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CYTOTOXICITY, CALMODULIN AND DNA LESIONS IN CELLS TREATED WITH STREPTOZOTOCIN

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Abstract—Streptozotocin is a nitrosourea-derivative containing a methyl group instead of a haloethyl group. Treatment of human adenocarcinoma cells with streptozotocin induces DNA lesions. The presence of the lesions is visualized during cell lysis in dilute alkali as changes in fragmentation of pre-labelled DNA. The increase in DNA fragmentation is paralleled by increased cytotoxicity. Furthermore prolonged duration of treatment reduces the level of DNA fragmentation. The repair of the DNA lesions is prevented by treating the cells with W-7, an inhibitor of calmodulin.

Streptozotocin (streptozocin) is an anti-neoplastic agent belonging to the nitrosoureas [1]. In its structure it contains a nitrosourea moiety with a methyl group attached to one end and a glucose molecule at the other. It is known to show antitumour activity in human colon adenocarcinoma and various endocrine-related tumours. Furthermore streptozotocin is known to produce hyperglycemia and it has a well-established diabetogenic effect [2, 3]. Compared to other nitrosoureas it has a less damaging effect on normal bone-marrow.

The mechanism of streptozotocin cytotoxicity is largely unknown. The action of streptozotocin on cells is independent of the stage of cell cycle [4]. The cytotoxicity of other nitrosoureas is probably due to formation of inter-strand DNA cross-links. The cross-links are believed to appear by a two-step mechanism in which O^6-alkylation of guanine via haloethyldiazonium hydroxide occurs first on one DNA strand and is followed by a reaction with the other DNA strand, thus forming a bridge [5]. In cells treated with streptozotocin one has so far detected O^6-methylguanine but no cross-links.

We have now examined the effect of streptozotocin on the growth of human colon adenocarcinoma cells and we show the presence of DNA lesions. The ultimate effect of streptozotocin on cells is a balance between the extent of damage inflicted on DNA and the ability of cell to repair it. We show here that in cells pretreated with W-7 (to inhibit the Ca^{2+}-binding protein calmodulin) repair of streptozotocin-induced DNA damage is reduced.

Calmodulin is a ubiquitous protein involved in cell proliferation and it is a potential target for anti-neoplastic agents used in the clinic. Furthermore calmodulin is known to be involved in the repair of DNA lesions induced by various anti-neoplastic agents, e.g. bleomycin and dacarbazine [6, 7, 8, 9].

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MATERIALS AND METHODS

Cells, culture methods, and labelling with ^{3}H-*thymidine.* A human colon adenocarcinoma cell line (WiDr) was grown as monolayers, as described earlier, in the presence of 10% fetal calf serum [10]. Growth-arrested cells were obtained by cultivation for two days in medium containing 0.5% fetal calf serum.

The out-growth experiments were performed as described by [11]. Portions of treated and untreated cells were incubated for 5 days with daily changes of medium. The level of cell survival in treated cells was measured by determining the difference in the number of cell doublings in untreated and treated cells.

For experiments involving pre-labelled DNA the cells were seeded in small culture dishes (35 × 10 mm) containing 3 ml medium with 50 μCi tritiated thymidine (20 Ci/mmole; Amersham). After 24 hr the medium was changed to fresh medium without thymidine and after another 24 hr the cells were used for drug-treatment experiments. For pulse-labelling experiments 100 μCi tritiated thymidine was added to the culture medium for the desired length of time.

Drugs. Streptozotocin was obtained from Sigma Chemicals (St. Louis, MO). W-7 and W-5 were obtained from Miles Inc., U.S.A.

Cell lysis. The incubation medium was drained off and the cells rinsed twice in cold phosphate-buffered saline. Cell lysis was performed in the dark at 0° by the addition of 2.25 ml of 0.03 M NaOH. After 30 min the solution was neutralized by the addition of 0.067 M HCl/0.02 M NaH_2PO_4. Finally the solution was made 1% with regard to SDS. For a more detailed description, see [10, 12].

Gel electrophoresis. The labelled DNA was separated in 0.75% agarose gels [13] using a LKB Multiphor electrophoretic system. When the separation was completed, the gels were sliced in 1-mm-thick slices, which were incubated in scintillation fluid

containing 3% Soluene-100 (Packard) and the radioactivity was measured in a scintillation counter (Packard).

RESULTS

To examine streptozotocin-induced lesions in DNA we use the following approach: cells are lysed in dilute alkali which removes macromolecules from the DNA and disrupts the base-pair structure of the DNA [10, 12]. However, the DNA strands cannot separate before enough time has elapsed to allow unwinding. The unwinding is initiated at gaps present in the DNA chains. Such gaps are known to exist during the process of synthesis of new DNA chains. However, the time needed to unwind the DNA is shortened when alkali-labile bonds are present in the DNA, since the cell lysis in alkali induces additional gaps in the DNA chains. Treatment of cells with X-rays or drugs as 5-Fu, ara-C introduces gaps and/or alkali-labile regions in the DNA. This results in increased numbers of points to initiate unwinding of the DNA. The amount of DNA that may be unwound at each point has been estimated as 20 kb.

When the alkaline solution is neutralized the high molecular weight DNA renatures and forms double-stranded DNA. Small DNA fragments, which are released during the unwinding, remain in solution as single-stranded DNA molecules. The fragments can then be separated from the high molecular weight DNA by agarose gel electrophoresis. We have earlier found that treatment of cells with 5-fluoropyrimidines, methotrexate or dacarbazine results in release of DNA fragments [12, 14]. Consequently it should be possible to detect whether streptozotocin induces alkali-labile regions in the DNA, since such damage should result in the generation of DNA fragments.

Treatment with streptozotocin

Although DNA-damaging agents can give rise to many different types of lesions in DNA, the damage often results in alkali-labile regions. The regions either arise through enzymatic strand scission as part of a repair process or through direct chemical alterations in the DNA molecule.

Cells with pre-labelled DNA were treated with streptozotocin (50 μg/ml) for 30 min (Fig. 1) and then either immediately analyzed or analyzed after incubation in fresh medium for 60 min or 24 hr. The results showed that immediately after treatment DNA fragmentation is detectable. During the post-incubation the level of DNA fragmentation increases and at 24 hr almost all DNA is fragmented. The initial level of DNA fragmentation is higher in cells treated for 60 min than in cells treated for 30 min (not shown).

Next, we examined cells with pre-labelled DNA and treated with streptozotocin (50 μg/ml) for 3, 6, 12 or 24 hr. Figure 2(A) shows that with increasing duration of the treatment there is less DNA fragmentation. In parallel one can detect increasing levels of high molecular weight DNA.

We also performed experiments to examine whether the level of DNA fragmentation is dose-dependent. Cells with pre-labelled DNA were treated for 30 min at 25 μg/ml, 50 μg/ml or 100 μg/ml and then immediately lysed. Figure 2(B) shows that by increasing the dose of the drug higher levels of DNA fragmentation are obtained.

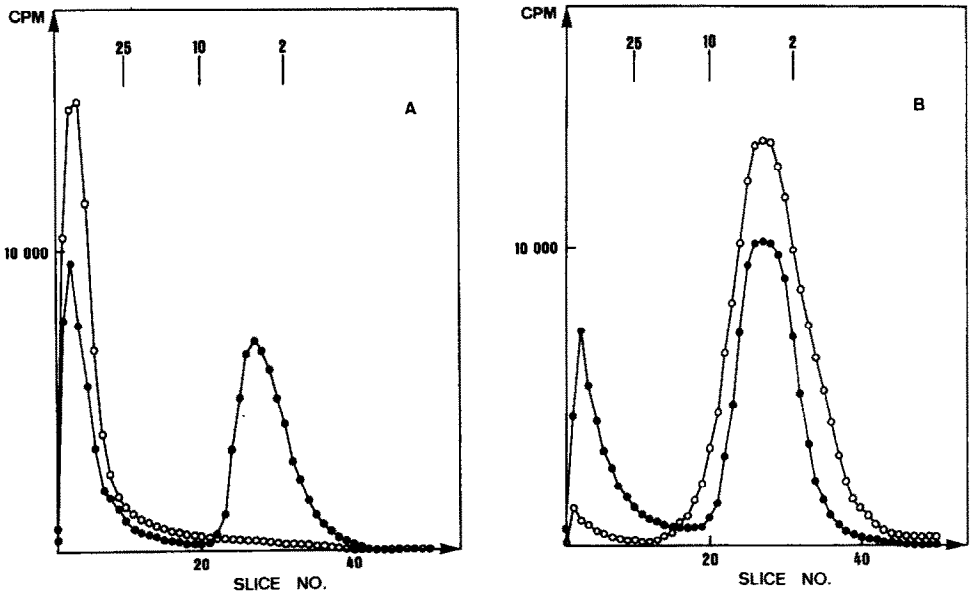

Fig. 1. Treatment with streptozotocin (50 μg/ml) for 30 min. (A) Cells with pre-labelled DNA were drug-treated (—●—) and then lysed in dilute alkali. Untreated cells (—○—). The DNA was then separated in 0.75% agarose gels. 25, 10 and 2 denote the location and size (in kb) of single-stranded DNA markers. (B) Cells drug-treated for 30 min and then incubated in fresh medium for 60 min (—●—) or 24 hr (—○—).

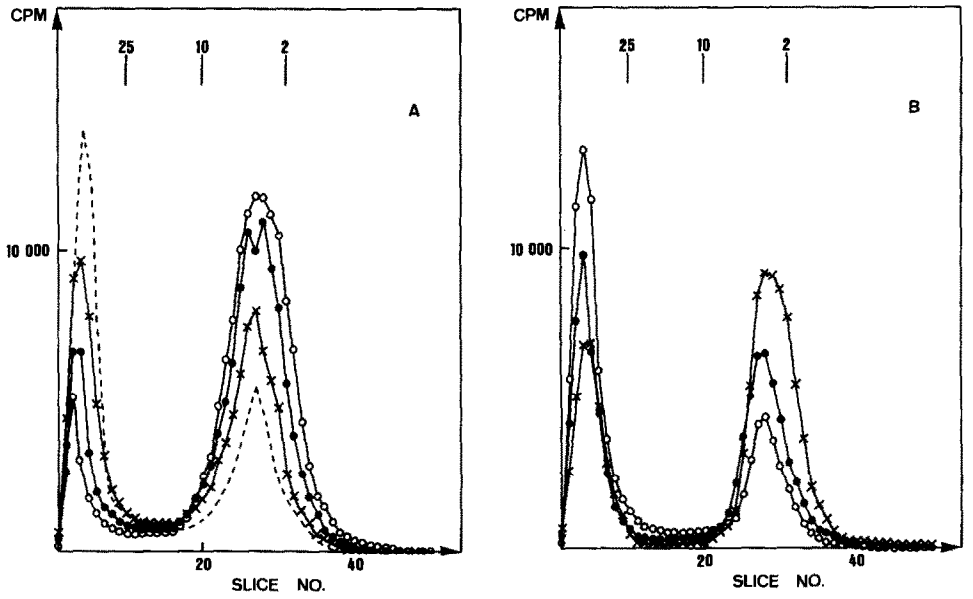

Fig. 2. (A) Cells with pre-labelled DNA were treated with streptozotocin (50 μg/ml) for 3 hr (—○—), 6 hr (—●—), 12 hr (—×—) or 24 hr (– – –) and then lysed in dilute alkali. The DNA was then separated in 0.75% agarose gels. 25, 10 and 2 denote the location and size (in kb) of single-stranded DNA markers. (B) Cells were treated with streptozotocin for 30 min at 25 μg/ml (—○—), 50 μg/ml (—●—) or 100 μg/ml (—×—).

Treatment with W-7 and streptozotocin

W-7 (a naphthalene sulfonamide derivative) is a highly specific inhibitor of calmodulin [15]. Calmodulin is involved in the repair of lesions induced in DNA by bleomycin and dacarbazine. When calmodulin is inhibited by W-7 repair of some drug-induced lesions is reduced, which is paralleled by potentiated cytotoxicity.

We therefore tested whether inactivation of calmodulin with W-7 influences the ability of the cells to repair the streptozotocin-induced DNA damage. W-7 induces DNA fragmentation in growing cells. Therefore the present experiments were performed in growth-arrest cells. Growth-arrested cells were obtained by cultivation for two days in medium containing 0.5% serum [9].

Cells with pre-labelled DNA were treated with W-7 (10 μM) for 30 min and then with W-7 and streptozotocin for 30 min. The cells were either lysed immediately or after incubation with W-7 for 24 hr (Figs 3 A and B). The results showed no DNA fragmentation, which contrasts with the almost complete DNA fragmentation in cells examined 24 hr after the incubation with streptozotocin alone.

As outlined earlier alkali-labile DNA may arise through enzymatic strand scission as part of a repair process. Since there is no fragmentation in cells treated with W-7, which is known to reduce the repair of DNA lesions, it is very likely that the absence of fragmentation is due to inhibited DNA repair although DNA lesions are formed in a normal manner.

To test this we have used the drug W-5, an analogue of W-7 with much less inhibitory effect on calmodulin [15]. We treated cells according to the same protocol as above but substituted W-5 for W-7. The results now showed a high level of DNA fragmentation (Fig. 3C).

Cytotoxicity analysis

Cytotoxicity was analyzed using the out-growth method [11]. Cells were treated with streptozotocin for either 30 min, 3 hr, 12 hr or 24 hr. In cells treated for 30 min cell growth was resumed after a delay of 2 hr. When the incubation was prolonged there was gradually a less efficient regrowth of the cells (Fig. 4A).

Dose–response experiments were also performed. Cells were incubated for 30 min with streptozotozine at 25 μg/ml, 50 μg/ml or 100 μg/ml. Figure 4(B) shows that with increasing dose there is less viability of the cells.

Furthermore, treatment with W-7/streptozotocin significantly increased cytotoxicity in comparison to treatment with streptozotocin alone (Fig. 4C). This increase was not seen in cells treated with W-5/streptozotocin. This implies, as stated above, that W-7 prevents the repair of the streptozotocin-induced DNA lesions.

DISCUSSION

Streptozotocin is a nitrosourea derivative [1]. The structure differs from other nitrosoureas in one important aspect, the presence of a methyl group instead of the haloethyl group thought to be responsible for DNA cross-linking induced by the haloethylnitrosoureas. In this paper we have analy-

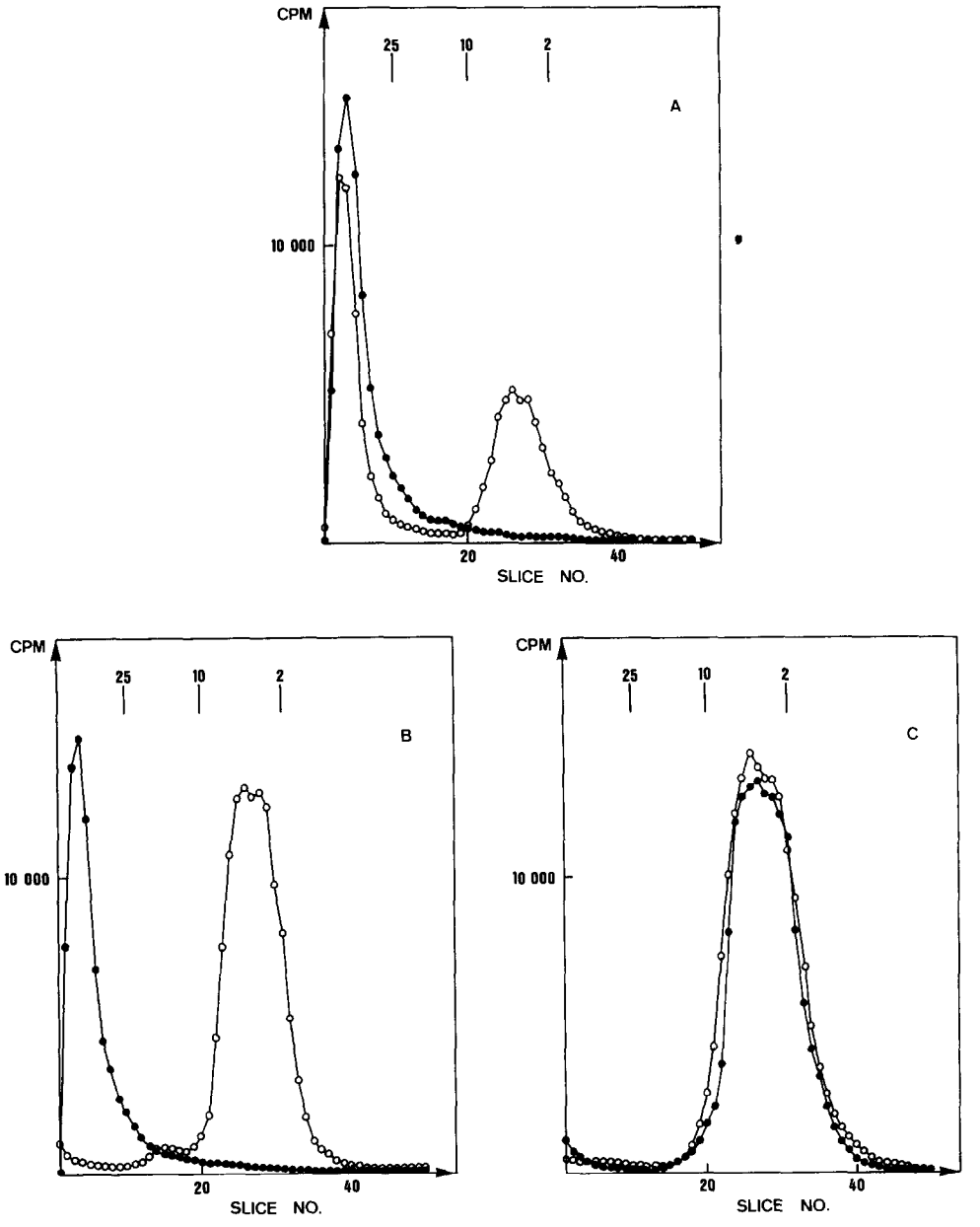

Fig. 3. Treatment with inhibitors of calmodulin. (A) Cells with pre-labelled DNA were treated with W-7 (10 μM) for 30 min and then W-7 and streptozotocin (50 μg/ml) for another 30 min (—●—). Cells treated with streptozotocin alone (—○—). The cells were lysed in dilute alkali and the DNA then separated in 0.75% agarose gels. 25, 10 and 2 denote the size (in kb) and location of single-stranded DNA markers. (B) Cells with pre-labelled DNA were treated with W-7 for 30 min, W-7 and streptozotocin for 30 min and W-7 for another 24 hr (—●—). Cells treated with streptozotocin for 30 min and then incubated in fresh medium for 24 hr (—○—). (C) Cells with pre-labelled DNA were treated with W-5 (10 μM) for 30 min, W-5 and streptozotocin for another 30 min and then with W-5 for 24 hr (—●—). Cells treated with streptozotocin for 30 min and then incubated in fresh medium for 24 hr (—○—).

zed the induction and the repair of lesions in DNA induced by streptozotocin. The level of lesions that are undergoing repair is correlated with the cytotoxicity induced by the drug.

DNA of drug-treated cells is often fragmented during cell lysis in dilute alkali. The fragments arise either through enzymatic strand scissions as part of a repair process or through direct chemical alterations in the DNA. In cells with pre-labelled DNA one can detect DNA fragmentation induced by a short incubation with streptozotocin. The amount of DNA fragments increases with increasing drug concentration as well as with increasing duration of the post-incubation period in fresh medium. This is paralleled by increasing cytotoxicity.

In contrast, when the duration of the drug-treatment is increased one detects lower levels of DNA fragments. Streptozotocin is known to produce O^6-

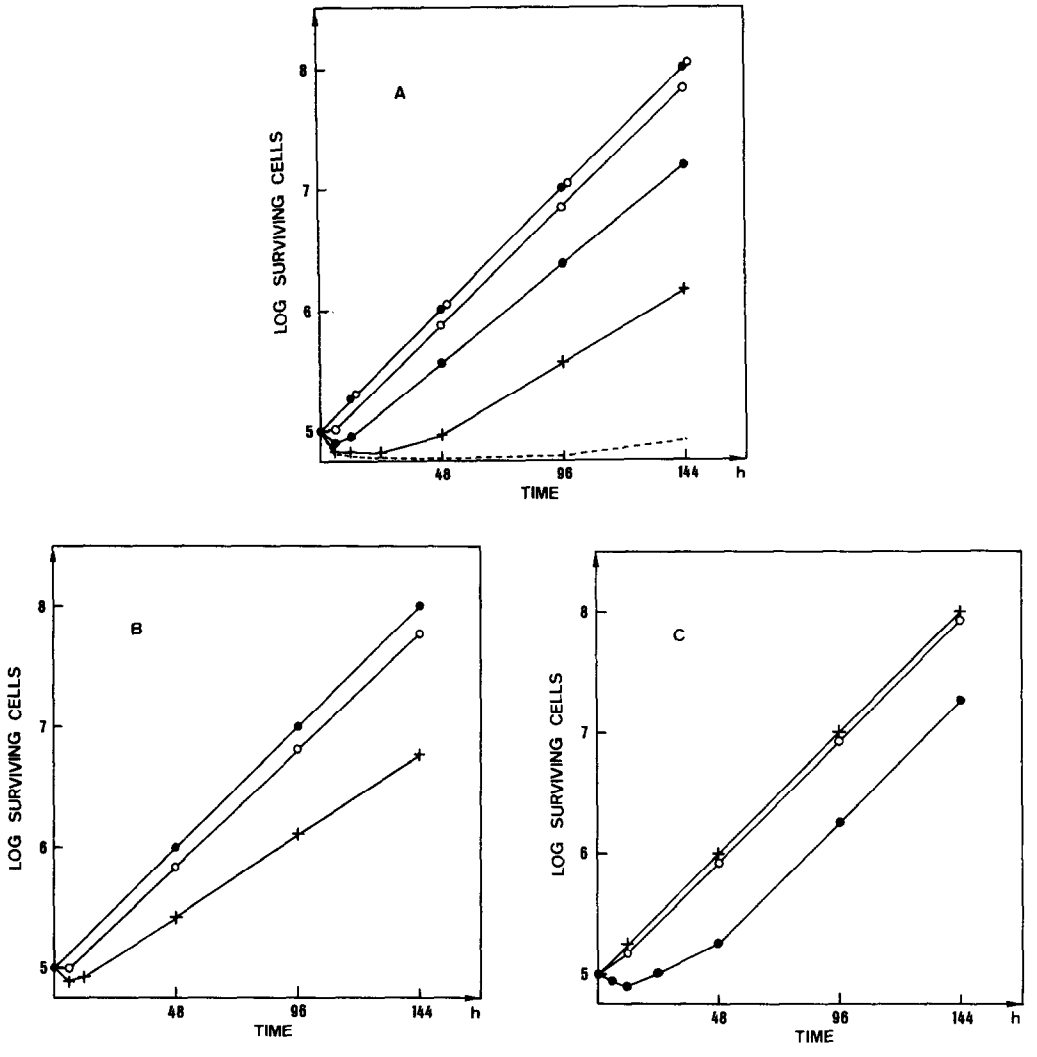

Fig. 4. Out-growth experiments. (A) Cells were treated with streptozotocin (50 μg/ml) for 30 min (—○—), 3 hr (—●—), 12 hr (—×—) or 24 hr (- - -). Control cells (—●○—). The cells were then washed free of the drug and their growth capacity was measured. (B) Dose–response experiments. Cells were treated with streptozotocin for 30 min at 25 μg/ml (—●—), 50 μg/ml (—○—) or 100 μg/ml (—×—). The cells were then washed free of the drug and their growth capacity was measured. (C) Cells were treated with W-7 (10 μM) for 30 min, W-7 and streptozotocin for 30 min and then W-7 for 24 hr (—●—). Cells treated with W-5 (10 μM) for 30 min, W-5 and streptozotocin for 30 min and then W-5 for 24 hr (—○—). Cells treated with streptozotocin for 30 min and then incubated in fresh medium for 24 hr (—×—). The cells were then washed free of drugs and 24 hr later the growth capacity of the cells was measured.

methylguanine that is reverted to guanine by the transfer of the methyl group from guanine to the enzyme alkyltransferase, whereby the enzyme is irreversibly inactivated [16–19]. It has been proposed that depletion of alkyltransferase by its reaction with O^6-methylguanine reduces the ability of the cells to repair cross-linking lesions induced by a subsequent treatment with alkylating agents [18]. The activation of alkyltransferase also occurs due to direct alkylation of the enzyme [17].

Treatment with streptozotocin introduces O^6-methylguanine. It therefore seems likely that during the prolonged treatment with streptozotocin there is depletion of alkyltransferases. When the cells are then examined after 12 or 24 hr of treatment there are no (or very few) functioning enzyme molecules left, resulting in little DNA fragmentation due to DNA repair. However, streptozotocin is less efficient in sensitizing the cells to cross-linking agents by inactivating alkyltransferase than other drugs (e.g. MNNG, MNU) that react with O^6-methylguanine [17, 18]. Therefore it is possible that this is not the only mechanism.

To modulate the level of DNA lesions we have used W-7, an inhibitor of calmodulin. Ca^{2+} and the Ca^{2+}-binding protein calmodulin are involved in the repair processes of DNA lesions induced by several anti-neoplastic agents (as e.g. bleomycin, vincristine,

antracyclines, dacarbazine). We have found that calmodulin is also involved in the repair of the streptozotocin-induced DNA lesions. Inhibition of calmodulin with W-7 results in reduced repair of the lesions. In agreement with this, in cells treated with W-7/streptozotocin, we observe increased cytotoxicity. This is a potential target mechanism for anti-neoplastic agents used in the clinical treatment of patients [7]. In contrast W-5, an analogue of W-7 with much less inhibitory effect on calmodulin, does not change the level of DNA lesions and the cytotoxicity induced by streptozotocin.

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