.3	13.0	11.6	10.4	10.2	10.4	9.2
1 MeA	16.1	14.7	15.4	14.9	11.2	9.6	8.5	10.2
6 MeA	4.0	3.2	6.2	4.3	4.8	4.6	5.8	5.8
Total cpm/culture in tRNA	4,300	4,500	3,900	5,100	9,200	15,000	29,000	37,000

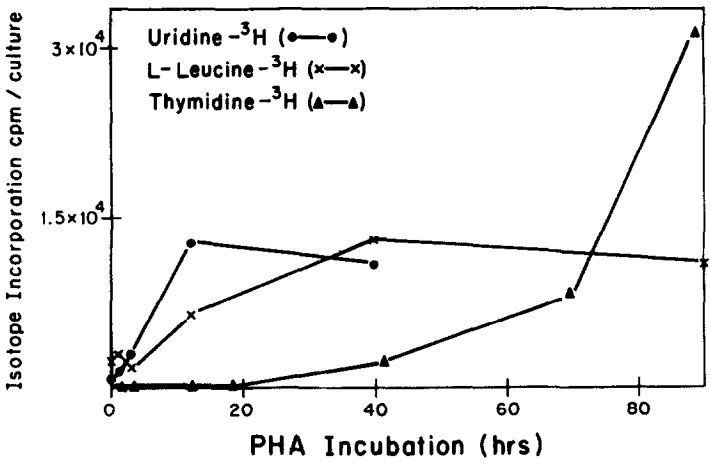

Figure 1. Changes in relative rates of RNA, protein and DNA synthesis during lymphocyte transformation. To cultures containing 1 ml of the cell suspension (7.5 x 10^5 lymphocytes) was added 50 µl of 0.15 M KCl containing 10 µC uridine-^{3}H, or 47 µC L-Leucine-^{3}H or 2.5 µC thymidine-^{3}H at the indicated time after stimulation by PHA. Incubation was for one hour at 37°C and incorporation was terminated by adding 10 µmoles of the corresponding unlabeled precursor. Incorporation into an acid-insoluble precipitate was determined as previously described (7).

Table 1. PHA-stimulated lymphocytes were cultured for the times indicated and then labeled for 3 hr with (methyl-^{3}H)-methionine. Cultures without PHA were incubated for 41 hr before exposure to methionine. For the isolation of tRNA, lymphocytes were homogenized in 2 ml of 0.1 M Tris HCl, pH 7.5 containing 1 M NaCl - 0.005 M EDTA and an equal volume phenol saturated with water. To the aqueous layer, 200 µg of E. coli B tRNA was added as carrier and the RNA was precipitated with 2.5 volume of 95% ethanol and 0.1 volume of 20% potassium acetate, pH 5.0. The RNA was dissolved in 0.1 M Tris, pH 7.5 and was loaded onto a DEAE cellulose column which was successively washed with 0.1 M Tris, 7.5 containing 0.1 NaCl, and then again with a similar buffer containing 0.3 M NaCl. The transfer RNA was finally eluted with 1 M NaCl and precipitated with 2.5 volumes of 95% ethanol. The RNA was incubated with 100 µg pancreatic deoxyribonuclease, free from ribonuclease (Worthington) in 0.1 M Tris HCl, 0.01 M $MgCl_2$, pH 7.5 at 37°C for 30 min, followed by 100 µg of Pronase (Calbiochem). The reaction mixture was deproteinized with an equal volume of phenol saturated with H_2O and then tRNA was precipitated from the aqueous layer with 2.5 volume 95% ethanol. The "stripping" of endogeneous amino acids was done by incubation in 1.8 M Tris HCl, pH 8.0 at 37°C for 90 min. All the radioactivity present in isolated tRNA could be rendered soluble in 5% trichloroacetic acid upon incubation with ribonuclease. The tRNA (50-75 µg) was hydrolyzed with 1 N HCl in a sealed Pyrex tube for 60 min at 100°C. The hydrolysate was dried in vacuo over KOH. It was taken up into 20 µl of water and spotted on a thin layer cellulose plate (Eastman Kodak), and the methylated constituents were separated by the procedure of Bjork and Svensson (6). The values have been expressed as percentage of radioactivity recovered and each sample was analyzed in duplicate. 1-methylguanine and 7-methylguanine were not adequately separated in most of the analyses, so they have been expressed collectively. The underlined values (____) are for emphasis. The abbreviations are: 1 MeG, 1-methylguanine; 7 MeG, 7-methylguanine; N^2MeG, N^2-methylguanine; N^2_2 DiMeG, N^2_2 Dimethylguanine; 1 MeA, 1-methyladenine and 6 MeA, 6 methyladenine.

active precursors into RNA, protein, and DNA was determined in parallel assays with lymphocytes from the same individual (Fig. 1). In these cells uridine incorporation into acid-insoluble material increases during the first few hours, while leucine incorporation starts to increase at a later time. In this experiment the increase in thymidine incorporation was not evident until 41 hr after stimulation. Thus, changes in methylation are an early event of lymphocyte transformation which coincides temporally with the increase in uridine incorporation.

The effect of Actinomycin D and Puromycin on the early alterations in the metabolism of tRNA during lymphocyte transformation is seen in Table II.

TABLE II

Effect of Puromycin on Transfer RNA Methylation During PHA-Stimulated Lymphocyte Transformation

Methylated Constituent	Control		Puromycin	
	-PHA	+PHA	-PHA	+PHA
	%	%	%	%
Methylated Cytidylic Acids	26.7	25.1	24.9	23.2
Methylated Uridylic Acids	9.5	9.1	10.7	10.1
1 MeG + 7 MeG	21.5	21.0	22.9	21.6
N^2 MeG	11.1	19.2	12.1	19.2
N_2^2 DiMeG	12.1	10.6	11.9	8.9
1 MeA	13.2	10.1	12.2	13.1
6 MeA	5.5	4.9	5.2	4.1

Culture conditions are detailed in text. With Actinomycin D less than 100 cpm was recovered in tRNA in either control or PHA-stimulated cultures.

Cultures of lymphocytes were incubated with Actinomycin D (0.06 μg/ml of culture) or puromycin (50 μg/ml of culture) or without an inhibitor for 30 min prior to the addition of PHA. The lymphocytes were then incubated for 3 hr with PHA, harvested, washed and labeled with (methyl-^{3}H)-methionine. Actinomycin reduced the rate of uridine incorporation into acid-insoluble material by greater than 90%, and prevented the methylation of tRNA in both PHA-stimulated and unstimulated cultures. These results suggest that the methylation occurs mainly on newly synthesized tRNA in both stimulated and unstimulated lymphocytes. In contrast, puromycin, in amounts sufficient to inhibit more than 95% of the leucine incorporation in PHA-stimulated cultures, did not affect the changes in tRNA methylation. These results suggest that the synthesis of new enzymes is not necessary for the early alterations in tRNA methylation.

Stimulation of human lymphocytes by PHA causes an increase in the rate of RNA synthesis and protein synthesis as well as alterations in the types of proteins synthesized. The change in rate of RNA synthesis as measured by the incorporation of radioactive uridine is not uniform for the different species of RNA. The species taking up the most label sediments at 4S and has been separated into different classes by gel filtration on Sephadex G-100 (8). Of these classes, the one most enriched with radioactive uridine was found not to be methylated and was considered a tRNA precursor. It is apparent from our studies that tRNAs synthesized during lymphocyte transformation are different, i.e. have different patterns of methylation, compared with tRNAs of unstimulated lymphocytes. Methylation of newly synthesized tRNA does not require synthesis of new enzymes, though the requirement for the synthesis of RNA is absolute. Riddick and Gallo (9) have reported an increase in the rate as well as in the extent of methylation of *E. coli* tRNA by extracts of human lymphocytes stimulated with PHA for 38 to 42 hr. Their results indicate that the changes in tRNA methylases were dependent on new RNA synthesis. By their procedures they were unable

to detect any change in methylases during early stages. This is in accord with our results which indicate that new synthesis of enzymes was not necessary for the methylation of tRNA during 1 to 3 hr after stimulation.

Alterations in tRNA methylation have been found in biological systems which are under regulatory control (10). From the studies of tRNAs in protein synthesis, some of the functions of methylated bases of tRNA are becoming clear. Methylation of tRNA is involved in amino acid acceptance (11), binding to ribosomes (12) and in codon response (13). It is not clear how the activity of tRNA methylases is regulated to enable them to achieve a different pattern of methylation. It is tempting to speculate, that the newly synthesized tRNA during 1 to 3 hr of contact with PHA with the altered distribution of methyl groups, behaves differently during the later stages of protein synthesis and exerts some controlling influence on subsequent events leading to lymphocyte transformation. It would be of interest to determine if similar changes in methylation of tRNA occur in other systems in which non-dividing cells are stimulated to enter a replicative phase.

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