MEASUREMENT OF UNSCHEDULED DNA SYNTHESIS ON ALKYLATING CARCINOGENS IN RAT INTESTINAL MUCOSAL CELLS FROM DIFFERENT SITES

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Abstract.....Unscheduled DNA synthesis (UDS) induced by *in vitro* exposure to five alkylating carcinogens was measured in the mucosal cells from three different sites of the rat intestine. The yield of UDS by N-methyl-N'-nitro-N-nitrosoguanidine or N-ethyl-N'-nitro-N-nitrosoguanidine or methylazoxymethanol acetate was higher in the cells of colon & rectum than in those of the jejunum or ileum. Conversely, UDS induction by N-methyl-N-nitrosourea or methyl-methanesulphonate was seen at a rather higher level in the cells from the small intestine compared with those from the colon & rectum.

Key words: Unscheduled DNA synthesis, Alkylating carcinogens, Rat intestine.

INTRODUCTION

Using many types of carcinogenic chemicals, various animal models for gastrointestinal (GI) carcinogenesis have been recently established. However, the reason for the organ specific carcinogenic activities of these chemicals is not yet completely understood. Several organotropic actions of alkylating agents such as in DNA damage or the relating DNA repair synthesis, are known (Kleihues and Margison, 1974, Montesano *et al.*, 1976, Stich and Kieser, 1974, Stich and Koropatnick, 1976, Swan and Magee, 1968). Age and species differences in the DNA repair levels of these carcinogens have also been reported (Ishikawa *et al.*, 1979). N-methyl-N-nitrosourea (MNU) is known to generate DNA methylation in several organs, including the intestines, which are target organs for carcinogenicity. Another alkylating carcinogen, N-

methyl-N'-nitro-N-nitrosoguanidine (MNNG), which effectively induces gastric cancers in experimental animals, also gives rise to DNA fragmentation and subsequent DNA repair in animal gastric epithelium. To clarify the relationship between the level of DNA damage and the carcinogenic susceptibility of these carcinogenic alkylating agents, it seems important to analyze the capability of DNA repair in the cells of some uniform organs like GI tracts. In the present study, we attempted to explain the organotropism of certain alkylating agents by *in vitro* measurement of unscheduled DNA synthesis (UDS), determined by the incorporation of tritiated thymidine in rat intestinal mucosal cells which had been obtained from different sites and exposed to the chemicals.

MATERIALS AND METHODS

Isolation of the intestinal mucosal cells was performed according to the method described by Freeman and San (1980). About 15 cm of the intestinal segments were removed from the 3 different sites of the intestines (upper jejunum, lower ileum and colon & rectum) of 3 months old, male ACI rats. These segments were immediately turned inside out by inserting a glass rod into the gut segments and kept in a solution of Eagle's MEM (Nissui Seiyaku Co., Tokyo) containing 0.1% pronase (Kaken Chem. Co., Tokyo), in a CO₂ incubator at 37°C for 45 min. To get enough mucosal cells, 2 rats were used and cells from each site were combined with cells from the respective site in the second rat. After incubation, the solution was centrifuged at 750 rpm to remove mucus elements and resuspended in the same medium without pronase. The isolated mucosal cells were then counted and assessed for viability by trypan blue exclusion. For carcinogens, MNNG, N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), MNU and methyl-methanesulphonate (MMS) (Nakarai Chem., Co., Osaka), were dissolved in dimethyl-sulfoxide (DMSO) and another carcinogen, methylazoxymethanol acetate (MAM acetate) (Ash Stevens Inc., Detroit, U. S. A) was dissolved in distilled water. The isolated mucosal cells were exposed to each of these carcinogens in the presence of ³H-thymidine (10 μ Ci/ml, 49 Ci/mmol; Amersham, U. K.) for 2 hr in the CO₂ incubator at 37°C. Following the incubation, cells were collected by centrifugation (1,000 rpm) and resuspended in 1% sodium citrate for 15 min to allow the nuclei to swell, then pelleted and fixed, following suspension of glacial acetic acid : ethanol (1 : 3) for 15 min. UDS in the cell nuclei of the mucosal cells was measured by quantification of the nuclear grains. Triplicate experiments were done using 5 carcinogens except in one experiment in which the assay was done with 4 carcinogens.

RESULTS

The approximate cell yield from an intestinal segment of one rat following the 45 min exposure to 0.1% pronase solution was 4×10^5 (jejunum), 3×10^5 (ileum) and 2×10^5 (colon & rectum) cells/cm of the length. Viability of the mucosal cells after the collection in trypan blue was 80-90%. The percentage of cells from the different sites of the intestines undergoing DNA replication (heavily labeled nuclei) in both the

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Table 1 UDS in Isolated Rat Intestinal Mucosal Cells Exposed to Each Carcinogen

Carcinogens	Percentage of UDS positive cells and each UDS level in the mucosal cells in :		
	Jejunum	Ileum	Colon & rectum
MNNG			
3×10^{-3} M	8(6.0 ± 2.0)	7(6.0 ± 1.9)	57(12.4 ± 4.6)
3×10^{-4} M	8(5.9 ± 1.8)	12(6.5 ± 2.4)	47(9.5 ± 4.4)
ENNG			
3×10^{-3} M	7(5.9 ± 1.8)	18(8.0 ± 3.0)	36(8.3 ± 3.2)
3×10^{-4} M	9(6.3 ± 2.1)	20(7.4 ± 2.5)	48(10.0 ± 3.6)
MAM acetate			
1 mg/ml	13(6.4 ± 2.0)	20(7.3 ± 2.8)	52(10.8 ± 4.0)
10^{-1} mg/ml	7(5.9 ± 1.9)	13(6.5 ± 2.3)	39(9.1 ± 3.4)
MNU			
3×10^{-3} M	66(12.3 ± 4.6)	73(14.7 ± 5.9)	40(9.6 ± 3.6)
3×10^{-4} M	34(7.5 ± 2.8)	34(7.6 ± 2.7)	23(6.6 ± 2.6)
MMS			
3×10^{-3} M	49(9.8 ± 3.4)	57(11.3 ± 4.1)	35(7.9 ± 2.5)
3×10^{-4} M	37(8.2 ± 2.7)	43(9.4 ± 3.6)	24(6.5 ± 2.2)
Solvent control			
DMSO ^a	1(4.6 ± 0.8)	1(4.5 ± 0.6)	2(4.8 ± 0.9)
Ditilled water ^b	1(4.6 ± 0.5)	2(4.5 ± 0.6)	1(4.7 ± 0.6)

^a Used for MNNG, ENNG, MNU and MMS. ^b Used for MAM acetate. The results are combined for 3 experiments except with MMS (2 experiments). In each experiment, 300 cells were scored in one representative slide. A UDS positive cell was defined as that with net counts of at least 4 grains/nucleus. The number in parenthesis is Mean $\pm$ S.D. (composite variance from the experiments) on the grains/nucleus of UDS positive cells. The percentages of UDS positive cells on each carcinogen are all significantly higher than on corresponding solvent controls ($p < 0.01$ by χ^2 test). Underlined values are also significantly higher than those of solvent controls on the grain levels ($p < 0.05$ by Students' t test). The percentage of UDS positive cells from colon & rectum is significantly higher than that from jejunum or ileum with the treatment of MNNG or ENNG or MAM acetate ($p < 0.001$). The percentage of UDS positive cells from the small intestine with MNU or MMS is also significantly higher than that of the colon & rectum ($p < 0.01$). A similar tendency was obtained for the grain levels of UDS positive cells ($p < 0.05$) except the case (•).

carcinogen-treated and solvent control cultures did not exceed 6%. Combined results from the experiments showing the percentage of UDS positive cells and grain level in the mucosal cells from each site are indicated in Table 1. In the solvent control, the percentage of UDS positive cells (with lightly labeled nuclei) was less than 2%, and the mean net count of grains/nucleus of UDS positive cells was less than 4.8 ± 0.9 . Intestinal mucosal cells exposed to the carcinogens indicated the percentage ranged between 7% (3×10^{-3} M of MNNG, ileum; 3×10^{-3} M of ENNG, jejunum; 10^{-1} mg/ml of MAM

acetate, jejunum) and 73% (3×10^{-3} M of MNU, ileum) and the corresponding grains/nucleus of UDS positive cells were between 5.9 ± 1.8 and 14.7 ± 5.9 . In general, the yield of UDS in the mucosal cells exposed to MNNG, ENNG or MAM acetate was similar in the colon & rectum and in the small intestine. However, in the cases of MNU and MMS, the level of UDS was rather higher in the cells from the small intestine than in those from the colon & rectum (Table 1).

DISCUSSION

In this assay, the standard deviation for the grain count indicating unscheduled DNA synthesis was fairly large. This may be due to the inclusion of early or late S-phase nuclei incorporating thymidine in short hour incubation. However this would have no significant influence on the averaged grain number as far as the UDS level of solvent controls is concerned. The reason for the small number of cells in replicating DNA synthesis whose nuclei were heavily labeled and distinguishable from the weakly labeled repairing nuclei probably is that the present method for cell isolation would release more easily non-dividing matured cells which had migrated from the bottom after the cessation of mitosis and were located at the upper part of the crypt (Lipkin, 1973). The UDS yield in the cell nuclei of the mucosal cells induced by the exposure of MNNG or ENNG or MAM acetate was clearly seen in the cells from colon & rectum compared with those in the cells from the jejunum or ileum. However, it was observed at rather a higher level in the cells from the small intestines with treatments of MNU or MMS. These results show a site difference in the extent of UDS induction in the mucosal cells of the intestines by different types of alkylating carcinogens. The data also suggests some relationship between DNA repair levels and carcinogenic susceptibility. It is already known that the enzymatic pattern in the mucosal cells, especially in the surface epithelium, is different according to sites in GI tracts (Kordovsky, 1976). These facts may be related to the chemical reaction for the DNA damage or to its repair process in the intestinal mucosal cells. Intravenous injection of MAM acetate or oral administration of cycasin, a precursor chemical of MAM, induces colonic or rectal tumors more frequently than small intestinal tumors (Hirono *et al.*, 1976 and Laqueur, 1964). The carcinogen 1, 2-dimethylhydrazine which is known to be metabolized to MAM, generates 0⁶-methylguanine extensively in the colon DNA (Montesano *et al.*, 1976). Swan and Magee (1968) reported that MNU makes 7-methylguanine at almost the same level of methylation of nucleic acids in both small and large intestines. Furthermore, MNNG is known to induce colo-rectal cancers at a high incidence if given to animals by rectal infusion (Narisawa *et al.*, 1971). Sugimura *et al.* (1969) reported the occurrence of colonic carcinomas in rats due to the oral administration of MNNG. Thus, the results of *in vitro* measurement of UDS on these chemicals seem to be in agreement with previous reports on organotropic activities in carcinogenicity or DNA methylation. In this study, UDS induction by MMS was mainly observed in the cells from the small intestine. MMS is a classic alkylating agent and its carcinogenic effect

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has been manifested in the nervous system (Kleihues *et al.*, 1972). Although it is known that MMS generates methylation in rat brain, it also alkylates DNA in most other organs (Cradock, 1975, Swan and Magee, 1968). The fact that MMS generates 7-methylation but not 0⁶-methylation (Cradock, 1973, Kleihues and Magee, 1973) provides some evidence for the current hypothesis that 0⁶-methylation rather than 7-methylation is a relevant lesion for carcinogenesis. However, one of significant things may be that the level of DNA damage will not always correspond to the level of the repair. Thus, the present demonstration concerning DNA repair will not be necessarily consistent with the carcinogenic susceptibility of these chemicals directly, but it may provide an important information for identifying the relationship between individual cell type and type of DNA damage or the extent of the damage which will be related to the carcinogenicity of these alkylating agents.

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