Differences between Thymic and Splenic Cells of the Rat: Biochemical and Physico-Chemical Investigations *in vitro* on DNA Topoisomerase II – Inhibitors and Thiyl Radicals

K. Tempel

Institut für Pharmakologie, Toxikologie und Pharmazie, Universität München, Königinstraße 16, D-80539 München, Bundesrepublik Deutschland

Z. Naturforsch. **48c**, 946–951 (1993); received June 18/August 30, 1993

Novobiocin, Nalidixate, Thiyl Radicals, Thymic Cells, Splenic Cells, Chromatin

Interactions of novobiocin (NB) and nalidixic acid (NA) with thiols were investigated *in vitro* in thymic (T-) and splenic (S-) cells of the rat, by determining nucleic acid synthesis as well as nucleoid sedimentation and viscosity of alkaline cell lysates. In T-cells NB, at concentrations of 0.35–1.4 mM, increased unscheduled DNA synthesis (UDS) and RNA synthesis (RNS), whereas S-cells underwent a dose-dependent inhibition of UDS and RNS following exposure to NB at concentrations >0.7 mM. Combining NA and thiols (*e.g.*, dithiothreitol) resulted in a slight stimulation of UDS in S-cells and in a highly significant increase of UDS in T-cells ascribed to a depletion of the cellular thymidine pool. In both cell types, neither NB nor NA exerted a significant effect on thiol-induced DNA damage. At a concentration of 1.4 mM, NB increased the viscosity of alkaline T-cell lysates; the opposite effect was observed in S-cells. From these results as well as from previous investigations we conclude that T- and S-cells differ in their state of chromatin conformation. This interpretation offers a simple model for the study of the influence of chromatin structure on cell-specific physico-and/or chemico-biological interactions.

Introduction

Splenic cells (S-cells) and thymic cells (T-cells) are used as model systems to investigate the effects of various agents on mammalian cells (see, *e.g.*, refs. [1–6]), including *in vivo*/*in vitro* systems for biological monitoring [7]. Significant differences exist, however, between both cells types [1–4].

As shown in previous investigations, the DNA topoisomerase II inhibitors novobiocin (NB) and nalidixic acid (NA) stimulate unscheduled DNA synthesis (UDS) without detectable DNA damage, the extent of stimulation being much higher in T- than in S-cells [8]. Since NB and – to a lesser extent – NA, at concentrations normally used to inhibit eukaryotic type II topoisomerase activity (100–1000 μg/ml) influence chromatin structure [3, 9, 10], and since chromatin structure is cell-specific (see, *e.g.*, refs. [11, 12]), the possibility must be considered that both substances change the accessibility of DNA within the chromatin complex in a cell-specific manner. To test this assumption, ribonucleic acid synthesis (RNS) as well as the effect of thiyl radicals [13–15] were investigated in T- and S-cells treated with NB or NA *in vitro* using biochemical and physico-chemical methods. The biochemical methods comprised scheduled DNA synthesis (SDS), UDS, and RNS. Physico-chemical procedures consisted of nucleoid sedimentation and viscometry of alkaline cell lysates.

Reprint requests to K. Tempel.

Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/93/1100–0946 $ 01.30/0

Materials and Methods

Chemicals

Chemicals were purchased from Merck (Darmstadt, Germany) and Sigma (München, Germany). Methyl-[^{3}H]thymidine (dT-^{3}H; 3 TBq/mmol) and 5.6-[^{3}H]uridine (U-^{3}H; 2 TBq/mmol) were from DuPont NEN Research Products (Dreieich, Germany) and Amersham Buchler (Braunschweig, Germany), respectively.

Cells

T- and S-cells were prepared in Hank's balanced (Mg^{2+}- and Ca^{2+}-free) salt solution as previously described [8]. Cell viability was assayed microscopically by trypan blue exclusion (0.01 ml 2% trypan blue to 0.1 ml cell suspension) throughout the

experimental period. Following incubation periods of 30, 60, and 90 min, 98.6 ± 5.1, 89.5 ± 8 and 77.1 ± 6.9% of the cells were viable. No significant differences existed between the two cell types.

Treatment of cells

Stock solutions of test substances were freshly prepared in Hank's solution. 0.1 ml of these solutions were added to 1 ml of the cell suspensions. To study the interactions between NB (or NA) and thiyl radicals, the cell suspensions were first incubated for 30 min at 37 °C with the topoisomerase II inhibitors. Thereafter, cells were washed three times in Hank's solution. The final cell sediment was resuspended in Hank's medium containing the thiols, *i.e.*, dithiothreitol (DTT), cysteine·HCl (CY-E), cysteamine·HCl (CY-A), and S-(2-Aminoethyl)-isothiuroniumbromide·HCl (AET) at concentrations of 1.3, 2.6, and 5.2 M.

Analytical procedures

Nucleic acid synthesis was measured by the uptake of dT-^{3}H (SDS, UDS) and U-^{3}H (RNS) into the perchloric acid (6%) precipitate of the cells as previously described [8]. In order to determine UDS, SDS was suppressed by the ribonucleotide reductase inhibitor hydroxyurea (HU, 10^{-2} M). Changes in the intracellular dT- and U-pool sizes were examined by dilution of the ^{3}H-labelled nucleosides with increasing molarity of (unlabelled) thymidine and uridine, respectively [16]. Depletion of the intracellular nucleoside pool sizes reduces the concentrations of unlabelled nucleosides necessary to inhibit the incorporation of labelled nucleosides into nucleic acids – and *vice versa*.

Viscometry of alkaline cell lysates and the *nucleoid sedimentation* technique were performed as described in previous papers (see refs. [4] and [8], respectively).

In general, the data points represent the average of at least 2 or 3 independent experiments which were done in triplicate.

Results

In a previous study in T- and S-cells, we found that NB inhibits and NA enhances SDS *in vitro* [8]. As shown by Table I, NB (0.35–1.4 mM) and NA (0.9–7.2 mM) stimulated UDS and RNS in T-cells. After exposure to NB, S-cells remained without significant effects (NB, 0.35 and 0.7 mM) or underwent a concentration-dependent inhibition of dT-^{3}H and U-^{3}H incorporation (NB, 1.4 and 2.8 mM). NA slightly enhanced UDS and RNS in S-cells (Table I). A parallelism between UDS and RNS follows therefore from the investigations in T- and S-cells treated with NB and NA.

In a further series of experiments, the effects on SDS, UDS, and RNS of dithiothreitol (DTT) alone or in combination with NB or NA were studied. Fig. 1 shows that the thiol depletes SDS of both cell types as well as UDS of S-cells. Combining NB (1.4 mM) and DTT (5.2 mM) resulted in a small stimulation of UDS in S-cells and in a dose-dependent increase in UDS up to 220% in T-cells (Fig. 1). Under the same conditions, RNS remained unchanged (1.3 and 2.6 mM DTT) or was slightly inhibited at a DTT concentration of 5.2 mM (results not shown). Similar results were obtained when DTT was substituted by other thiols, *i.e.*, CY-E, CY-A, and AET at equimolar

Table I. Mean (± standard deviation) incorporation of dT-^{3}H (UDS) and U-^{3}H (RNS) into the PCA-insoluble fraction of T- and S-cells following exposure to NB and nalidixic acid. Incorporation rates in per cent of the controls (= 100%).

Novobiocin	Thymic Cells				Splenic Cells				
	0.35	0.70	1.4	2.8	0.35	0.70	1.4	2.8	mM
UDS	131 ± 10	205 ± 21	230 ± 28	105 ± 18	103 ± 6	93 ± 5	43 ± 11	10 ± 5	%
RNS	139 ± 20	197 ± 28	273 ± 27	81 ± 17	108 ± 7	100 ± 10	84 ± 14	58 ± 17	%
Nalidixic Acid									
	0.90	1.8	3.6	7.2	0.90	1.8	3.6	7.2	mM
UDS	115 ± 17	141 ± 10	169 ± 28	205 ± 20	100 ± 10	116 ± 12	133 ± 8	123 ± 13	%
RNS	121 ± 7	167 ± 17	204 ± 16	222 ± 27	108 ± 7	122 ± 9	130 ± 17	146 ± 11	%

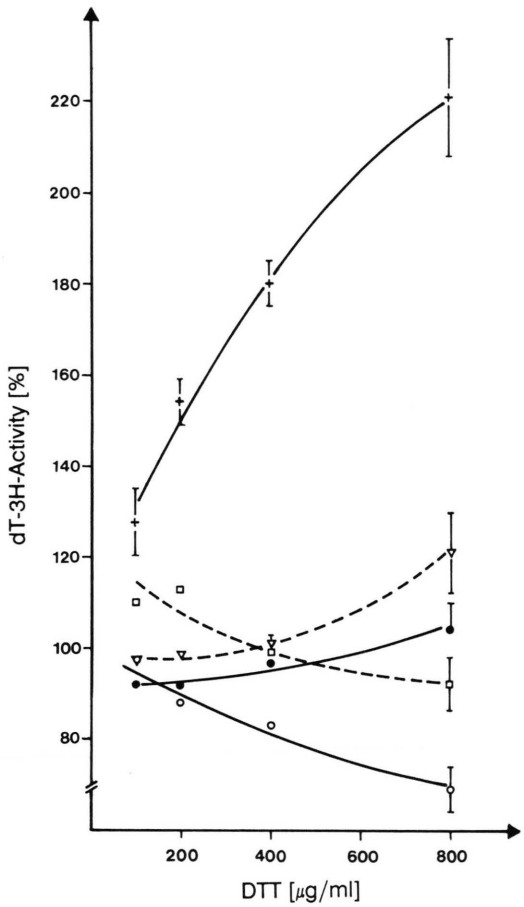

Fig. 1. Mean (± standard deviation) incorporation of dT-^{3}H into the perchloric acid (PCA) – insoluble fraction of T- and S-cells following exposure to dithiothreitol (DTT) alone or in combination with novobiocin (NB). Incorporation rates in per cent of the controls (= 100%). —●—: UDS in T-cells without NB-pretreatment, —+—: UDS in T-cells pretreated with 1.4 mM NB, —○—: SDS in T-cells without NB-pretreatment, – – –□– – –: UDS in S-cells without NB-pretreatment, – – –▽– – –: UDS in S-cells pretreated with 1.4 mM NB.

concentrations. Treatment of T- and S-cells with NA and thiols did not influence UDS significantly (results not shown).

NB has a marked effect on mitochondria and lowers the intracellular ATP/ADP ratio [17]. Possible changes in the cellular nucleic acid precursor pools must be considered, therefore. The results obtained by isotope dilution (Fig. 2) suggest a depletion of the thymidine- and an expansion of the uridine-pool in T-cells after exposure to the combined action of NB and DTT.

Physico-chemical investigations

DNA damage was measured by nucleoid sedimentation and viscometry of alkaline cell lysates (Table II). At a concentration of 2.6 mM, DTT diminished nucleoid sedimentation in T-cells by about 60 and in S-cells by almost 10%. NB alone or in combination with the thiol had no significant effects.

The viscosity of alkaline cell lysates was slightly decreased by DTT in T-cells whereas an increase by about 10% was observed in S-cells.

NB elevated the viscosity of alkaline lysates of T-cells. The opposite effect was seen in S-cells (Table II). Like NB, other agents interfering either directly or indirectly with chromatin, *e.g.*, 3-aminobenzamide, Hoechst 33258, and hyperthermia, revealed significant differences between T- and S-cells (Table III).

Discussion

At the expense of ATP hydrolysis, DNA topoisomerases II modify DNA topology "*in vivo* by passing an intact nucleic acid helix through a transient double-stranded break generated in a second helix" [18] (for reviews see, *e.g.*, refs. [19–21]).

Table II. Nucleoid sedimentation (SED) and viscosity of alkaline lysates (VIS) of T- and S-cells following exposure to NB (1.4 mM), DTT (2.6 mM), and a combination of both substances. Values in per cent of the controls (= 100%).

Treatment	Thymic Cells		Splenic Cells		
	SED	VIS	SED	VIS	
Novobiocin	100 ± 8	141 ± 7	100 ± 6	51 ± 9	%
Dithiothretol	38 ± 10	87 ± 9	88 ± 10	111 ± 12	%
Combination	50 ± 8	97 ± 7	92 ± 5	53 ± 11	%

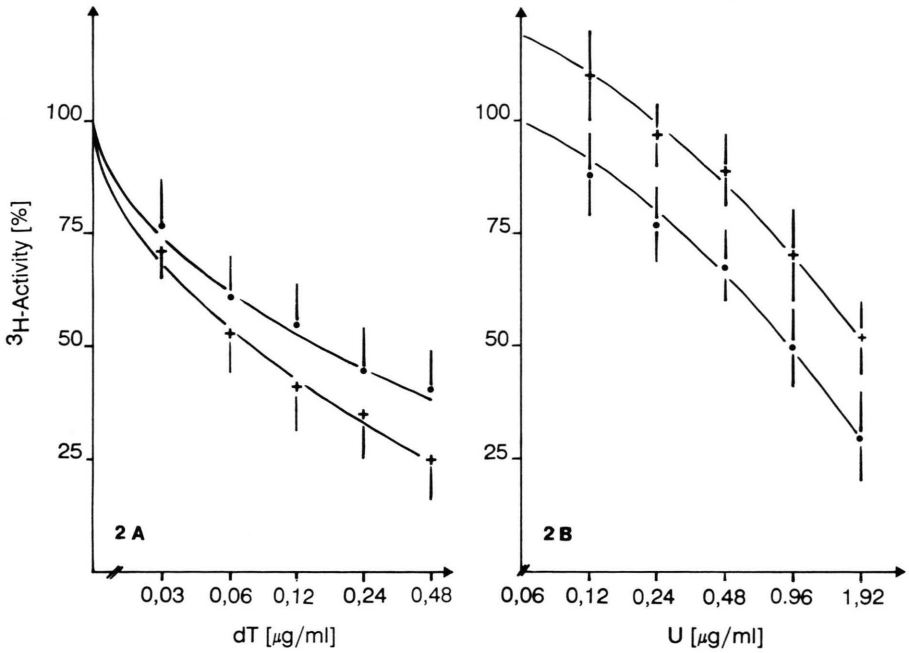

Fig. 2. Mean (± standard deviation) incorporation of dT-^{3}H (Fig. 2A) or U-^{3}H (Fig. 2B) into the PCA-insoluble fraction of T-cells following exposure to 1.4 mM NB (—●—) or 1.4 mM NB and 2.6 mM DTT in combination (—+—). Incorporation rates in per cent of the controls (= 100%).

Table III. Viscosity of alkaline lysates of T- and S-cells following exposure to NB, 3-aminobenzamide (3-AB), Hoechst 33258, and hyperthermia. Concentrations of the substances used: I, II, III, and IV, resp.: NB: 0.35, 0.7, 1.4, and 2.8 mM, 3-AB: 1.25, 2.5, 5.0, and 10.0 mM, Hoechst 33258: 25, 50, 100, and 200 µg/ml, resp. – Mechanism(s) of action: NB: See text, 3-AB: Inhibition of the enzyme poly(ADP-ribose)polymerase which, through ribosylation of various nuclear acceptor proteins, adds negative charges to basic proteins (*e.g.*, histones), resulting in a less condensed chromatin [23–25], Hoechst 33258: DNA minor groove intercalation [26, 27], hyperthermia: Increase of the nuclear matrix protein mass [28, 29].

	Thymic Cells				Splenic Cells			
	I	II	III	IV	I	II	III	IV
Novobiocin	100 ± 8	107 ± 7	130 ± 9	98 ± 9	98 ± 8	90 ± 6	51 ± 9	11 ± 5 %
3-Aminobenzamide	102 ± 9	108 ± 10	102 ± 11	109 ± 6	70 ± 8	54 ± 8	38 ± 6	27 ± 7 %
Hoechst 33258	114 ± 17	118 ± 18	143 ± 10	143 ± 14	173 ± 13	192 ± 33	231 ± 27	259 ± 25 %
Hyperthermia	60 min / 43 °C 87 ± 12 30 min / 45 °C 110 ± 14				60 min / 43 °C 48 ± 5 30 min / 45 °C 28 ± 5			

Apart from its effect on DNA topoisomerase II, NB interacts with histones and is able, therefore, to modulate chromatin conformation through a topoisomerase II – independent mechanism [9, 10]. The increase in viscosity of alkaline cell lysates of NB-treated T-cells [3] suggested that NB lowers, within a definite concentration-range, chromatin compactness so that the DNA becomes more accessible to exogenic (*e.g.*, thiyl radicals) and/or endogenic (*e.g.*, transcription factors) agents. Apparently consistent with this assumption are the findings that thiols stimulate UDS in

NB-pretreated T-cells (Fig. 1) and NB as well as NA elevate, particularly in T-cells, UDS and RNS (Table I) without detectable DNA damage [8] (Table II). With respect to stimulation of UDS various mechanisms, however, especially DNA damage and changes in the cellular nucleic acid precursor metabolism [16], must be considered. DNA damage following exposure of T-cells to DTT could not be enhanced by NB-pretreatment (Table II). Whereas neither nucleoid sedimentation nor viscometry of alkaline cell lysates support, therefore, the assumption of higher DNA accessibility to thiyl radicals, the results obtained by isotope dilution (Fig. 2) suggest a depletion of the thymidine pool in T-cells after the exposure to the combined action of NB and DTT. In other words: The thiol-induced stimulation of unscheduled dT-^{3}H incorporation into the DNA of NB-pretreated T-cells may be due to variations in the cellular thymidine pool size. In this context, one should notice that thymocytes contain very small quantities of deoxyribonucleotides and ribonucleotides, pool sizes of, *e.g.*, ATP, GTP, dATP, and dTTP being less than 10% of the pool sizes commonly observed in mammalian cells [22].

Apart from the cell-specific peculiarities regarding a possible koergism between NB and thiols, viscosity of alkaline lysates of NB-treated cells showed another striking difference between both cell types (Table II): Whereas NB increased the viscosity of T-cell lysates at a concentration of 1.4 mM, the opposite effect was observed in S-cells thus confirming previous investigations [3]. To explain some S- and T-cell – specific responses to chromatin-interactive agents, we propose various chromatin-transition states (Table V). In this context, it is suggested that T-cells are characterized by a high amount of negatively supercoiled DNA whereas in S-cells positively supercoiled DNA prevails. In support of this conclusion, it could be shown that various agents interfering either directly (*e.g.*, Hoechst 33258, hyperthermia) or indirectly (*e.g.*, 3-aminobenzamide) with chromatin reveal also significant differences between T- and S-cells (Table III).

Though chromatin structure of T- and S-cells must be studied by more sophisticated methods, we conclude from previous investigations and from the present results that the – hypothetically assumed – higher accessibility of "dynamic chromatin" may be limited to endogenous rather than to exogenous factors and that both cell types offer a simple model to investigate the influence of chromatin conformation on cell-specific reactions to physical and/or chemical agents.

Acknowledgements

The author is indepted to Mrs. H. Kortenbeutel for her excellent technical assistance. This study was partly supported by the Ernst and Berta Grimmke-Stiftung, Düsseldorf, Germany.

Table IV. Schematic representation of the proposed transition states of chromatin conformation in T- and S-cells after treatment with NB at increasing concentrations. As to the terms "static" and "dynamic chromatin" see ref. [10].

Novobiocin at increasing concentrations (> 0.35 M)			
Chromatin Conformation I	Chromatin Conformation II	Chromatin Conformation III	Chromatin Conformation IV
"Static Chromatin"	"Dynamic Chromatin"	"Static Chromatin"	Highly condensed Chromatin
DNA negatively supercoiled	Relaxed DNA	DNA positively supercoiled	Depletion of, *e.g.*, nucleic and protein synthesis
Prevails in T-cells	Stimulation of, *e.g.*, UDS and RNS	Prevails in S-cells	

A transition of conformation III into conformation IV is suggested in 3-aminobenzamide- and/or hyperthermia-treated S-cells whereas Hoechst 33258 seems to elevate the amount of conformation II in the same cell species (Table III).

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