Acetylation and Methylation of Histones in Pernicious Anemia

By Lawrence Kass

Acetylation and methylation of erythroid histones was investigated in two patients with pernicious anemia before and after treatment with vitamin B₁₂ in vivo. It was found that vitamin B₁₂ facilitated both acetylation and methylation of histones. Electrophoresis of amino acids obtained from histone hydrolysates prior to treatment with vitamin B₁₂ demonstrated that lysine was present as L-lysine. After treatment with vitamin B₁₂, most of the lysine appeared as methyl-L-lysine. Whether this biochemical lesion is specific for vitamin B₁₂ deficiency or also occurs in folic acid deficiency awaits future studies.

This report describes acetylation and methylation of histones in untreated pernicious anemia bone marrow cells after they have been exposed to vitamin B₁₂ in vivo.

MATERIALS AND METHODS

Sternal or iliac bone marrow was obtained in a heparinized glass syringe from two patients with severe untreated pernicious anemia. In both cases a sample was also obtained 18-20 hr after the initial treatment of the patient's pernicious anemia consisting of 1000 μg of vitamin B₁₂ (cyanocobalamin) given intramuscularly. Films of bone marrow flecks were made between methanol-cleaned glass cover slips and stained with Wright's stain for conventional light microscopic examination.

Since bone marrows were composed largely (80%-90%) of erythroid precursors and because the amount of material was limited by that obtained in a routine aspiration, no attempts to fractionate the bone marrow samples were made. The sample of bone marrow containing approximately 3-5 ml sinusoidal blood and 30-80 mg of bone marrow flecks was divided equally into two plastic disposable culture tubes (Falcon Plastics, Los Angeles). Into one tube were placed 5 μCi of tritium-labeled L-methionine, specific activity 2.6 Ci/Mmole. To the other tube 5 μCi of tritium-labeled acetic acid sodium, specific activity 1.3 Ci/Mmole (Schwarz-Mann, Orangeburg, N.Y.) were added. The samples of bone marrow containing the isotopes were incubated at 37°C for 1 hr, then in three successive washes of Hanks' solution (BBL, Cockeysville, Md.) for 30 min each. A solution of 3% saponin in 0.162 N NaCl was added to hemolyze erythrocytes, and the marrow particles were washed twice in Hanks' solution. Lysine-rich and arginine-rich histones were extracted from the bone marrow particles by methods described previously.1 Lysine-rich histones were extracted at pH 1.8 and arginine-rich histones at pH 1.0. Approximately 400 μg of total histone (arginine rich and lysine rich) was obtained. Total protein count was determined on the histone fractions, and radioactivity was counted in each as described previously.

It was essential to determine whether the 3H-sodium acetate was incorporated as acetyl groups attached to histone. Accordingly, histone powders containing tritium-labeled sodium acetate were treated with methanol-chloroform-ether 3:1:1 to remove lipids. Separate histone powders were boiled in 10% TCA (trichloracetic acid) for 30 min to remove nucleoproteins and polysaccharides.
The insoluble histone powders were recovered, washed in acetone, and dissolved in distilled water. Both the organic solvent-treated histone and TCA-treated histone were subjected to total protein determination and liquid scintillation counting as above. The results were compared with the original untreated histone powders. Because of the minute amount of material obtained in these acetate samples and the loss of sample after solvent and TCA extractions, further studies on them could not be carried out.

Methylation of histones was also evaluated in detail. Samples of histones labeled with 3H-L-methionine and extracted from vitamin B12-treated bone marrows were hydrolyzed for 24 hr in 6 N HCl and evaporated to dryness in a rotary evaporator. Approximately 300 µg of amino acids were recovered, and half of this material was used for each of two electrophoretic runs. Amino acid electrophoresis was carried out on Whatman No. 3 filter paper for 105 min in a high-voltage electrophoresis apparatus (Gilson Medical Electronics, Oberlin, Ohio) using Varsol (Enco Oil Company) and 6.2% formic acid, pH 2.0, as buffer at 4000 V, 175 mA for 85 min. Simultaneously in the electrophoresis, methylated derivatives of lysine and arginine were run as standards. These included N-epsilon-methyl-L-lysine hydrochloride, N⁰-monomethyl-L-arginine, N⁰,N⁰-dimethyl-L-arginine, and N⁰,N⁰-dimethyl-L-arginine, (Calbiochem, San Diego, Calif.). The electrophoretic strips were dried and stained with collidine stain (60 ml 0.5% ninhydrin, 240 ml ethanol, 100 ml acetic acid, and 4 ml 2,4,6 trimethylpyridine) to identify amino acid spots. A tritium probe (Eberline Co., Santa Fe, N.M.) was passed over each of the spots to detect radioactivity.

RESULTS

Prior to treatment with vitamin B12, Wright’s stained films of the bone marrows showed severe megaloblastic erythropoiesis. Proerythroblasts constituted approximately 50% of the marrows, and 30% of the marrow cells were large intermediate megaloblasts. The remainder were giant metamyelocytes, aberrant-appearing polymorphonuclear leukocytes, and megakaryocytes. Approximately 18–20 hr after treatment with vitamin B12, the proerythroblasts largely disappeared, and the marrows contained primarily early intermediate macronormoblasts.

Table 1 shows dpm per mg protein (disintegrations per minute per milligram protein) in the histone samples. This isotopic study shows striking uptake of both tritiated methionine and sodium acetate into histone fractions obtained from pernicious anemia bone marrows after they had been exposed to vitamin B12. This uptake ranged from threefold to as high as 30-fold over the untreated control samples. Incorporation of 3H sodium acetate was greatest in the arginine-rich (pH 1.0) fractions. It was approximately equal in both lysine-rich and arginine-rich fractions in the case of 3H-L-methionine. After treatment of the acetylated histones with either organic solvents or boiling TCA, greater than 83% of the radioactivity remained in both instances. This indicated specific

<table>
<thead>
<tr>
<th>Table 1. Disintegrations per Minute per Milligram Protein</th>
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<tbody>
<tr>
<td>pH 1.8</td>
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<tr>
<td>--------</td>
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<tr>
<td><strong>3H-sodium Acetate Labeled Histones</strong></td>
</tr>
<tr>
<td>pH 1.8</td>
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<tr>
<td>pH 1.0</td>
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<tr>
<td><strong>Control</strong></td>
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<tr>
<td>300,228 (a), 61,670 (b)</td>
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<tr>
<td>71,620 (a), 58,790 (b)</td>
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(a) and (b) represent two different patients.
pH 1.8, lysine-rich histone fraction; pH 1.0, arginine-rich histone fraction.
HISTONES IN PERNICIOUS ANEMIA

Fig. 1. Electrophoretic pattern of amino acids obtained from pH 1.8 erythroid histone (lysine rich) prior to vitamin B₁₂ therapy. The lysine in the sample (arrow) corresponds in location to the l-lysine standard (met, methyl-l-lysine standard). The electrophoretic pattern of amino acid at the bottom of the figure is a mixture of known amino acids.

incorporation of the labeled acetate radical into the histone molecule rather than into impurities such as lipids, polysaccharides, or nucleoproteins.

In histones extracted from untreated pernicious anemia erythroid precursors to which tritium-labeled l-methionine was added, the electrophoretic mobility of lysine in the predominately erythroid histone (both pH 1.8 and pH 1.0 fractions) appeared to coincide with the l-lysine standard (Fig. 1). After treatment with vitamin B₁₂, most of the lysine was found to have an electrophoretic mobility identical to the methyl-l-lysine standard (Fig. 2), and small amounts of

Fig. 2. Electrophoretic pattern of amino acids obtained from pH 1.8 erythroid histone 24 hr after treatment with cyanocobalamin. The majority of the lysine in the sample (arrow) corresponds in mobility to the methyl-l-lysine standard. Scant amounts of l-lysine were also observed in some instances. The electrophoretic pattern at the bottom represents a mixture of l-lysine and methyl-l-lysine as a reference standard.
l-lysine were usually observed. Using a tritium probe, small amounts of radioactivity were detected only in the methyl-l-lysine spot. The remainder of the amino acids identified by the electrophoresis did not appear to change in type or mobility after treatment with vitamin B₁₂.

**DISCUSSION**

These observations extend earlier cytochemical² and biochemical¹ studies of histones in pernicious anemia and indicate that lysine appears to have been methylated after exposure of the megaloblasts to vitamin B₁₂. To date, most of the available evidence in man³ and in bacteria⁴ indicates that those methylation reactions which involve vitamin B₁₂ also involve folate. In the present experiments, however, the relationship between vitamin B₁₂ and folate could not be examined because of the unavailability of patients with severe untreated folate deficiency.

Acetylation and methylation of histones have been studied by others using a variety of tissues. Currently, histone methylation is believed to occur prior to the combination of histones with DNA⁵ and to play a role in the regulation of gene transcription.⁶ Methylated arginine derivatives are formed in the process of histone methylation,⁷ and epsilon-N-methyllysine is formed by the transfer of methyl groups from methionine to the free epsilon amino group of lysine residues.⁸ Histone acetylation is believed to be involved in the activation of genes⁹,¹⁰ and acetyl groups have been found to be N-terminal substituents of lysine-rich calf thymus histone.¹¹ Histone acetylation precedes nucleoprotein synthesis,¹² and most of the ¹⁴C acetate in isotopic experiments has been recovered as epsilon-N-acetyl lysine in the arginine-rich fractions.¹²,¹³

At present the role of acetylation and methylation of histones in the pathogenesis of the megaloblastic lesion in pernicious anemia must remain speculative. Both vitamin B₁₂ and folate participate in methyl transfer reactions in a variety of animal and bacterial systems, and methyl B₁₂ is required for acetate synthesis in *Clostridium thermoaceticum*.¹⁴ Tidwell et al.¹⁵ have recently suggested that methylation of histones correlates with condensation of chromatin and curtailment of nucleic acid synthesis. In this regard, the delicately fenestrated chromatin of the megaloblast could result from several abnormalities including defects in DNA synthesis,¹⁶ decreased biosynthesis of arginine-rich histones,¹ and failure of acetylation and methylation of histones as shown in the present study. Since methylated histones are involved in functional and structural nuclear changes prior to mitosis,¹⁵ it is conceivable that the abnormal cellular divisions and aberrant mitotic figures often seen in untreated megaloblastic anemia erythroid cells may be due in part to failure of methylation of histones.

**ACKNOWLEDGMENT**

The assistance of Robert D. Farnsworth is acknowledged.

**REFERENCES**

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