Effect of treatment in vivo with N,N-dimethylnitrosamine or methyl methanesulphonate on the cytoplasmic DNA polymerase of regenerating rat liver.

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ABSTRACT

A 10-16 fold increase in rat liver cytoplasmic DNA polymerase (DNA polymerase- α) was observed by 24 hrs after two thirds partial hepatectomy. Injection of either N,N-dimethylnitrosamine (DMN) or methyl methanesulphonate (MMS) at 6 or 12 hrs after partial hepatectomy completely inhibited this increased production of polymerase, but when given at 20 hours they had less effect. Neither compound altered the molecular size distribution of the enzyme. These data indicate that the lowered levels of DNA polymerase- α could play a major role in the reduction in DNA synthesis which occurs after carcinogen treatment.

INTRODUCTION

Whilst DMN is a potent liver carcinogen when administered chronically with the diet¹, single doses have failed to produce tumours in adult rats unless given after the liver has been stimulated into proliferation by partial hepatectomy^{2,3}. Similar results indicating the greater susceptibility of regenerating liver, have also been obtained with other carcinogens, e.g. urethane^{4,5} and with other nitroso compounds, e.g. N-methyl-N'-nitro-N-nitrosoguanidine² and N-methyl-N-nitrosourea⁶. These results are consistent with the hypothesis that changes incurred during a period of active DNA synthesis may be of special significance for tumour initiation. MMS, like the nitroso compounds previously mentioned, readily methylates hepatic nucleic acids, but fails to produce liver tumours even when administered after partial hepatectomy². This apparent discrepancy between the effects of these methylating agents can be explained in terms of differences in the methylation patterns produced in rat liver DNA by MMS and DMN. Thus, following administration of MMS the 'promutagenic' base, O⁶-methyl-guanine^{7,8} was virtually undetectable but was present in readily

measurable quantities after treatment with DMN^{9,10}. A difference in the relative production of phosphotriesters by MMS or DMN has also been established¹¹.

A detailed survey of the effects of these two agents on DNA synthesis in regenerating rat liver has been carried out by Craddock¹². An injection of DMN (9mg/kg) or MMS (66mg/kg) at 6 hrs after partial hepatectomy almost abolished the wave of DNA synthesis normally occurring at 20-24 hrs after the operation. DMN given at this early time gave a low yield of tumours but when given during the wave of DNA synthesis (22 hrs) the yield of tumours was higher. MMS gave no tumours in either case. These findings raise the question of whether the inhibition of DNA synthesis after early treatment is due to the presence of altered DNA or to changes in the activity or the production of enzymes involved in DNA synthesis. DMN, for instance, is known to interfere with hepatic protein synthesis^{13,14} and may, therefore, exert an effect at the level of enzyme production, but we are unaware of similar reports relating to the effects of MMS. There are, however, reports which demonstrate the effects of other agents on DNA polymerase. Bollum *et al*¹⁵, found that the build up of DNA polymerase after partial hepatectomy was inhibited by whole body irradiation given at an early stage during regeneration and Giudice and Novelli¹⁶ showed that actinomycin D had the same effect.

DNA polymerase- α from regenerating rat liver has a molecular size of 10.5S as determined on glycerol gradients at low ionic strength, but it is reversibly converted to a 6-8S form at higher ionic strengths; a small but reproducible peak of activity is always obtained at 3.4S¹⁷. The purpose of these experiments was to investigate the effect of DMN or MMS administration on the cytoplasmic DNA polymerase after partial hepatectomy, with respect to the molecular size distribution, and the activity of the enzyme.

MATERIALS AND METHODS

³H-dTTP (30 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks. Calf thymus DNA, rabbit muscle lactic dehydrogenase, bovine liver catalase and E.coli β -galactosidase were all supplied by the Sigma Chemical Co., Ltd., London and human haemoglobin by Koch Light Laboratories Ltd., Colnbrook, Bucks. Deoxyribonucleoside triphosphates were from the Boehringer Corporation (London)

Ltd., MMS was prepared from silver methanesulphonate by reaction with methyl iodide, and DMN was from K and K Laboratories, Inc., Plainview, N.Y., U.S.A.

Treatment of Animals

Male Wistar rats (240-300 gms) were used throughout. Adequate drinking water and Normal Rat Diet (Oakes Ltd., Congleton, Cheshire) were provided at all times. Two thirds partial hepatectomy was by the method of Higgins and Anderson¹⁸. At 6, 12 or 20 hrs after the operation intraperitoneal injections of either DMN (9mg/kg) or MMS (80mg/kg) were given and at 24 hrs the animals were sacrificed and the livers used to prepare cytoplasmic DNA polymerase. At these doses a 90% recovery of the animals could be expected as determined by toxicity tests. In control experiments the build up of DNA polymerase after partial hepatectomy was monitored by comparing cytoplasmic DNA polymerase levels in liver from non-partially hepatectomised rats and from rats killed at 6, 12, 20 and 24 hrs after partial hepatectomy.

Preparation of Cytoplasmic DNA Polymerase

Each liver was used to prepare a 105,000xg supernatant by the method of Chang and Bollum¹⁹. Each of these supernatants was dialysed extensively against buffer containing 50 mM K phosphate (pH 7.4), 1 mM mercaptoethanol, 20% glycerol, and stored at 4°C.

DNA Polymerase Assay

Standard incubation mixtures for assay of polymerase activity contained 20 mM K phosphate (pH 7.4), 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM each of dATP, dGTP, dCTP and 0.1 mM ^3H -dTTP (0.4Ci/nmole), 15µg activated calf thymus DNA, 50µg Bovine serum albumin and enzyme, in a total volume of 0.1 ml. Incubation was for 1 hr at 37°C and the reaction was stopped by the addition of ice cold 2% (w/v) $Na_4P_2O_7 \cdot 10H_2O$ 5% (w/v) TCA solution. Acid insoluble products were collected by filtration on Whatman GF/C discs, washed with the same solution and finally with 95% ethanol. Discs were air dried and the radioactivity was counted in toluene phosphor.

Glycerol Density Gradient Centrifugation

For application to glycerol gradients, portions of the crude cytoplasmic DNA polymerase were dialysed against 50 mM K phosphate (pH 7.4), 1 mM mercaptoethanol, and 0.2ml samples (approx 3mg protein) were applied to 14 ml 10-30% linear gradients containing 50 mM K phosphate (pH 7.4), 1 mM mercaptoethanol. Gradients were centrifuged for

47 hrs at 25,000 rpm in a 6 x 10.5 swing out rotor of an MSE 65 MkII ultracentrifuge. The gradients were fractionated and samples (40 μ l) assayed for polymerase activity. The molecular size of the enzyme was determined from gradients calibrated with standard protein markers (Fig 1).

Protein Determinations were by the method of Lowry *et al*²⁰.

Figure 1. Calibration of glycerol gradients using standard proteins (β -galactosidase, 15.9S; Catalase, 11.3S; Lactic Dehydrogenase, 7.0S; Haemaglobin, 4.1S); sedimentation is from right to left.

RESULTS

The major DNA polymerase from regenerating rat liver cytoplasm is a high molecular weight species which sediments in glycerol gradients at 10.5S in low salt concentration. Also present in the cytoplasmic extract is a small but relatively constant level of a 3.4S DNA polymerase species. Fig.2. shows that in non partially hepatectomised livers and at 6 and 12 hrs after the operation there is a very low level of high molecular weight polymerase in the cytoplasm and this is spread heterogeneously over the 6-11S region on glycerol gradients; some 3.4S activity is also detectable. During the period 12 - 24 hrs after partial hepatectomy, however, there is a massive increase in 10.5S activity (10-16 fold) with little increase in the 3.4S enzyme. These results are in good agreement with those of Hwang *et al*²¹ for the build

Figure 2. Increase in rat liver DNA polymerase- α after partial hepatectomy. A. Non partially hepatectomised rats (○—○); B. Rats killed at 6 hours (○) or 12 hours (●), and C. killed at 20 hours (○) or 24 hours (●), after partial hepatectomy. Gradients were aligned using an 11.3S Catalase marker (E280, —) and sedimentation was from right to left (% glycerol, x—x). DNA polymerase activity (picoles/μl fraction) has been expressed as activity/g protein applied to the gradient to take account of small differences in the protein concentrations of different preparations.

up of polymerase- α activity after partial hepatectomy, and also with Chang and Bollum¹⁹, although the latter authors reported the high molecular weight peak to be at 6-8S after sedimentation in the presence of 0.1M K phosphate. Fig.3. shows the effect of giving DMN or MMS at 6, 12 or 20 hrs after partial hepatectomy, on the level of enzyme activity found at 24 hrs. The results clearly show that administration of either compound at 6 or 12 hrs completely blocks the normal production of DNA polymerase. Administration at 20 hrs, however, has less effect. Neither compound causes a detectable shift in molecular size distribution from that seen in control animals.

Figure 3. Effect of DMN (9mg/kg; ●) or MMS (80mg/kg; ○) on DNA polymerase- α in livers of rats killed 24 hrs after partial hepatectomy. Compounds were administered at A. 6 hours, B. 12 hours, and C. 20 hours, post operatively. Gradient conditions, the position of the Catalase marker and the expression of DNA polymerase activity are as described for Figure 2.

DISCUSSION

From the results obtained it seems most likely that the low levels of polymerase seen after carcinogen treatment at 6 and 12 hrs result from an inhibition of DNA polymerase- α production following the injection. At 20 hrs the block is incomplete probably because at this time enzyme synthesis is occurring at a very fast rate during the period when the compounds are being assimilated. Additionally, some protein which was produced in the earlier period (12-20 hrs) may mature and become active after further protein synthesis is blocked. At no time was the molecular distribution of the enzymes from DMN or MMS treated animals detectably different from that of the controls killed at an equivalent time. These results are strongly indicative that the prevention of DNA polymerase- α production after DMN or MMS injection early during liver regeneration is an important factor in the inhibition of DNA synthesis which occurs as a result of these treatments¹². However, in spite of the magnitude of the effect observed at the

polymerase level other factors such as a reduction in the template activity of modified DNA, or a decrease in other associated enzymes (e.g. nucleoside or nucleotide kinases), may also play an important role and cannot be excluded.

It will be of interest to determine how widespread the inhibition of DNA polymerase- α after carcinogen-treatment in vivo proves to be. A number of other carcinogens, e.g. 7,12-dimethylbenz(α) anthracene²², N-hydroxy-2-fluorenylacetamide²³, and diethylnitrosamine¹² have been reported to inhibit DNA synthesis when administered to rats after partial hepatectomy and similar results have also been observed after urethane treatment^{24,25}. However, it should be noted that Hwang et al.²⁵, assayed for DNA polymerase- α activity after the urethane treatment during liver regeneration and found no alteration in the level of activity.

Although the results obtained in this study give no indication of the way in which the two agents manifest their effect on DNA polymerase production, their similarity to the results obtained for the blocking of polymerase production by actinomycin D¹⁶ suggest that an inhibition of DNA transcription should be considered in addition to the well established inhibitory effects of DMN on protein synthesis¹³ which is mediated by an interference in the initiation of m-RNA translation¹⁴.

It now seems likely that DNA replication is necessary for the fixation of precarcinogenic lesions in DNA. If DMN is administered early after partial hepatectomy, DNA synthesis is inhibited and tumour yield is very low^{12,2}. It may be, therefore, that the polymerase-mediated delay in DNA synthesis allows a greater opportunity for the repair of lesions before replication commences. By 20 hrs after partial hepatectomy, however, DNA synthesis is already underway and lesions in DNA can be quickly fixed. Thus, the available data support the current hypothesis that the commitment of a cell to malignant transformation depends upon both the type and amount of modification to DNA, and on the time available for its repair before the cell enters the next round of DNA synthesis.

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Note: We have recently received confirmation of these findings from Dr V. Craddock who has found decreased levels of DNA polymerase activity in liver extracts of rats treated with DMN or MMS during the regenerative phase²⁶.

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