INHIBITORS OF NUCLEAR ADP-RIBOSYL TRANSFERASE RETARD DNA REPAIR AFTER *N*-METHYL-*N*-NITROSO-UREA

Further evidence for the involvement of $(ADP\text{-ribose})_n$ in DNA repair

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1. Introduction

ADP-ribosyl transferase is a nuclear enzyme that transfers ADP-ribosyl residues from NAD^+ to chromatin proteins, to form mono-, oligo- and poly(ADP-ribose) (reviews [1–4]). The enzyme is totally dependent on DNA [5] and is markedly stimulated by fragmentation of the DNA [6–9]. Evidence has accumulated over the last 5 years that $(ADP\text{-ribose})_n$ participates in the cellular recovery from DNA damage [10,11]. Both radiation and alkylating agents lower the cellular NAD content [6,12–14] and thereby interfere with glycolysis. This drop in cellular NAD is mediated by nuclear ADP-ribosyl transferase [10,12]. Poly-(ADP-ribose) in intact cells is increased after DNA damage [15].

The rejoining of DNA strand breaks induced by exposure to dimethyl sulphate is retarded by representatives of all 4 classes of inhibitors of ADP-ribosyl transferase [10,11]. Furthermore, these same enzyme inhibitors also potentiate the cytotoxicity of dimethyl sulphate [10,11,16]. Finally, lowering the cellular NAD level by nutritional deprivation of nicotinamide totally prevents DNA repair after dimethyl sulphate [10]. All this evidence argues forcefully that $(ADP\text{-ribose})_n$ participates in DNA repair.

However, the above experiments examined repair of damage induced by dimethyl sulphate. It is known that the spectrum of damage induced in DNA depends on the chemical properties of the damaging agent. For example, dimethyl sulphate produces rather small proportions of O-6 methyl guanine. By contrast, *N*-methyl-*N*-nitroso-urea (MNU) produces a substantially greater proportion of O-6 methyl guanine [17].

Here we describe experiments using MNU. We show that both the enzyme inhibitors, thymidine and 5-methylnicotinamide retard DNA strand-rejoining after MNU damage as assessed by alkaline sucrose gradient analysis. These data indicate that $(ADP\text{-ribose})_n$ biosynthesis is a necessary requirement for repair of DNA damage induced by a range of alkylating agents.

Thymidine not only inhibits ADP-ribosyl transferase but also interferes with the biosynthesis of deoxycytidine. However, the inhibition of deoxycytidine biosynthesis can be circumvented by adding deoxycytidine to the medium. Under these conditions there is no major distortion of nucleotide levels and L1210 cells show a cloning efficiency in soft agar of >95% [16]. Any residual consequences of the thymidine are probably not due to interference with nucleotide metabolism.

2. Methods

2.1. *Cells*

Mouse leukaemia (L1210) cells were grown in RPMI 1640 medium supplemented with 10% horse serum, penicillin (50 units/ml) and streptomycin (37.5 units/ml) at 37°C in 95% air, 5% CO_2. Cell number was estimated using a model B Coulter counter or a haemocytometer.

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2.2. *Alkylating reagent*

A solution of MNU was prepared immediately before each experiment in 100 mM citrate buffer (pH 4.5).

2.3. *Radioactive labelling of DNA*

DNA was labelled with radioactive thymidine; $1-2 \times 10^5$ cells/ml was incubated for ~1.5 generation times (18 h) in 1.0 μCi/ml, 1 μM (18.2 Ci/mmol) [^{3}H]thymidine. Under these conditions the cells incorporated [^{3}H]thymidine into DNA at a constant rate for at least 12 h but the growth rate was reduced by ~20% due to perturbations in the cell cycle by the radioactive DNA precursor [18].

2.4. *Exposure of cells to alkylating agent*

After washing in fresh medium and centrifugation (1000 rev./min for 5 min in a MSE bench centrifuge) to remove the radioactive precursors, the cells were incubated for 20 min with 1 mM MNU in the presence or absence of the enzyme inhibitor. After the MNU treatment the cells were washed twice in phosphate-buffered saline at 4°C then resuspended in medium pre-warmed to 37°C and incubated further in the presence or absence of enzyme inhibitor.

2.5. *Alkaline sucrose gradient analysis of DNA repair*

Samples of 1 ml were taken at various times after treatment, resuspended in phosphate buffered saline at $\sim 5 \times 10^5$ cells/ml. $1-2 \times 10^4$ Cells were analysed on alkaline sucrose gradients. Linear alkaline sucrose gradients contained 4.8 ml 5–20% (w/v) sucrose, 100 mM NaOH and 100 mM NaCl overlaid with a lysis layer of 100 μl 2% (w/v) sodium dodecyl sulphate (SDS), 20 mM EDTA [19]. The gradients were centrifuged at 25 000 rev./min at 20°C in a SW 50.1 rotor in a Beckman L5.65 centrifuge. Fractions (12 drops) were collected on filter strips and the trichloroacetic acid-insoluble radioactivity was estimated by scintillation spectrometry.

3. Results

The effect of thymidine and of deoxycytidine on L1210 cell growth was examined. Deoxycytidine at 3 mM did not slow down cell growth at all (fig.1). Thymidine at 10 mM completely blocked cell reproduction and was evidently lethal since the cell number began to decline after 40 h. The combination of

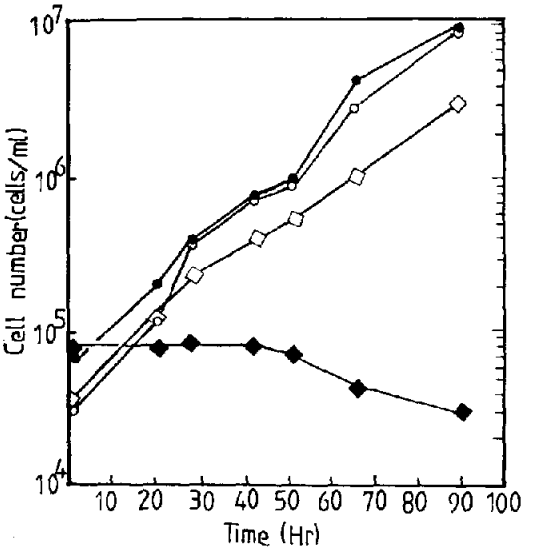

Fig.1. Effect of thymidine and deoxycytidine on growth of L1210 cells. Exponentially growing cells were resuspended into medium containing the nucleosides at 0 h and after a second dilution at 45 h. Additions to the medium: (○) 3 mM deoxycytidine; (◆) 10 mM thymidine; (◇) 3 mM deoxycytidine + 10 mM thymidine; (●) control.

3 mM deoxycytidine with 10 mM thymidine had no adverse effect on cell reproduction. With this combination the effect of thymidine on DNA repair can be examined. In [16], 2 mM thymidine combined with 3 mM deoxycytidine permitted a relative cloning efficiency of L1210 cells of >95%. The molecular mass of the DNA in alkaline sucrose gradients was not altered by preincubation in a mixture of 10 mM thymidine and 3 mM deoxycytidine (not shown).

The effect of thymidine on DNA strand-rejoining was examined in alkaline sucrose gradients. Prelabelled cells were exposed to 1 mM MNU for 20 min and then incubated for 30–60 min to permit repair. During this recovery period some cell cultures contained 10 mM thymidine and others 10 mM thymidine plus 3 mM deoxycytidine. The course of strand-rejoining in the absence of enzyme inhibitors is shown by the solid lines in fig.2. By 60 min extensive repair had occurred. By contrast, in the samples containing thymidine (fig.2d–f) or thymidine and deoxycytidine (fig.2a–c) (–), there was hardly any increase in the size of the DNA. A marked inhibition of strand-rejoining is evident. It was observed in a similar parallel experiment that 3 mM deoxycytidine did not significantly retard strand-rejoining (fig.3).

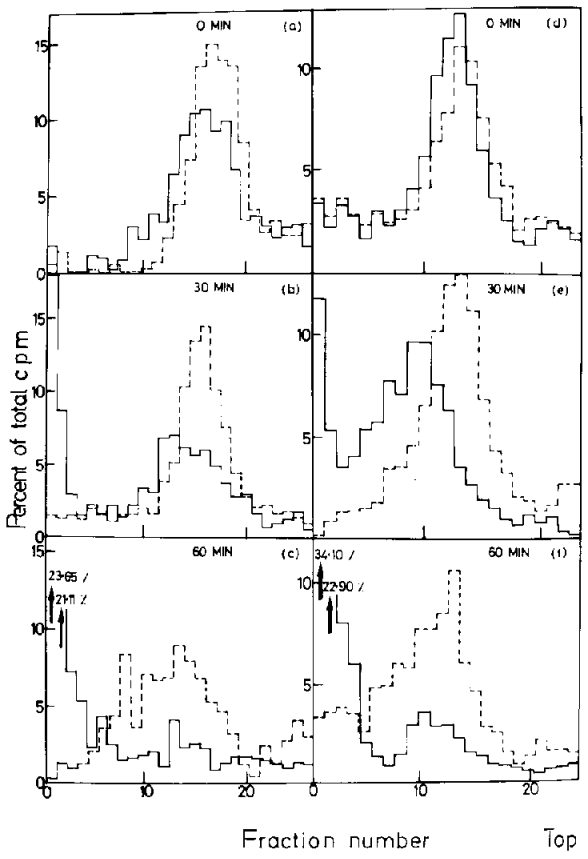

Fig.2. Inhibition of DNA strand-rejoining by the ADP-ribosyl transferase inhibitor (thymidine or thymidine and deoxycytidine) after alkylation by MNU. Samples were taken at 0, 30 and 60 min after treatment with 1 mM MNU for 20 min (——). The discontinuous lines (– – –) indicate cultures with enzyme inhibitor present; (a–c) contained 10 mM thymidine and 3 mM deoxycytidine; (d–f) contained only 10 mM thymidine. The enzyme inhibitors were present both during the 20 min alkylation period as well as during the recovery period. The gradients were centrifuged at 25 000 rev./min for 60 min. Sedimentation is from right to left. Ordinate: % total radioactivity recovered.

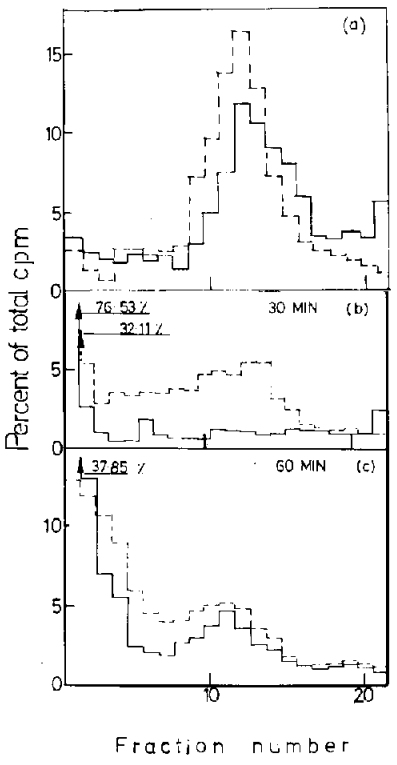

Fig.3. Deoxycytidine does not inhibit excision repair of MNU-induced DNA damage in L1210 cells. Cells were treated with 1 mM MNU for 20 min (——); or additionally with 3 mM deoxycytidine during both the damage and recovery periods (– – –).

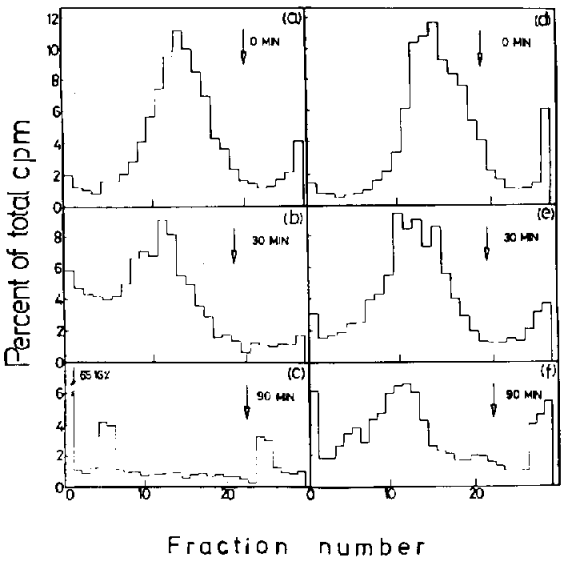

Fig.4. Inhibition of DNA strand-rejoining after MNU damage by the ADP-ribosyl transferase inhibitor, 5-methyl-nicotinamide: (a–c) 1 mM MNU alone; (d–f) 1 mM MNU plus 5 mM 5-methyl-nicotinamide present during 20 min exposure as well as during the recovery period.

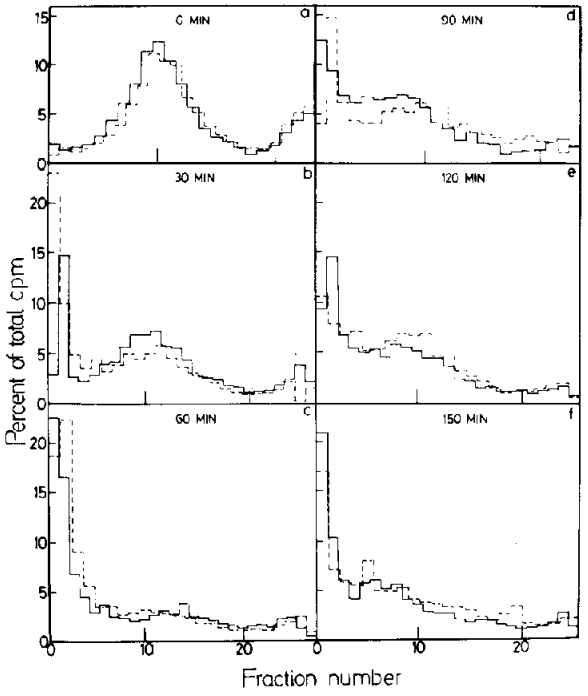

Fig.5. Sodium nicotinate does not inhibit excision repair of MNU-induced DNA damage in L1210 cells. Cells were treated with 1 mM MNU for 20 min (— —); or additionally with 5 mM sodium nicotinate during both the damage and recovery periods (– – –). At the indicated times samples of cells were taken and the DNA was analysed on alkaline sucrose gradients.

An alternative inhibitor of ADP-ribosyl transferase was examined as an inhibitor of DNA strand-rejoining. 5-Methyl-nicotinamide is a powerful inhibitor of ADP-ribosyl transferase, while the related compound, sodium nicotinate, is not at all inhibitory [20]. 5-Methylnicotinamide at 5 mM clearly slows down DNA strand-rejoining after MNU damage (fig.4). After 90 min there is substantial inhibition of DNA strand-rejoining (cf. fig.4c,f). By contrast 5 mM sodium nicotinate has no inhibitory effect at all on DNA repair (fig.5).

4. Discussion

Here, the rejoining of single-strand breaks in DNA induced by 1 mM MNU was monitored by alkaline sucrose gradients. This procedure displays both metabolic breaks as well as those produced by the alkaline conditions of the gradient. We observed that the DNA can be effectively repaired after 1 mM MNU treatment. This conversion of the DNA to higher molecular mass was clearly retarded by 10 mM thymidine, either alone or in combination with 3 mM deoxycytidine (fig.2). In combination with the deoxycytidine the cells grew normally in the presence of thymidine (fig.1); therefore the effect of the thymidine is probably not mediated by a distortion of deoxynucleotide metabolism. Thymidine is known to be a powerful inhibitor of ADP-ribosyl transferase [20] and we suggest that this property explains its ability to retard DNA repair.

This view is supported by the observation that 5-methyl-nicotinamide also retards DNA strand-rejoining (fig.4) although neither 5 mM sodium nicotinate (fig.5) nor 3 mM deoxycytidine (fig.3) by themselves inhibited DNA repair. Thus, two potent inhibitors of ADP-ribosyl transferase are shown to retard rejoining of single-strand breaks in DNA after treatment with 1 mM MNU for 20 min. This adds further support to the conclusion that $(ADP\text{-ribose})_n$ participates in DNA repair. This is the first report of inhibition of strand-rejoining after exposing cells to MNU by inhibitors of ADP-ribosyl transferase.

All 4 classes of ADP-ribosyl transferase inhibitors retard DNA repair after exposing cells to dimethyl sulphate [10,11]. These results are now extended to include damage by methylnitrosourea. These 2 methylating agents produce rather different populations of methylated products. Despite this, the inhibitors of ADP-ribosyl transferase retard strand-rejoining after exposure to both MNU and DMS. We have so far only tested small methylating agents and we do not yet know whether the repair of larger alkylating groups also requires $(ADP\text{-ribose})_n$.

Acknowledgements

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References

[1] Hayaishi, O. and Ueda, K. (1977) Annu. Rev. Biochem. 46, 95–116.
[2] Hilz, H. and Stone, P. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1–58.

[3] Purnell, M. R., Stone, P. R. and Whish, W. J. D. (1980) Biochem. Soc. Trans. 8, 215–227.
[4] Smulson, M. and Sugimura, T. (eds) (1980) Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Elsevier/North-Holland, Amsterdam, New York.
[5] Tsopanakis, C., Leeson, E., Tsopanakis, A. and Shall, S. (1978) Eur. J. Biochem. 90, 337–345.
[6] Whish, W. J. D., Davies, M. I. and Shall, S. (1975) Biochem. Biophys. Res. Commun. 65, 722–730.
[7] Berger, N. A., Weber, G. and Kaichi, A. S. (1978) Biochim. Biophys. Acta 519, 87–104.
[8] Halldorsson, H., Gray, D. A. and Shall, S. (1978) FEBS Lett. 85, 349–352.
[9] Benjamin, R. and Gill, M. (1980) J. Biol. Chem. 255, 10493–10508.
[10] Durkacz, B. W., Omidiji, O., Gray, D. A. and Shall, S. (1980) Nature 283, 593–596.
[11] Durkacz, B. W., Nduka, N., Omidiji, O., Shall, S. and Zia'ee, A. A. (1980) in: Novel ADP-Ribosylations of Regulatory Enzymes and Proteins (Smulson, M. and Sugimura, T. eds) Elsevier/North-Holland, Amsterdam, New York.
[12] Skidmore, C. J., Davies, M. I., Goodwin, P. M., Halldorsson, H., Lewis, P. J., Shall, S. and Zia'ee, A. A. (1979) Eur. J. Biochem. 101, 135–142.
[13] Jacobson, M. K., Levi, V., Juarez-Salinas, H., Barton, R. A. and Jacobson, E. L. (1980) Cancer Res. 40, 1797 1802.
[14] Goodwin, P. M., Lewis, P. J., Davies, M. I., Skidmore, C. J. and Shall, S. (1978) Biochim. Biophys. Acta 543, 576–582.
[15] Juarez-Salinas, H., Sims, J. L. and Jacobson, M. K. (1979) Nature 282, 740–741.
[16] Nduka, N., Skidmore, C. J. and Shall, S. (1980) Eur. J. Biochem. 105, 525–530.
[17] Newbold, R. F., Warren, W., Medcalf, A. S. C. and Amos, J. (1980) Nature 283, 596–599.
[18] Ehmann, U. K., Williams, J. R., Nagle, W. A., Brown, J. A., Belli, J. A. and Lett, J. T. (1975) Nature 258, 633–636.
[19] McGrath, R. A. and Williams, R. W. (1966) Nature 212, 534–536.
[20] Preiss, J., Schlaeger, R. and Hilz, H. (1971) FEBS Lett. 19, 244–246.