DNA topoisomerase activities in concanavalin A-stimulated lymphocytes

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Topoisomerase activities have been measured in nuclear extracts of concanavalin A-stimulated lymphocytes. In parallel with the wave of DNA synthesis, type II topoisomerase activity was considerably increased. After 72 h treatment, this activity was stimulated approx. 20-fold over the activity in untreated cells. In contrast, type I topoisomerase was poorly stimulated after 24 h treatment, and 4–5-fold after 72 h. These findings, together with our previous results on regenerating rat liver, suggest a major role of topoisomerase II in DNA replication.

Mitogenic agent *DNA replication* *Lymphocyte* *Topoisomerase*

1. INTRODUCTION

Circumstantial evidence for the involvement of topoisomerases in DNA replication has been demonstrated in a variety of experimental systems [1–3], especially in prokaryotes and bacteriophages. In these organisms, the concerted use of specific inhibitors [4,5] and conditional lethal mutants [6] led to the conclusion that gyrase (topoisomerase II) was involved in DNA replication, in particular in the initiation stage. This conclusion was also supported by studies on an in vitro replication system using the *Escherichia coli* origin of replication (ori C) [7]. In this system, the ω protein (topoisomerase I) was also necessary [3]. One hypothesis is that ω and gyrase may regulate DNA replication (as well as other genetic processes) by precisely controlling the level of superhelicity of the DNA.

In eukaryotes, very little is known on the function of topoisomerases. Since the discovery of a nicking-closing (topoisomerase I) activity in mammalian cells [8], a common idea was that this type of enzyme represents the hypothetic swivelase necessary to remove the positive supercoils generated ahead of the replication fork [8]. However, topoisomerase I activity was found remarkably constant in a variety of systems, including cultured cells in S phase, virus-infected cells or regenerating liver [9,10]. In contrast, we have shown that eukaryotic type II topoisomerase, a ubiquitous enzyme initially discovered for its catenation properties [11] was considerably increased in regenerating liver [10]. Recently, a type II topoisomerase activity was reported to be induced in fibroblasts by the action of epidermal growth factor (EGF) [12]. Moreover, it has been suggested that the EGF receptor was itself a topoisomerase [13].

Here, we describe another experimental system in which type II topoisomerase is considerably increased: cultured guinea pig lymphocytes

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Abbreviations: Con A, concanavalin A; EGF, epidermal growth factor

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stimulated by the mitotic agent Con A. This system is useful for studying the sequence of events during the transition of a cell from a resting to an active growth state.

2. EXPERIMENTAL

2.1. *Cell cultures and thymidine incorporation*

The preparation of guinea pig lymph node cell suspensions and cell cultures was performed as previously described [14]. The cells were cultured at a concentration of $1-2 \times 10^6$ cells/ml in tissue culture flasks (Falcon) in a volume of 12 ml per flask. Cultures were incubated at 37°C, in a humidified atmosphere containing 5% CO_2 in air. The culture medium was RPMI 1640 [15] supplemented with 100 U/ml penicillin and 5% guinea pig serum. Cultures were stimulated by the addition of 7.5 μg/ml of Con A (Miles-Yeda). [^{3}H]thymidine (CEA, Saclay) incorporation was measured with 24-h pulses as in [14].

2.2. *Preparation of nuclear extracts*

Cultured lymphocytes (10^8 cells) were centrifuged at 650 × *g* for 10 min, washed in 10 ml of 50 mM Tris–HCl, pH 7.5, 25 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$ (TKCM buffer) containing 0.25 M sucrose and 1 mM PMSF (from Sigma) and resuspended in 1 ml of the same buffer. The cells were disrupted in a Potter homogenizer at 0°C, and centrifuged at 2000 × *g* for 10 min. The nuclear pellet was suspended in 1 ml of TKCM buffer containing 0.25 M sucrose, layered over a 0.4 ml cushion of TKCM buffer containing 0.6 M sucrose, and centrifuged at 2000 × *g* for 10 min. The nuclei were then washed in TKCM containing 0.25 M sucrose and 0.5% Triton X-100. At this stage, aliquots of nuclei suspension, dispersed in Triton X-100–TKCM buffer, were easily counted in a Malassez chamber under the microscope [10]. The nuclei were collected by centrifugation at 2000 × *g* for 10 min, washed twice with TKM–0.25 M sucrose buffer (identical to TKCM, but containing 5 mM $MgCl_2$ instead of 3 mM $MgCl_2$, and lacking $CaCl_2$) and finally resuspended in 0.3 ml of TKM.

The nuclear extracts were prepared as previously described [10] by lysis at high ionic strength followed by polyethylene glycol (PEG 6000) precipitation of nucleic acids. The final supernatant was referred to as Fraction II and utilized to measure topoisomerase activities.

2.3. *Preparation of the DNA substrates*

Kinetoplast DNA (k.DNA) was purified from sarkosyl extracts of *Trypanosoma cruzi* cultures kindly provided by A. Berneman and H. Eisen (Département d'Immunologie, Institut Pasteur). After extraction of proteins with phenol, the k.DNA was purified by cesium chloride–ethidium bromide density centrifugation [16].

Supercoiled plasmid pBR 322 was prepared from *E. coli* strain HB 101 as in [10].

2.4. *Topoisomerase assays and quantitation of activities*

Detection of topoisomerase activities in lymphocytes has been performed on serial dilutions of the nuclear extracts (fraction II), as described in the case of regenerating liver [10]. Dilution of the extract has several advantages: as shown in the upper part of fig.1, relaxation of the DNA was severely reduced with pure fraction II from lymphocytes (lane 2), but was already apparent with dilutions 1/2 (lane 3), 1/4 (lane 4) or with more dilute fractions. This phenomenon, also found in rat liver [10] and in mouse L cells [17] was first described in the case of calf thymus and Hela cells [18]. Dilutions ranging from 1/8 to 1/200 were used here, so that the effect of 'crude fraction' was abolished. Moreover, the possible interference with nuclease activities (although these are not prominent in our lymphocyte extracts) was also minimized by dilution. As shown in an agarose gel containing chloroquine (fig.1, lower part), relaxation of the DNA, even with the slightly diluted fraction II (1/4 or 1/2), gave covalently closed DNA without apparent increase in form II.

Both topoisomerases I and II were present in the same extracts. Separate determination of each type of enzyme was performed by using specific assays, as previously described in the case of regenerating rat liver nuclei [10].

Topoisomerase I was measured by the relaxation of supercoiled DNA (pBR 322) in the absence of ATP and magnesium ions. Topoisomerase II was measured by the decatenation of the natural networks of kinetoplast DNA (k.DNA) in the presence of ATP and magnesium ions. In both cases, serial dilutions of the extracts were used and

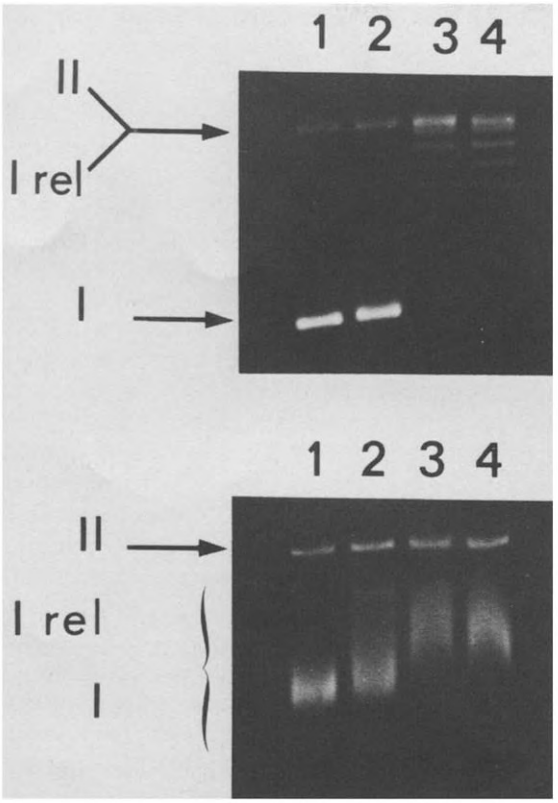

Fig.1. Relaxation of pBR 322 DNA by various dilutions of fraction II from lymphocytes. The DNA (0.4 μg) was incubated with 2 μl of fraction II in the presence of ATP and magnesium, as in [10]. In these conditions, both topoisomerase I and II are active [10]. After incubation, each sample was separated in two halves and analyzed in two agarose gels. Upper: gel without chloroquine. Lower: gel run in the presence of 3 μg/ml chloroquine. Lane 1, pBR 322 control; lanes 2–4, dilutions 1/1, 1/2, 1/4 of fraction II.

the products of incubations were analyzed by electrophoretic migration in 1% agarose gels, essentially as previously described [10]. After migration and staining with ethidium bromide, photographs of the gels were taken under short-wave (254 nm) UV light. Densitometric profiles of the negatives were made using a Vernon densitometer and peak areas of the supercoiled and relaxed DNA (in the case of relaxation) and of the free minicircles (in the case of decatenation) were directly integrated. In each case, peak areas obtained with the various dilutions of the extracts were compared to standards of fully supercoiled and fully relaxed DNA on one hand, and of fully decatenated k.DNA (100% minicircles) on the other.

One unit of topoisomerase I is defined as the amount of enzyme necessary to relax 50% of the 0.4 μg input supercoiled DNA in 30 min at 37°C.

One unit of topoisomerase II is defined as the amount of enzyme necessary to decatenate 50% of the 0.3 μg input k.DNA in 30 min at 30°C.

In some experiments, the DNA was analyzed in agarose gels containing 3 μg/ml chloroquine, a drug which changes the torsion of the DNA, allowing a clear separation of nicked (form II) DNA from covalently closed forms.

3. RESULTS AND DISCUSSION

Fig.2 illustrates a gel analysis of topoisomerase II activities in Con A-treated lymphocytes: decatenation of kinetoplast DNA gave rise to 1.48 kbp minicircles appearing as a rapidly migrating band in agarose gels (lanes 1,8). Topoisomerase II was present at low levels in untreated lymphocytes (lanes 2–4) and considerably stimulated in lymphocytes cultured for 72 h with Con A (lanes 5–7).

The quantitative aspects of the present study are represented in table 1 and fig.3. The number of enzyme units per 10^8 nuclei (table 1) and the stimulation ratio (fig.3) are expressed as a function of time

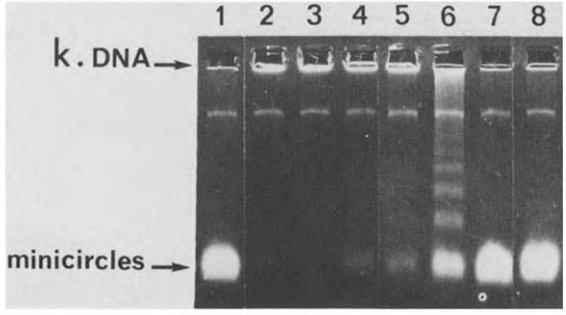

Fig.2. Decatenation (topoisomerase II) activities in lymphocyte nuclei after 72 h of culture. Kinetoplast DNA (0.3 μg) was incubated in the standard reaction mixture with serial dilutions of the nuclear extracts (fraction II) for 30 min at 30°C, and analyzed in an agarose gel. Lane 1 and 8: decatenated k.DNA control. Lanes 2–4, activities in untreated lymphocytes with dilutions 1/8, 1/4, and 1/2. Lanes 5–7, activities in Con A-treated lymphocytes with dilutions 1/48, 1/32, 1/16.

Table 1

Topoisomerase activities in lymphocytes after various stimulation times

Enzyme	Topoisomerase I (10^3 units/ 10^8 nuclei)			Topoisomerase II (units/ 10^8 nuclei)		
Time of culture (h)	24	48	72	24	48	72
Treated by Con A	5.7	12.8	24.5	760	1340	3900
Untreated	5.3	4.8	4.5	217	245	224

Enzymatic activities, assayed as described in section 2, are expressed in 10^3 units/10^8 nuclei (topoisomerase I) or in units/10^8 nuclei (topoisomerase II). Each value is an average of 3–5 independent preparations of lymphocytes

of culture. Treatment of lymphocytes with Con A resulted in a wave of DNA synthesis as shown by [^{3}H]thymidine incorporation (fig.3A) up to 3 days of culture.

Type II topoisomerase activity exhibited a considerable increase during this period (fig.3B). This increase closely paralleled the stimulation of DNA polymerase activity previously described in this experimental system [19] or in closely related systems [20,21]. After 72 h of treatment, the stimulation ratio was respectively about 13 and 18 for DNA polymerase [19] and topoisomerase II. In contrast, topoisomerase I activity was poorly stimulated after 24 h of treatment and 4–5-fold after 72 h (fig.3B and table 1). In untreated control lymphocytes, thymidine incorporation (fig.3A) as well as topoisomerases I and II activities (table 1) did not change appreciably with the time of culture, as also reported in the case of DNA polymerase.

The experiments reported here indicate in the first place that both topoisomerases are present in unstimulated lymphocytes, though at a low level in the case of type II enzyme. These results are consistent with the situation found in other resting cells [10]. Upon stimulation of the cells, the increase of type II topoisomerase is precisely parallel to the rise of DNA replication rate and of DNA polymerase activity [19,20], as we have recently reported in regenerating liver [10]. These findings suggest that type II topoisomerase, as DNA polymerase, plays a major role in DNA replication or in related processes such as chromatin decondensation/condensation in mammalian cells. Recent studies on topoisomerase II thermosensitive mutants in yeast [22] indicate that the enzyme is probably necessary for the segregation of chromosomes at the termination of DNA replication. Similar results were reported by authors in [23] in *E. coli* gyrB ts mutants, where daughter nucleoids remained catenated at the non-permissive temperature. Studies of chromatin assembly in *Xenopus* oocytes suggest the possible involvement of type II topoisomerase in this process.

We also demonstrate that type I topoisomerase

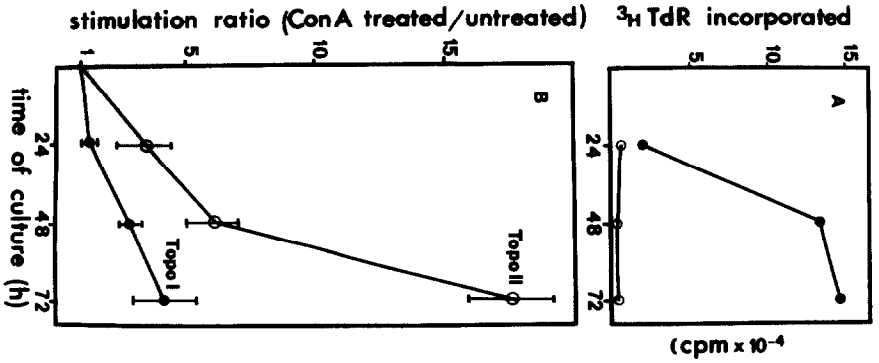

Fig.3. Stimulation of topoisomerases in Con A-treated lymphocytes. (A) [^{3}H]Thymidine incorporation into lymphocytes after various culture times: (●—●), lymphocytes treated with Con A; (○—○), untreated control. (B) The ratio of topoisomerases activities plotted against the culture time: (●—●), topoisomerase I; (○—○), topoisomerase II. The vertical bars represent the extreme variations observed in various lymphocyte preparations.

is increased in stimulated lymphocytes, though at a moderate level. This result is in agreement with [24] in the case of synchronized lymphocytes, but contrasts with data reported [9,10] in several other biological systems, including cell cultures, virus-infected cells and regenerating liver where topoisomerase I activity remains constant. This situation is perhaps restrained to lymphocytes where repair and recombination capacity is high [20], and may correlate with increase in topoisomerase I activity, if, as has been suggested [25], this enzyme is involved in recombination.

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