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Escherichia coli DNA synthesis in vitro: insensitivity of ATP-dependent DNA repair to inhibition by novobiocin

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ABSTRACT

Novobiocin, an effective inhibitor of DNA replication in Escherichia coli, is shown to have no effect on the ATP-dependent DNA repair carried out by toluenized cells after ultraviolet irradiation. Therefore novobiocin can be considered a selective inhibitor of replicative DNA synthesis in vitro.

INTRODUCTION

Novobiocin and the related drug coumermycin are effective inhibitors of semiconservative DNA replication in nucleotide-permeable E. coli cells (1,2) and in cell-free systems for the replication of small plasmids (3,4). On the other hand, the "repair-like" DNA synthesis carried out by DNA polymerase I in unirradiated cells (5) is not affected by these drugs.

Cells deficient in DNA polymerase I still can carry out an ATP-dependent nonconservative DNA repair which is induced by ultraviolet radiation (6,7). There appear to be two ATP-dependent steps in this repair pathway, one required for incision and one associated with DNA resynthesis (8). The repair synthesis is distinct from ATP-dependent replicative DNA synthesis in that it persists at the restrictive temperature in dnaB and polC(dnaE) mutants thermosensitive in DNA replication (9). Furthermore, arabinosyl nucleotides and nalidixic acid which inhibit DNA replication in permeabilized cells have little or no effect on the ATP-dependent DNA repair (10,11). It was therefore of interest to investigate whether this mode of repair synthesis is also insensitive to novobiocin or whether this drug inhibits ATP-dependent DNA synthesis in general.

The results presented in this paper indicate that novobiocin is a selective inhibitor of replicative DNA synthesis.

MATERIALS AND METHODS

Preparation of toluenized cells

E. coli TG 169 (F^- *polA1 dnaB thy his malA* str^r) was kindly provided by Dr. P. C. Hanawalt, Stanford. 1-l cultures of this strain were grown in M-9 medium containing 1 mCi ^{3}H-thymidine at 32°C. At an $O.D._{600}$ of 0.5 the cells were collected by centrifugation at room temperature and resuspended in one third of the original volume of M-9 medium without a carbon source. The culture was then divided into two portions, one portion served as unirradiated control, 15-ml samples of the other portion were exposed in 9.5 cm petri dishes to ultraviolet light (600 ergs/mm^2). All steps after irradiation were performed in yellow light. Cells were concentrated 50-fold from the original density in 50 mM potassium phosphate buffer pH 7.5 and treated with 1% toluene for 2 min at 30°C.

Assay of DNA synthesis

0.2 ml of the toluenized cell suspension (3 x 10^9 cells) were directly pipetted into a prewarmed assay mixture containing a final concentration of 40 mM potassium phosphate pH 7.5, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM NAD, and 25 µM each of dATP, dCTP, dTTP, and ^{32}P-dGTP (specific activity 0.5 Ci/mmol corresponding to about 1000 cpm/pmol) in a total volume of 0.5 ml. Incubations were carried out at 33°C and 44°C respectively. At the times indicated 0.05 ml aliquots were removed and pipetted into 1-ml of 0.5 M NaOH - 0.5% sodium dodecylsulfate - 10% saturated sodium pyrophosphate. After a 15 min incubation at 44°C the samples were placed in an ice bath and 4 ml of cold 2 M trichloroacetic acid were added. Acid-insoluble radioactivity was determined as described by Wirtz & Hofschneider (12).

Isopycnic analysis

Standard assay mixtures (0.5 ml) containing bromodeoxyuridine triphosphate (BrdUTP) instead of dTTP were incubated for 30 min and incorporation was stopped by addition of an equal volume of

0.1 M EDTA and cooling in an ice bath. Cells were washed once with 50 mM Tris-HCl pH 8.0 - 1 mM EDTA - 0.1 M NaCl, resuspended in 1 ml of the same buffer, and incubated with 0.5 mg/ml lysozyme for 30 min at $0^{o}C$. Cells were lysed by addition of 0.1 ml 5% sarkosyl and the viscosity of the lysate was reduced by repeated shearing with a syringe. Total lysates were then subjected to equilibrium centrifugation in a Spinco Ti-50 rotor as described previously (13).

Chemicals

Novobiocin was from Sigma Chemical Co., St. Louis. Oxolinic acid was obtained from Gödecke AG., Freiburg. ^{32}P-dGTP (2.5 Ci/mmol) was purchased from The Radiochemical Centre, Amersham. The sources of all other materials have been described previously (13).

Results and Discussion

The amount of ATP-dependent repair synthesis in toluene-treated preparations of UV-irradiated cells is small (less than 5%) compared to the amount of semiconservative DNA replication performed by unirradiated control cells under identical conditions (6). Studies on this mode of DNA repair are therefore facilitated by employing double mutants deficient in polymerase I and thermosensitive for DNA replication. As shown in Fig. 1 unirradiated cells of strain TG 169 (*dnaB*) give only a low background incorporation during incubation at the restrictive temperature ($44^{o}C$). Upon UV-irradiation a significant incorporation of label is observed which levels off after about 10 min. This incorporation is nearly unaffected by novobiocin at a concentration which reduces replicative DNA synthesis by more than 90% (1).

Table 1 summarizes the results observed with various drug concentrations. The ATP-dependent DNA repair is only slightly affected even after increasing the novobiocin concentration to 500 µg/ml. A similar amount of inhibition is also observed in the presence of high concentrations of the quinolone antibacterial agents nalidixic acid and oxolinic acid (14,15). This is probably due to secondary effects unrelated to the mechanism

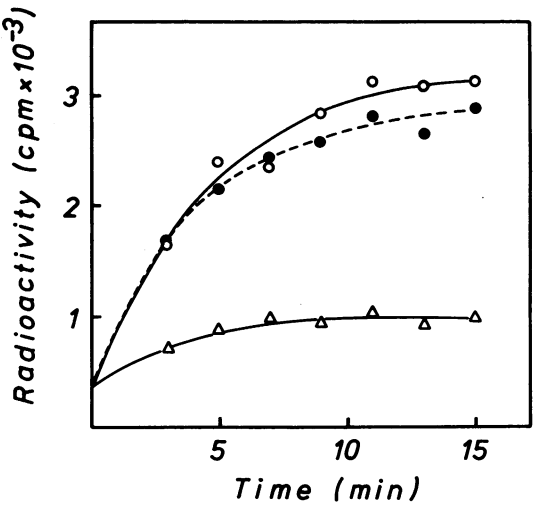

Fig. 1: Kinetics of ^{32}P-dGMP incorporation by toluenized E. coli TG 169 cells
Standard incubation mixtures (Materials and Methods) were incubated at 44°C. At the times indicated 0.05 ml aliquots were removed and assayed for acid-insoluble radioactivity.
(o——o), UV-irradiated cells; (●---●), UV-irradiated cells plus novobiocin (100 µg/ml); (Δ——Δ), unirradiated control

Table 1: UV-stimulated repair synthesis in toluenized E. coli cells
The complete system is the standard incubation mixture (0.5 ml) described in Materials and Methods. Incubation was carried out for 30 min at 44°C. 100% activity corresponds to 24.6 pmol dGMP incorporated by 3 x 10^9 cells.

Additions or omissions	^{32}P-dGMP incorporation
	%
Complete system	100
- ATP	22
+ nalidixic acid (100 µg/ml)	91
+ oxolinic acid (50 µg/ml)	94
+ novobiocin (20 µg/ml)	94
+ novobiocin (100 µg/ml)	88
+ novobiocin (500 µg/ml)	85

by which these drugs interfere with DNA replication.

These conclusions are confirmed by pycnographic analysis of the newly synthesized DNA. Since the repair patches are small

compared to the molecular weight of approximately 20 x 10^6 dt found for unsonicated DNA fragments (16), the bromodeoxyuridine incorporated should not appreciably alter the buoyant density of the fragments carrying the repaired regions. Repair synthesis is therefore clearly distinguishable from replicative synthesis, which yields DNA of hybrid density. Control experiments show that the semiconservative DNA replication observed in the unirradiated TG 169 cells at the permissive temperature (Fig. 2A) is completely blocked by addition of 100 µg/ml of novobiocin (Fig. 2B) or raising the temperature to 44°C (Fig. 2C). The UV-stimulated repair synthesis detectable at 44°C (Fig. 2D) is reduced to the background level of the unirradiated control by omission of ATP (Fig. 2E), but unaffected by addition of novobiocin (Fig. 2F).

To investigate whether novobiocin has any effect on the size distribution of the repair patches, the DNA from the parental density region of the neutral CsCl gradients shown in Fig. 2 was extensively sheared by sonication to a single strand molecular weight of about 0.5 x 10^6 and analysed by equilibrium centrifugation in alkaline CsCl gradients (Fig. 3). It can be seen that the density profile of the repaired DNA was unaffected by novobiocin. However, in contrast to the size heterogeneity of repair patches produced *in vivo* (16) only short patch repair is observed in the toluenized cell system. This probably reflects the limited extent of the repair reaction under the *in vitro* conditions employed (8).

Recent work by Gellert et al. (4,17) has shown that novobiocin inhibits DNA gyrase, an enzyme that introduces negative superhelical turns into closed circular DNA in an ATP-dependent reaction. These authors have suggested that DNA gyrase might act as a swivel removing positive superhelical turns introduced by replication. Since such a swivel function is obviously not required for repair synthesis after excision of damaged DNA, this would explain the selective inhibition of replicative DNA synthesis by novobiocin.

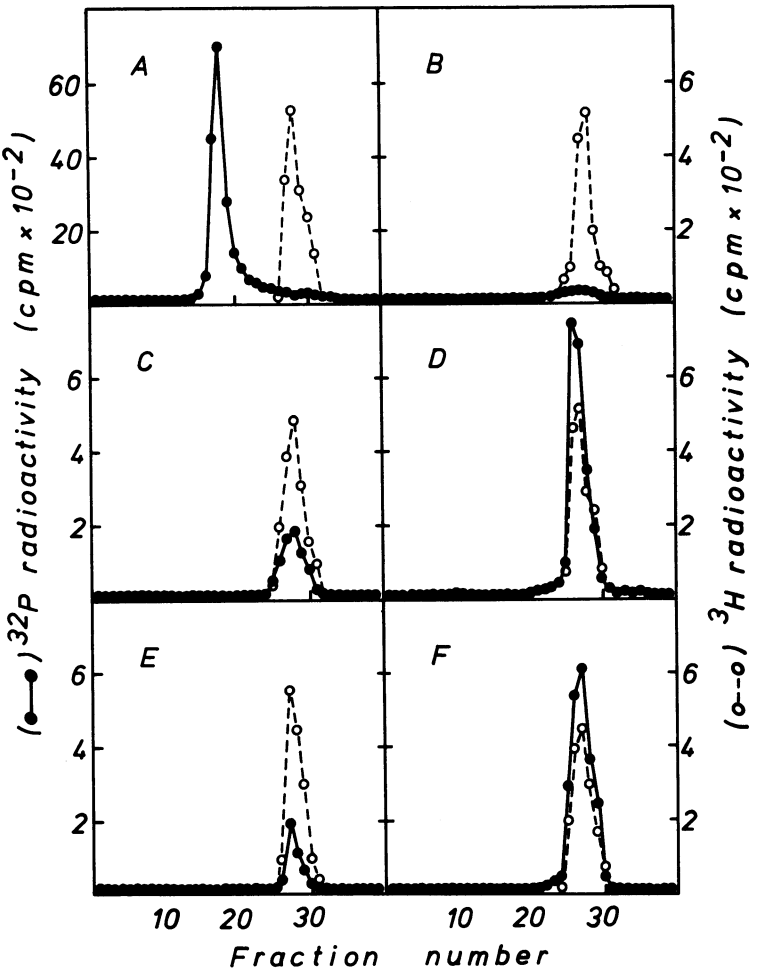

Fig. 2: Pycnographic analysis in neutral CsCl
Standard incubation mixtures (0.5 ml) containing BrdUTP instead of dTTP were incubated for 30 min at 33°C (A and B) or 44°C (C - F). Novobiocin was added (B and F) at a concentration of 100 µg/ml. Cells were lysed and the newly synthesized DNA analysed by buoyant density centrifugation in CsCl as described in Methods. (A) Unirradiated cells incubated at 33°C, (B) unirradiated cells incubated at 33°C plus novobiocin, (C) unirradiated cells incubated at 44°C, (D) UV-irradiated cells incubated at 44°C, (E) UV-irradiated cells incubated at 44°C without ATP, (F) UV-irradiated cells incubated at 44°C in the presence of novobiocin. Density increases from right to left. (●——●), ^{32}P-labeled newly synthesized DNA; (o---o) ^{3}H-prelabeled DNA

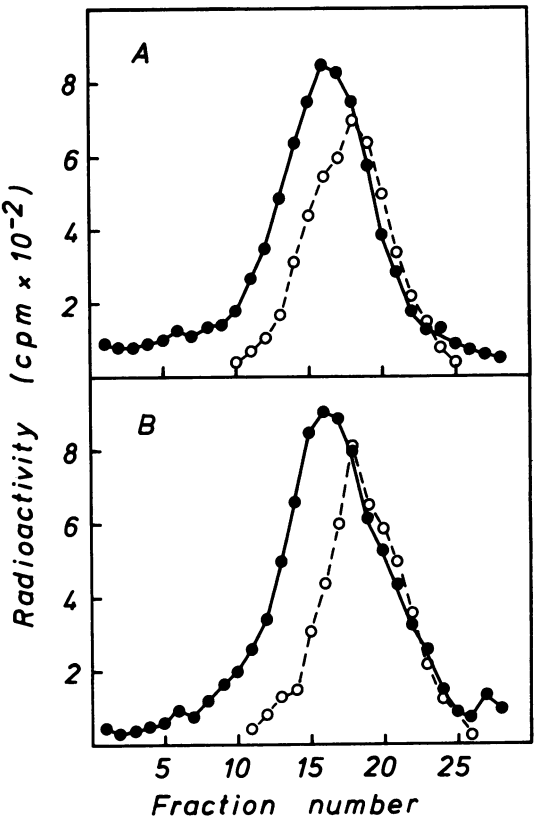

Fig. 3: Pycnographic analysis in alkaline CsCl of extensively sheared repaired DNA
UV-irradiated toluenized cells (0.4 ml) were incubated in a standard incubation mixture (total volume 1.0 ml) containing dBrUTP for 30 min at 44°C in the absence (A) or presence (B) of novobiocin (100 g/ml). Repaired DNA of parental density was isolated as described in Fig. 2 and extensively sheared by 2 min of sonication with a Branson Instruments sonifier at maximum power setting (16). The extent of shear was estimated from the sedimentation coefficient of the DNA fragments determined by alkaline velocity sedimentation (18). The sheared DNA was centrifuged to equilibrium in a CsCl gradient containing 0,2 N NaOH (13). Density increases from right to left. (●——●), ^{32}P-labeled repaired DNA; (o---o) ^{3}H-prelabeled DNA

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