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METHYLATION-LIKE REACTION OF [^{3}H-METHYL]-*N*-5-TRIMETHYLLYSINE (TML) TO CHROMATIN COMPONENTS

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Abstract—To elucidate the mechanism of action of *N*-5-trimethyllysine, an endogenous amino acid derivative with stimulatory action on cell proliferation, the binding of [^{3}H-CH_3]-*N*-5-trimethyllysine (TML) to DNA, histones and non-histones was investigated in NK/Ly ascites tumour cells. Radioactivity was detected in all of the chromatin components, particularly in the non-histone fractions. Chromatin proteins separated on polyacrylamide gel electrophoresis revealed the preferential binding of TML to histones H1 and H4, and also to two groups of non-histones with molecular weights around 60×10^3 and 12×10^3 daltons. The binding of TML to DNA was mainly associated with adenine and in a purified nuclei assay system could be increased by manganese and inhibited by magnesium.

It has been reported that treatment with TML causes cell proliferation in resting human peripheral lymphocytes, and shortening of the cell cycle time in slowly growing tumour cell populations such as the NK/Ly ascites tumour at an advanced growth stage [1–4]. In human lymphocyte systems the actions of TML and PHA were similar in the sense that in both cases mitosis was preceded by increased RNA and DNA synthesis [5]. The initial appearance of [^{3}H]-thymidine in nascent DNA after pulse labelling and only later in the longer segments, together with inhibition of [^{3}H]-thymidine incorporation in cells by hydroxyurea and an increased DNA content per cell measured by cytophotometry have all been presented as evidence that TML can trigger normal replication of DNA ([4], Zs. Suba, G. Gyapay, M. Bencsáth, B. Szende, A. Jeney and K. Lapis, in preparation). Previous studies on the mechanism of action of TML have demonstrated the appearance of radioactivity derived from [^{3}H-CH_3]-TML in RNA, DNA and proteins and also the conversion of TML to metabolic products [6]. This latter observation, in conjunction with those reported by Cox and Hoppel [7], makes the decomposition of TML by the beta-oxidation mechanism and the formation of free methyl groups together with certain mono- and di-methylated alkylamine compounds very likely. Radioactivity in proteins following administration of labelled TML is the result of both protein methylation and incorporation of intact TML. However, the cellular function of TML is not well understood [8].

Since these studies have indicated that nucleic acids and proteins may be methylated by TML *in vivo* and because chemical post synthetic modifications of chromatin components are concerned with gene regulation, the binding of TML (or its metabolic products) to chromatin components has been investigated.

MATERIALS AND METHODS

[^{3}H-CH_3]-TML was synthesized by reacting [^{3}H-CH_3]-dimethylsulphate with L-lysine–HCl as previously described [6]. The specific activity of the methyl-labelled TML was 221.35 mCi/mmole. NK/Ly ascites tumour-bearing female CFLP mice transplanted with 5×10^6 cells 10 days previously were treated with 50 mg/kg[^{3}H-CH_3]-TML i.p. for 2 and 24 hr. Mice were killed by cervical dislocation, tumours removed, centrifuged [600 *g* 10 min, 4°) and the pelleted cells washed with cold 0.9% NaCl–10 mM KPO^-_4 (pH 7.4) buffer solution. Chromatin components were isolated from purified nuclei and separated on a hydroxylapatite column as described by MacGillivray *et al.* [9]. The histone and the two combined non-histone protein fractions from the hydroxylapatite were concentrated by dialysing against 30% polyethyleneglycol and analysed by SDS–polyacrylamide gel electrophoresis according to the method described by Weber and Osborne [10]. The gels stained with Coomassie Brilliant Blue G-250 were scanned in a Joyce–Loeble Chromoscan densitometer at 575 nm. To measure radioactivity the mechanically sliced gels were placed into scintillation vials and dissolved in 0.4 ml of 30% H_2O_2 at 50° for 24 hr. Binding to DNA was further studied *in vitro*, when nuclei were purified as before and 5×10^7 nuclei were incubated in 5 ml of 10 mM NaCl–10mM Tris–HCl (pH 7.4) buffer without or with various concentrations of magnesium or manganese (1–10 mM) for 30 min at 37° in the presence of 55 μmoles/l. of [^{3}H-CH_3]-TML. After terminating the reaction DNA was isolated by the method of Kirby [11] and electrophoresed on 10 cm disc gels of 2.8% acrylamide prepared as described by Loening [12]. DNA was located on the gel with Joyce–Loeble UV Scan and radioactivity measured in the gel slices. DNA was hydrolysed in formic acid and

Table 1. Radioactivity derived from [^{3}H-CH$_3$]-trimethyllysine in chromatin components of NK/Ly ascites tumour cells

Duration of *in vivo* treatment with 50 mg/kg [^{3}H-CH$_3$]-TML	Histones	Non-histones I	Non-histones II	DNA
2 hr	16 ± 4.24	25.3 ± 4.3	6.3 ± 2.1	5.8 ± 0.2
24 hr	19.5 ± 5.0	50.5 ± 14.6	10.0 ± 1.3	25.5 ± 2.12

* All data expressed as 10^3 × dpm per mg protein or DNA (± S.D.). Chromatin was isolated and fractionated on a hydroxylapatite column as described by MacGillivray *et al.* [9]. Histones, non-histone I, non-histone II and DNA fractions in this experiment correspond to those designated as HAP1, HAP2, HAP3 and HAP4, respectively, in the publication of MacGillivray *et al.* [9].

radioactivity measured in the eluted bases following MF300 cellulose thin layer chromatography [13]. The amounts of DNA and protein were measured using the diphenylamine and Folin reagents, respectively [14, 15]. Radioactivity measurements were performed in a Beckman LS 100 C spectrometer by using a 2-ethoxyethanol containing toluene based scintillation liquid [6].

RESULTS

Table 1 shows the radioactivity level of chromatin components 2 and 24 hr after injection of [^{3}H-CH$_3$]-TML into NK/Ly ascites tumour-bearing mice. All the components contained radioactivity, although the specific activities of these fractions was not the same and changed differently with time. Two hours treatment resulted in higher labelling in histone and non-histone I fractions relative to DNA and non-histone II fraction. For 24 hr the specific activity increased in all chromatin components except histones. The radioactivity showed a remarkable increase in DNA between 2 and 24 hr. The other noteworthy observation in this experiment was that the two non-histone fractions eluted from the hydroxylapatite column with 50 and 200 mM phosphate buffer, respectively, differed from each other in their capacity to attract methyl groups from TML.

To demonstrate the intimate association of the radioactivity representing methyl groups to chromatin proteins and to investigate whether the methylation-like reaction is random or whether certain proteins preferentially react with TML, chromatin proteins were separated according to their molecular weights by SDS–polyacrylamide gel electrophoresis. Radioactivity measurements on the gel slices revealed that two groups of non-histone proteins with molecular weights around 60×10^3 and 12×10^3 dalton had the highest level of methylation. Likewise, histones behaved also differently, as only histones H1 and H4 contained substantial radioactivity.

Reaction of [^{3}H-CH$_3$]-TML to DNA was studied in more detail in purified nuclei systems in order to minimize the possibility that the radioactivity methyl group from TML enter the 'one-carbon pool' and are incorporated into DNA through *de novo* nucleotide synthesis. Using the nuclei assay system it was established that reaction of [^{3}H-CH$_3$]-TML with DNA was very similar between pH 6 and 8 and

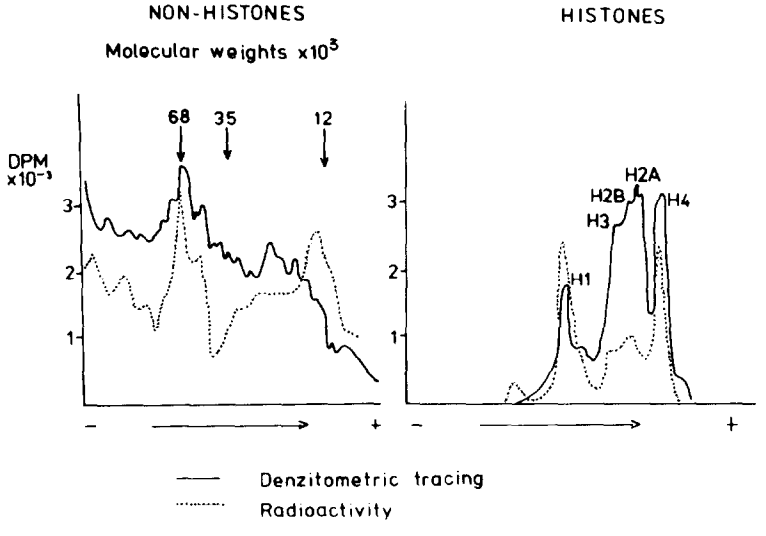

Fig. 1. Electrophoretic pattern of chromatin proteins from NK/Ly ascites tumour treated with [^{3}H-CH$_3$]-TML for 2 hr. Experiments were performed as described in Materials and Methods. Molecular weight standards were bovine serum albumin, pepsin and cytochrome *c*.

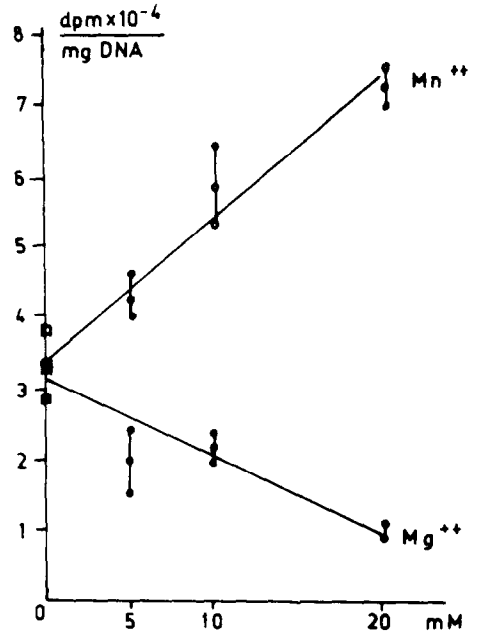

Fig. 2. The ion dependent reaction between [^{3}H-CH$_3$]-TML and DNA. Experiments were performed as described in Materials and Methods.

showed only a slight increase upon increasing the incubation temperature from 5° to 37° (data not shown). At the same time some divalent cations influenced this reaction. Calcium had no effect at all, but reaction was decreased by magnesium and increased by manganese (Fig. 2).

The [^{3}H-CH$_3$]-TML-derived radioactivity appeared on the polyacrylamide gel electropherogram of DNA at the same position as the main peak of the absorbance profile measured at 260 nm, providing the nuclei had been exposed to [^{3}H-CH$_3$]-TML for 30 min (Fig. 3).

The hypothesis that a chemical reaction takes place between [^{3}H-CH$_3$]-TML and DNA obtained further support in the experiment when radioactivity was detected on DNA bases. Interestingly, the distribution of radioactivity among the bases was not even

Table 2. Detection of [^{3}H-CH$_3$]-trimethyllysine derived radioactivity in DNA bases*

Bases	Total DPM recovered from the bases
Cytosine	655
Adenine	1530
Guanine	615
Thymine	210

* Isolated nuclei were treated with [^{3}H-CH$_3$]-TML as in experiments shown in Fig. 2. DNA was isolated and after hydrolysis in formic acid the bases were separated on MN 300 cellulose thin-layer, and isopropanol–HCl–water [65:16.37:18.3) was applied as solvent. For radioactivity measurements the bases identified by using standards were located under a u.v. lamp, the appropriate part of the plate was scraped and radioactivity was measured in the bases after elution with 0.01 M HCl.

because adenine contained 50 per cent of the total radioactivity from DNA and thymine showed only a low level of radioactivity (Table 2).

DISCUSSION

The labeling of chromatin components (DNA, histones and non-histones) with [^{3}H-CH$_3$]-*N*-5-trimethyllysine occurs at a dose which is far below the toxic value (LD_{50} > 2000 mg/kg) but which is sufficient to stimulate DNA replication in 10-day-old NK/Ly ascites tumour cell population in which proliferation is much lower than in early transplants [16].

The chemical nature of the reaction between TML and chromatin component has not yet been clarified. The only essential point which we have in this context is the fact that the TML applied in this study was labelled exclusively in the methyl groups. Previous studies demonstrated that methyl groups were released from TML, which was also metabolized by beta oxidation, thus producing a not yet identified monomethylated alkylamine compound [6, 8]. The purpose of the present experiments has been to indicate the probable site of action of TML at the chromatin level and to investigate the correlation

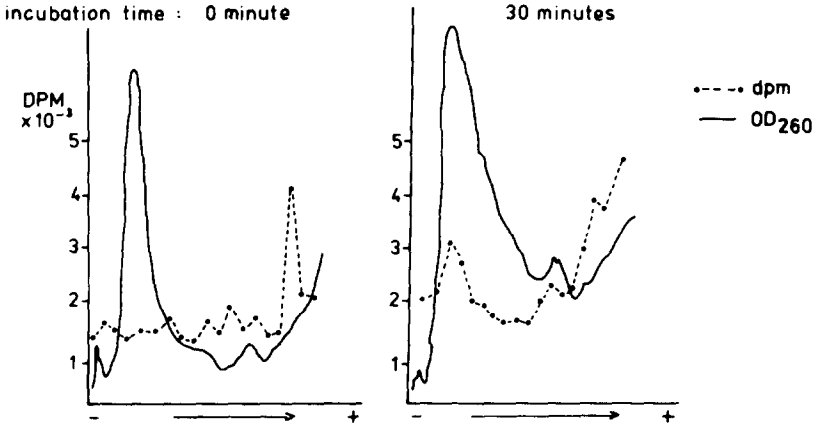

Fig. 3. Electrophoretic patterns of DNA isolated from [^{3}H-CH$_3$]-TML-treated NK/Ly ascites nuclei. Experiments were performed as described in Materials and Methods.

between the modification of chromatin components and the stimulation of cell proliferation induced by TML. The chemical post-synthetic modification of chromatin components (such as methylation) and its relevance to gene regulation has been well documented in many laboratories [17]. According to available data in eukaryotic DNA, methylation under physiological circumstances takes place only in cytosine and a certain regulatory function has been assigned to this reaction [18, 19]. On the other hand, methylation of guanine at the O^6 position is generally regarded as an initiation stage in carcinogenesis and alkylation of other groups—such as N^7 in guanine and N^3 in adenine—being mainly responsible for the cytotoxicity induced by antitumour alkylating agents [20–22]. Of the chromatin proteins, so far only the methylation of histone H3 and histone H4 have been reported (without any drug treatment) as a result of the action of methyltransferase. The interesting aspect of this postsynthetic enzymatic methylation is that it occurs exclusively in the G_2 phase of the cell cycle and, in contrast to phosphorylation, causes chromatin condensation [17, 23].

The reaction between DNA and TML was not temperature dependent and proceeded in isolated nuclei as well, which makes the involvement of methyltransferase most unlikely. Among the DNA bases adenine contained 50 per cent of all the radioactivity of DNA and thymidine was only slightly labelled. This observation excludes the possibility that the labelled methyl groups from TML entered the formate pool and were subsequently incorporated into DNA as a result of *de novo* nucleotide synthesis. The ion dependency of the binding in isolated nuclei indicates the importance of chromatin structure in the reaction between DNA and TML. In the presence of manganese the structure of chromatin may have been altered in such a way as to allow more access of TML to DNA. It is also conceivable that the substantial increase in binding to DNA from 2 to 24 hr after *in vivo* TML treatment is a result of structural changes in chromatin that occur during the cell cycle. If treatment time is prolonged more cells may enter into a phase in which chromatin reaction with TML is more extensive than in other phases of the cell cycle. One sign of selectivity was observed in the methylation of chromatin proteins. One of the two non-histone fractions revealed a 5-fold higher specific activity; it is noteworthy that this particular non-histone fraction has been reported to be underphosphorylated [9]. Of the histones, the H1 and H4 fractions showed the highest reactivity with TML.

These studies indicate a methylation-like reaction to certain chromatin components, which could be a novel way of post-synthetic chemical modification by an external agent that has no carcinogenic or cytotoxic action, at least in the experimental systems applied in numerous studies during the last few years. Therefore, it appears that TML reaction to adenine either does not cause cellular damage or it is subjected to fast repair process. The possibility that methylation of adenine is the molecular base for the stimulation of cell proliferation induced by TML, while unlikely, should be investigated further. It seems more likely that chemical modification of chromatin proteins may be connected with the stimulation of cell proliferation.

Future experiments are aimed at elucidating the structural and functional alterations of chromatin following methylation of histones H1 and H4 and certain non-histones generated by TML and its relevance to cell proliferation. Present studies, besides demonstrating the reaction between TML and certain chromatin components, are regarded as an indication that TML-induced chemical modification of chromatin offers a method for the selective modification of chromatin and also a conceptual approach for the investigation of gene regulation.

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