EFFECT OF THE ALKYLATING AGENT TRIETHYLENEIMINOBENZOQUINONE ON THE SYNTHESIS OF DNA AND ON THE INCORPORATION OF [^{3}H]LYSINE INTO NUCLEAR PROTEINS OF EHRLICH ASCITES TUMOR CELLS

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1. Introduction

Alkylating antitumor agents are known to cause a rapid decrease in the incorporation of labeled thymidine into the DNA of tumor cells (Wheeler [1,2]). This effect has been interpreted as an inhibition of DNA synthesis by the alkylating agent. However, we have been able to demonstrate that despite the inhibition of the incorporation of labeled thymidine into DNA, the rate of DNA synthesis remains unaltered after administration of 6×10^{-7} moles/kg of triethyleneiminobenzoquinone (Trenimon) [3]. Considering the tight coupling of DNA and histone synthesis [4–7] one should expect the incorporation of labeled precursors into histones to be unaffected under conditions where DNA synthesis proceeds normally. Busch et al. [8,9] have reported that the biosynthesis of the histones is relatively resistant to aminouracil mustard. According to their studies the synthesis of the non-histone proteins is most sensitive to alkylating agents. A high sensitivity of the non-histone nuclear proteins to alkylating agents has also been described by Nyhan [10]. The present studies demonstrate that – contrary to all expectations – concentrations of the alkylating agent Trenimon which do not affect the biosynthesis of DNA in Ehrlich ascites cells strongly inhibit the incorporation of [^{3}H]lysine into histones whereas [^{3}H]lysine incorporation into the chromosomal non-histone proteins and the total cellular proteins is hardly affected.

2. Materials and methods

2,3,5-Triethyleneiminobenzoquinone (Trenimon) was donated by Farbenfabriken Bayer AG, Leverkusen, Germany. 4,5-[^{3}H]lysine (250 Ci/mol) was obtained from the Radiochemical Centre, Amersham, England. Ehrlich ascites tumor cells were grown and harvested 5–6 days after transplantation as described previously [11].

The preparation of nuclear proteins was performed as described elsewhere [12] with the exception that the acid soluble nuclear proteins were extracted immediately after the washings with 0.14 M NaCl–0.01 M sodium citrate. The extraction with 2 M NaCl was omitted. Determination of the total cellular protein was performed by precipitating an aliquot of a suspension of Ehrlich ascites cells by addition of $HClO_4$ to a final concentration of 0.7 N. The precipitate was washed once with 0.2 N $HClO_4$ and solubilized in 0.1 N NaOH.

Protein determinations were performed according to Lowry et al. [13] with bovine albumin as a standard. The radioactivity of the solubilized protein fractions was determined in a scintillation spectrophotometer by pipetting an aliquot of the protein solution into the scintillation fluid described previously [12]. Gel electrophoresis was performed by a modification of the procedure described by Bonner et al. [14]. The modification consisted in replacing the *N,N*-methylene-bis-acrylamide by ethylene diacrylate. The use of the latter renders the gels soluble in 1 N NH_4OH and 'Nuclear Chicago Solubilizer' (NCS) [15]. The gels were stained with Amido Black 10 B

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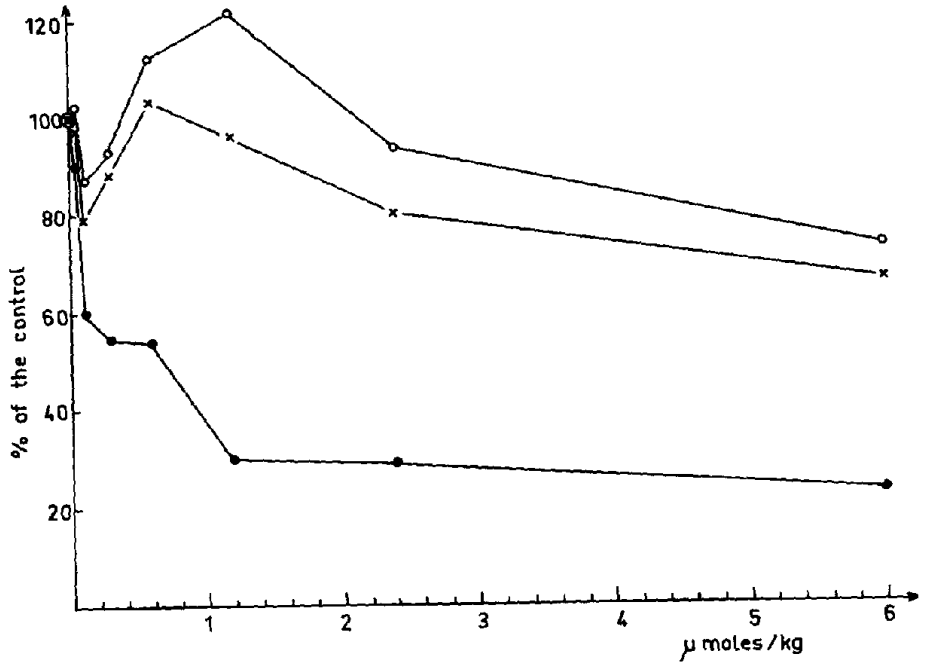

Fig. 1. Effect of triethyleneiminobenzoquinone (Trenimon) on the incorporation of [^{3}H]lysine into proteins of Ehrlich ascites tumor cells: (o—o—o) Total cellular protein; (×—×—×) nuclear non-histone proteins; (•—•—•) acid soluble nuclear proteins. Tumor bearing animals received various concentrations of Trenimon in 0.14 M NaCl i.p. Controls received NaCl only. Four hours later each animal was injected i.p. with 30 μCi of [^{3}H]lysine. After an additional hour, the tumors were harvested. Preparation and determination of the protein fractions are described under Materials and methods. For each determination, pooled tumors from 4 animals were employed.

and destained electrophoretically. The protein content of the stained proteins on the gel was determined densitometrically. The gels were scanned with a Gilford photometer equipped with a densitometer attachment and the areas of the tracings were measured with a planimeter. In a range between 5 and 50 μg of protein, a linear relationship was obtained between the amount of protein applied to the gel and the values obtained densitometrically. For the determination of the radioactivity, the individual bands were cut out with a razor blade. The gel slices were solubilized overnight in 1 N NH_4OH and NCS as described by Cain and Pitney [15] and after addition of 10 ml of scintillator [15] the radioactivity was measured in a scintillation spectrophotometer. DNA was determined according to Keck [16] with calf thymus DNA as a standard.

3. Results

Fig. 1 shows the effect of various doses of Trenimon on the incorporation of [^{3}H]lysine into the acid soluble nuclear proteins, the nuclear non-histone proteins and the total proteins of Ehrlich ascites tumor cells. There is a strong dose dependent decrease in the incorporation of the labeled lysine into the acid soluble nuclear proteins whereas the labeling of the total cellular proteins and the nuclear non-histone proteins is much less affected. Gel electrophoretic separation of the acid soluble proteins obtained 4 hr after treatment with the drug showed no significant changes in the relative or absolute amounts of the different histone fractions and revealed that the four histone fractions which are separated on the gel are affected to about the same extent (fig. 2, table 1). The slightly smaller inhibition of the lysine incorporation into the FI-fraction may be due to contaminating non-histone proteins. The time course of the effect of

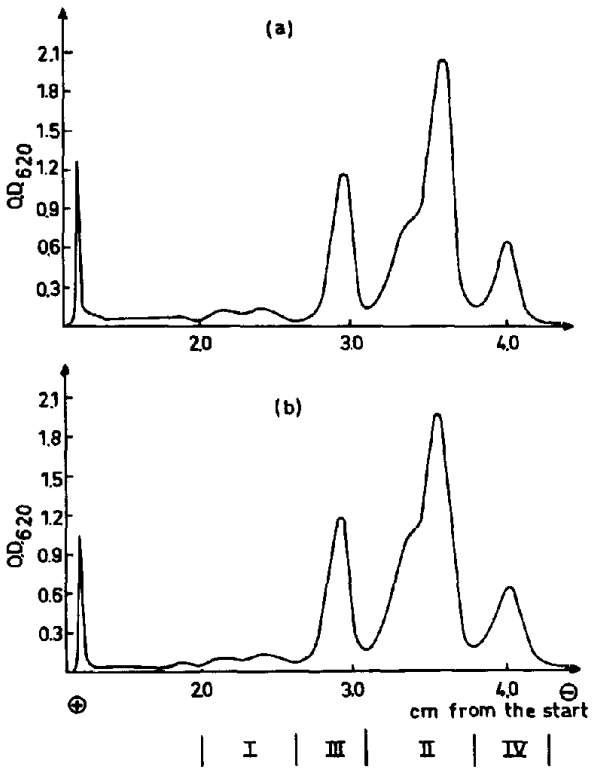

Fig. 2. Densitometer scans of polyacrylamide gel electrophoreses of acid soluble nuclear proteins from Ehrlich ascites tumor cells: a) control; b) material obtained 4 hr after treatment of tumor bearing mice with 6×10^{-7} moles/kg Trenimon. The vertical lines underneath the abscissa indicate the positions where the gels have been cut for radioactivity determinations. The gel slices were designated by Roman numerals corresponding to the migration of the individual histone fractions. Histones IIa and IIb are not separated on this gel and represent fraction II. For experimental details see Materials and methods.

a single dose of the alkylating agent (6×10^{-7} moles/kg) on the incorporation rate of lysine into the acid soluble nuclear proteins reaches a minimum 4 hr after administration of the drug. Although there is a slight decrease in the inhibitory effect during the subsequent 20 hr the marked depression of the labeling of the acid soluble nuclear proteins can still be observed 24 hr after exposure to the alkylating agent. Table 2 demonstrates that despite the marked inhibition of the incorporation of labeled lysine into the acid soluble nuclear proteins, the amount of DNA which is synthesized during the 24 hr-interval following the administration of

Table 1

Effect of triethyleneiminobenzoquinone (Trenimon) on the incorporation of [^{3}H]lysine into the histones of Ehrlich ascites tumor cells.

Fraction	Control dpm/μg	+ Trenimon dpm/μg	% of the control
I	408 ± 60.3	146 ± 29.3	35.8
III	404 ± 14.8	108 ± 3.6	26.7
II	196 ± 5.8	53 ± 1.8	27.0
IV	144 ± 13.3	44 ± 5.0	30.6

4 Tumor bearing mice received 3×10^{-7} moles/kg Trenimon i.p. After 4 hr 100 μCi of [^{3}H]lysine was injected i.p. into each Trenimon-treated animal and into 4 untreated mice which served as controls. One hour later the animals were killed. The tumors of each group (the Trenimon-treated group and the control group) were pooled and the acid soluble nuclear proteins were prepared as described under Materials and methods. The histone fractions were prepared and analyzed as described in the legend to fig. 2. The values represent the means of 5 determinations ± s.d.

Table 2

Effect of triethyleneiminobenzoquinone (Trenimon) on DNA synthesis and cell multiplication of Ehrlich ascites tumor cells.

	Time: 0	Time: 24 hr	
		Controls	+ Trenimon
Tumor cells/animal ($\times 10^{-8}$)	4.3 ± 0.3	7.1 ± 0.2	3.9 ± 0.1
Tumor DNA/animal (mg)	6.6 ± 0.4	10.1 ± 0.3	9.2 ± 0.6
DNA/tumor cell (pg)	15.5 ± 0.6	14.1 ± 0.7	21.3 ± 1.4

Treminon (6×10^{-7} moles/kg) was injected i.p. into tumor bearing mice 5 days after transplantation of the tumor. Control animals received the corresponding volume (0.2 ml) of 0.14 M NaCl. Immediately following the injection, ten of the control animals were killed and the tumor cells collected from each individual animal. After 24 hr another ten of the Trenimon-treated and another ten of the control animals were killed. Care was taken to collect the tumor cells quantitatively from each individual animal by excessive rinsing of the peritoneal cavity and the intestine with 0.25 M sucrose. Cell counts were performed microscopically with the aid of a Neubauer counting chamber. DNA was determined according to Keck [16]. The values represent means of 10 determinations ± s.d.

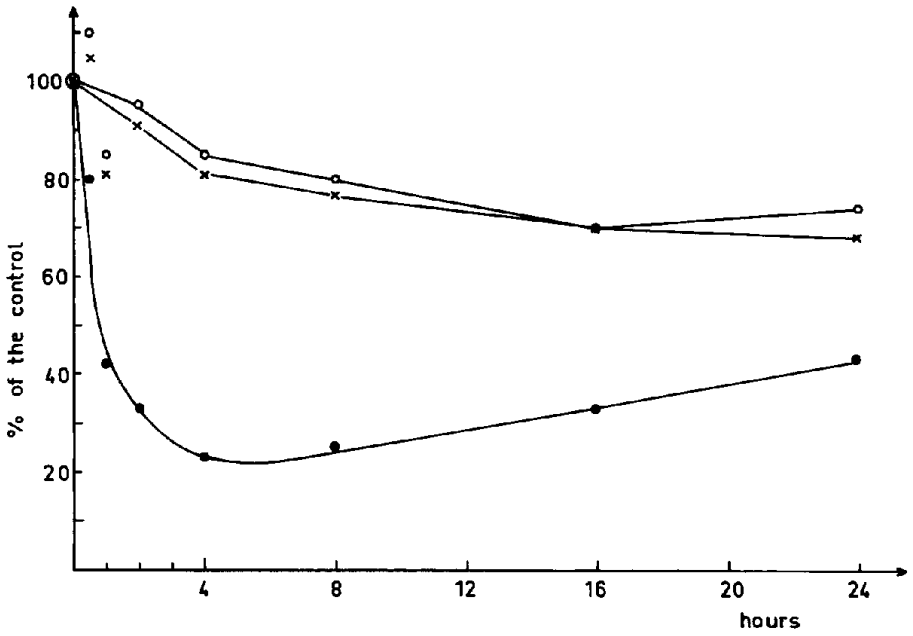

Fig. 3. Effect of triethyleneiminobenzoquinone (Trenimon) on the incorporation of [^{3}H]lysine into proteins of Ehrlich ascites tumor cells as a function of time after exposure to the drug. Tumor bearing mice received 6×10^{-7} moles/kg Trenimon in 0.14 M NaCl i.p. Control animals received NaCl only. One hour before harvesting the tumors, each animal was injected with 40 μCi of [^{3}H]lysine. Groups of 4 animals were killed at the time points indicated, the tumors pooled and the protein fractions isolated as described under Materials and methods.

6×10^{-7} moles/kg Trenimon is about the same in Trenimon-treated as well as in untreated populations of Ehrlich ascites cells.

4. Discussion

Busch et al. [8,9] have reported that aminouracil mustard decreases the rate of labeling of the non-histone nuclear proteins at earlier times and at lower doses of the drug than the labeling of histones or cytoplasmic proteins. Further studies with various alkylating agents have lead to the conclusion that the suppression of labeling of the non-histone nuclear proteins (called acidic proteins by these authors) is the only constant feature of the effects of antitumor agents on protein labeling [17]. The data shown in this paper demonstrate that this statement cannot be generalized. Our results show that all concentrations of the ethyleneimino compound used and at all time points studied, the incorporation of [^{3}H]lysine into the acid soluble nuclear proteins was suppressed to a much greater extent than the labeling of the non-histone nuclear proteins or the total cellular proteins. Although the acid soluble nuclear proteins contain some other proteins besides histones, the extent of the inhibitory effects observed as well as the data shown in table 1 permit the conclusion that the decrease in the labeling of the acid soluble nuclear protein is due to an inhibition of the incorporation of the [^{3}H]lysine into the histones. At a Trenimon dose of 6×10^{-7} moles/kg – which as shown in table 2 completely blocks cell multiplication – the labeling of the non-histone protein is hardly affected whereas the [^{3}H]lysine incorporation into the nuclear histones is markedly suppressed (fig. 1). The decrease in the incorporation of labeled lysine into the nuclear histones lasts for at least 24 hr after a single dose of the alkylating agent. However, despite the decreased labeling of the histones, the net increase in the amount of the DNA determined 24 hr after injection of 6×10^{-7} moles/kg Trenimon is about the same as in untreated controls. This is in accordance with previous findings from our laboratory which demonstrate that this

Trenimon concentration – although it decreases the incorporation of labeled thymidine into the DNA – does not affect the synthesis of DNA during the first 24 hr following the injection of the drug [3]. This was also found to be the case during the first 8 hr-interval after administration of the drug, a time period in which the suppression of histone labeling is at its maximum [3].

The data demonstrate that the alkylating agent is capable of dissociating the synthesis of DNA and the increase in the amount of the histones in the cell nucleus, two processes which are normally tightly coordinated. The reason why this effect has not been observed so far is probably due to the fact that most investigators used the incorporation of labeled thymidine into DNA as a measure for DNA synthesis. As mentioned above, this procedure can be misleading. The same discrepancy between thymidine incorporation and DNA synthesis has been described as occurring after X-irradiation [18]. The differential effects on thymidine incorporation and DNA synthesis may be due to alterations in pool sizes of the thymidine nucleotides [19]. The prevailing inhibition of the incorporation of [^{3}H]lysine into the histones, however, cannot be explained by changes in the intracellular lysine pool or an impairment of the lysine transport into the cell as both mechanisms would equally affect the non-histone proteins and the majority of the total cellular proteins. Our data do not permit a decision on whether the suppression of the histone labeling is caused by an inhibition of histone synthesis or by a block in the transport of the preformed histones from the cytoplasm into the nucleus. However, the latter possibility seems unlikely as the transport of the non-histone proteins – of which the majority is also synthesized in the cytoplasm [20] – is obviously not affected.

During the preparation of this manuscript a publication by Riches and Harrap appeared on the effects of chlorambucil on the chromatin of Yoshida ascites cells [21]. Their results are very similar to our findings with Trenimon by Ehrlich ascites cells. According to Riches and Harrap chlorambucil does not affect the synthesis of DNA during the first 24 hr after treatment, but leads to a progressive decrease in the histone/DNA ratio.

Acknowledgements

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