Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 304-311

DISTRIBUTION OF DNA REPAIR AND THE EXTENT OF ENZYMATIC DNA METHYLATION IN ALKYLATED HUMAN LYMPHOCYTES CARRYING THEIR DNA SYNTHESIS IN THE PRESENCE OR ABSENCE OF HYDROXYUREA.

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Received February 28,1980

Summary. The distribution of "repair-label" into DNase II sensitive and resistant regions of chromatin in human lymphocytes exposed to nitrogen mustard indicates that in the regions most accessible to this enzyme the number of newly inserted label molecules was about 3.5 times greater than that in resistant regions with only slight preference for the sequences considered to be transcriptionally active. With the progression of the extent of digestion this proportion becomes gradually lower. Pyrimidine tracts analysis did not reveal any significant differences between damaged and undamaged cells. In repair-inserted sequences no more than 1 per 50-60 cytosines was methylated while in undamaged lymphocytes 1 per about 15 cytosines was modified. The presence or absence of hydroxyurea during the course of repair synthesis did not seem to affect any of the parameters studied by us.

Introduction. The recognition of the differences in the efficiency of damage and repair between various functionally distinct regions of DNA may help to elucidate the role of these processes in mutagenesis and carcinogenesis. Several data suggest that there is a nonuniform distribution of DNA repair synthesis in chromatin after treating the cells with X rays, UV-irradiation or alkylating agents. The preferential sensitivity of repaired regions to micrococcal nuclease digestion indicates that at least at early hours after damage the great proportion of the total repair synthesis is located in the linker portion of the nucleosome /1-3/. On the other hand a somewhat higher level of repair synthesis has been found in DNase I digestion products /3,4/ and a reduced level in satellite DNA as compared to main band DNA /5/ what could suggest the slightly increased repair activity in the transcriptionally active chromatin. Such experiments were usually

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Abbreviations used: NM - nitrogen mustard; HU - hydroxyurea; TdR - thymidine; CdR - deoxycytidine; C - cytosine; 5MeC - 5-methylcytosine.

0006-291X/80/130304-08$01.00/0

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performed on damaged cells carrying their DNA synthesis in the presence of HU, assuming that under these conditions most of the label results from the repair process. Several studies have found little, if any, difference between HU-treated and untreated cells either in the extent of damage removed /6,7/ or in the amount of repair synthesis, particularly when the undividing cells were used /6,8/. However recent observations indicate that the presence of HU during repair synthesis results in chromosome decondensation and accumulation of single-strand gaps /9,10/.

Bearing this in mind in the present study which aimed to compare the distribution of newly synthetized DNA in chromatin of intact and alkylated cells in the DNase II sensitive and resistant regions and in the pyrimidine deoxyribonucleosides varying in length, we performed the parallel examination of damaged cells carrying their DNA synthesis in the presence or absence of HU.

In view of the data suggesting the enzymatic methylation of the repair-inserted DNA sequences /11/ we also examined the proportion of methylated cytosines in newly synthetized DNA.

As the experimental model we employed the human peripheral lymphocytes exposed to NM. The results of Lieberman et al. /12, 13/ indicate that such cells react to a variety of carcinogens and alkylating agents very much like other eukaryotic cells and when are exposed to such compounds repair DNA synthesis occurs, manifesting by stimulation of TdR and CdR incorporation in the presence of HU.

Experimental procedure.

Human peripheral lymphocytes were isolated from defibrinized venous blood by gelatin sedimentation /14/. After washing with saline the cells were resuspended in Eagle's medium supplemented with 10% calf serum, at a density of 5 x 10^5 cells/ml.

To induce the repair synthesis of DNA the cells were exposed to NM for 30 min at 37°C, washed twice with fresh medium and finally resuspended in fresh medium with or without 5 mM HU. At suitable times appropriate radioactive precursor was added. The control lymphocytes were treated similarly except that the pretreatment with NM was omitted.

DNA was isolated according to Butterworth /15/: the cellular pellets were homogenized and shaken in the solution containing 0,15 M NaCl;0,1 M EDTA /pH 8/ and 2% SDS then 5 M $NaClO_4$ was added to a final concentration of 1 M. Deproteinization was performed by several treating with chloroform-isoamyl alcohol/24:1/. Thus obtained DNA preparations were further purified by digestion with pronase and RNase.

Fractionation of chromatin by DNase II digestion was performed according to Gottesfeld et al./16,17/: nuclei isolated in buffered sucrose-Triton 100 were lyzed by suspension in 0,2mM EDTA /pH 7/ and sodium acetate was added to final concentration of 25 mM /pH 6,6/. After suitable time of incubation with DNase II /at 5 units per A_{260} unit at 24°C/ the pH of the sample was raised to 7,5 with 0,1 M Tris-HCl /pH 10/, the digested products separated by centrifugation and further fractionated by the addition of $MgCl_2$.

Degradation of DNA to pyrimidine oligonucleotides was performed according to Burton /18/. Chromatographic separation of pyrimidine isopliths according to chain length was performed on a DEAE-cellulose column /19/: after adsorption of the oligonucleotides the column was washed with 0,01 M Li acetate /pH 5/ to remove free purines; the nucleotides were then eluted with an increasing linear gradient of LiCl up to 0,35 M in the same Li-acetate buffer. After elution the column was washed with 1 M LiCl. Absorbance of fractions were read at 270 nm and their radioactivity measured in Tri-Carb scintillation counter.

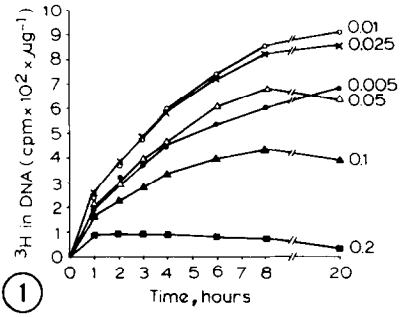
The proportion of 5MeC in DNA labeled with /U-^{14}C/deoxycytidine was determined on 12 N $HClO_4$ hydrolysates after separation of bases by 2-dimensional paper chromatography on Whatman no 1 paper with solvent system propan-2-ol-12 M HCl-water /85:22:18/ and methanol-12 M HCl-water /91:26:13/ /20/.

DNA was estimated by diphenylamine method of Burton /21/.

Results and discussion.

Effect of NM treatment on /^{3}H/TdR incorporation into total DNA.

Untreated lymphocytes incorporate labeled TdR in relatively high degree due to the presence of some cells in S phase. This replicative DNA synthesis is deeply supressed in the presence of HU. Such supression permits observations of DNA repair synthesis in cells exposed to damaging agents as revealed by subsequent TdR incorporation in the presence of HU. Fig. 1 shows the NM dose-response curves determined in the presence of HU. Since the 0,01 mM NM concentration causes the most marked increase in TdR incorporation when compared to lower and higher concentrations, this concentration was used in further experiments to induce DNA repair synthesis. As can be seen in Fig. 2 0,01 mM NM alone supressed TdR incorporation when compared with "replicative" incorporation in unalkylated lymphocytes. This supressed incorporation is however higher than that in NM-treated cells incubated with HU. The measurement of the time-course of "repair" incorporation in alkylated cells incubated in the presence of HU yielded an initial rapid phase during the first 2-3 h after damage and a later slower phase which practically completes in about 8-9 h. Similar distinction of the repair activity on "fast" and "slow"



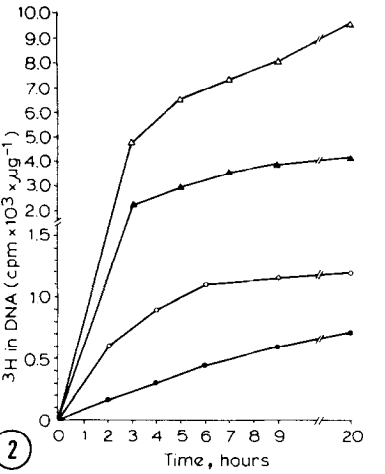


Fig. 1. Effect of various concentrations of NM on the rate of DNA repair synthesis measured by /^{3}H/TdR incorporation in the presence of 5 mM HU. Lymphocytes were treated with the indicated concentration /mM/ of NM for 30 min and then exposed to the radioactive label. At suitable times aliquots of cell suspension were taken for the estimation of /^{3}H/-specific activity of DNA.

Fig. 2. Time-course of /^{3}H/TdR incorporation into DNA of unalkylated and alkylated /0,01 mM NM, 30 min exposition/ lymphocytes incubated in the absence or presence of 5 mM HU. △—△ unalkylated incubated without HU; ▲—▲ alkylated incubated without HU; ○—○ alkylated incubated with HU; ●—● unalkylated incubated with HU.

phases was reported by Smerdon et al./4/ during UV-induced DNA repair in human fibroblasts.

Effect of NM treatment on /^{3}H/TdR incorporation into DNase II sensitive and resistant regions.

The DNase II procedure offers the possibility to separate the chromatin regions accessible to nuclease digestion into transcriptionally active and inactive sequences, due to the selective precipitation of digested inactive chromatin with magnesium ions /16,17/. Fig. 3 shows the time-course of chromatin fractionation of undamaged lymphocytic nuclei. Closely similar kinetics of digestion was observed in alkylated lymphocytes. Table 1 shows the results of DNase II digestion of nuclei of intact and alkylated cells labeled in various periods after damage and in the presence or absence of HU. It can be seen that while in the undamaged cells the proportion of DNA and radioactivity is roughly parallel in all fractions recovered, the data in alkylated cells are quite

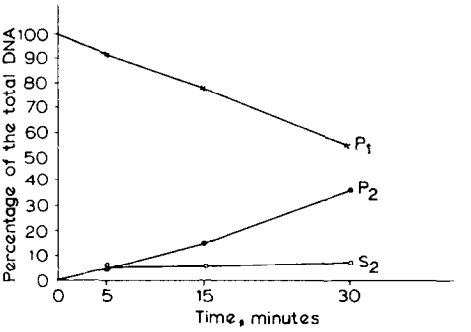


Fig. 3. Time-course of chromatin fractionation by DNase II digestion. After suitable time of digestion chromatin was separated into 1-st supernatant /S_1/ and pellet /P_1/ by centrifugation. The S_1 was further fractionated by the addition of $MgCl_2$ to 2 mM and the supernatant of Mg^{++}-soluble chromatin /S_2/ and Mg^{++}-insoluble pellet /P_2/ were recovered by centrifugation. Aliquots of each fraction were taken for DNA estimation.

distinct: in the digestion point where only about 7-8 % of the nuclear DNA becomes released into digested fraction /P_2+S_2/, the total radioactivity rendered soluble is about 25%. This disproportion becomes gradually less significant as the extent of the digestion progressed, reaching in the point of about 60% of digested DNA about 80% of digested radioactivity. The analysis of the data for the fraction P_2 and S_2 separately indicates moreover that this disproportion concerns both the DNA sequences considered to be transcriptionally active /S_2/ and inactive /P_2/, with only slight preference for the fraction S_2. The mutual relationships between the proportion of digested DNA and the radioactivity remains closely similar in cells pulse labeled either immediately after damage /0-2 h/ or later /4-9 h/. No differences could be seen between damaged cells carrying their DNA synthesis in the presence or absence of HU.

Effect of NM treatment on /^{3}H/TdR incorporation into pyrimidine deoxyribooligonucleotides varying in length.

Unalkylated and exposed to NM lymphocytes were incubated for 9 hours with /^{3}H/TdR in the presence or absence of HU and their DNA was separated into pyrimidine isopliths comprising from 1 to 5 and above 5 oligonucleotides. The obtained data indicate that the distribution of the label in the separated isopliths did not reveal any significant feature to distinguish newly synthetized DNA in damaged and undamaged cells.

Table 1

Comparison of the removal of /^{3}H/TdR-labeled products in DNase II sensitive and resistant regions of DNA of unalkylated and alkylated lymphocytes incubated in the presence or absence of HU.

Hours of /^{3}H/TdR labeling	Per cent of DNA in:			Per cent of ^{3}H in:			$\%^3$H/% DNA in:		
	P_1	P_2	S_2	P_1	P_2	S_2	P_1	P_2	S_2
Unalkylated lymphocytes									
0 - 2	90.5	5.8	3.7	90.5	5.9	3.6	1.00	1.02	0.97
0 - 2	89.2	6.9	3.9	88.4	7.1	4.5	0.99	1.03	1.15
0 - 2	78.8	14.8	6.4	78.7	14.0	7.3	1.00	0.95	1.14
0 - 2	57.2	35.7	7.1	55.3	35.7	9.0	0.97	1.00	1.25
4 - 9	88.9	6.0	4.1	89.8	5.4	4.8	1.00	0.90	1.17
4 - 9	76.8	16.4	6.8	75.2	16.8	8.0	0.98	1.02	1.18
Alkylated lymphocytes /HU absent/									
0 - 2	92.1	5.4	2.5	75.7	15.6	8.7	0.82	2.89	3.48
0 - 2	89.2	7.0	3.8	76.9	10.9	12.2	0.86	1.56	3.21
0 - 2	87.6	11.2	1.2	69.3	26.6	4.1	0.79	2.38	3.42
0 - 2	78.4	17.1	4.5	58.5	32.0	9.5	0.75	1.87	2.11
0 - 2	67.5	26.3	6.2	43.9	42.2	13.9	0.65	1.60	2.24
0 - 2	58.5	35.8	5.7	29.0	58.9	12.1	0.50	1.65	2.12
0 - 2	38.7	51.4	9.9	17.1	70.7	12.2	0.44	1.38	1.23
4 - 9	90.9	6.3	2.8	75.4	15.5	9.1	0.83	2.46	3.25
4 - 9	79.9	14.4	5.7	59.2	27.3	13.5	0.74	1.90	2.37
4 - 9	54.8	37.2	8.0	30.1	53.6	16.3	0.55	1.44	2.04
Alkylated lymphocytes /HU present/									
0 - 2	93.1	3.0	3.9	75.4	10.4	14.2	0.81	3.47	3.64
0 - 2	92.4	3.3	4.3	75.8	10.3	13.9	0.82	3.12	3.23
0 - 2	90.5	4.3	5.2	77.0	9.1	13.9	0.85	2.12	2.67
0 - 2	87.7	8.6	3.7	69.6	12.3	18.1	0.79	1.43	4.89
0 - 2	43.9	50.3	5.8	24.5	60.2	15.3	0.56	1.20	2.59
0 - 2	47.5	46.5	6.0	24.7	64.2	11.1	0.52	1.38	1.85
0 - 2	32.2	57.1	10.7	15.4	64.9	19.7	0.48	1.14	1.84
4 - 9	88.3	6.1	5.6	69.8	11.7	18.5	0.79	1.92	3.30
4 - 9	51.9	38.7	9.4	32.8	50.6	16.6	0.63	1.31	1.77

Unalkylated and exposed to NM lymphocytes were pulse labeled with /^{3}H/TdR either immediately /0-2 h/ or 4 hours after treating /4-9 h/ with the alkylating agent. Isolated labeled nuclei were then digested for various periods of time with DNase II. The following fractions were recovered and measured for DNA and ^{3}H content: undigested residue /P_1/, Mg^{++}-insoluble portion of digested products /P_2/ and Mg^{++}-soluble portion of digested products /S_2/. The values are expressed as the percentage of the respective total quantity recovered in each sample. Each point is the average of two parallel determinations.

Table 2
Proportion of 5MeC in DNA of unalkylated and alkylated lymphocytes, measured by the extent of deoxycytidine incorporation.

Exp. No	Unalkylated	Alkylated /HU absent/	Alkylated /HU present/
1	6.9	1.6	2.1
2	6.7	2.0	2.2

Unalkylated and exposed to NM lymphocytes were incubated for 9 hours with deoxy/U-^{14}C/cytidine in the presence or absence of 5 mM HU. DNA was isolated and procedured for ^{14}C measurement in C and 5MeC as described in experimental procedure. The results are expressed as $\frac{5MeC}{C+5MeC} \times 100$. Each value is the average of two determinations.

Effect of NM treatment on the extent of enzymatic DNA methylation

According to the data by Drahovsky et al./11/ human lymphocytes damaged by NM and incubated in the presence of HU are able to incorporate methyl residue of methionine into 5MeC of DNA. In our experiments we aimed to compare the extent of DNA methylation in intact and alkylated cells by the measurement of the proportion of methylated cytosines, based on the calculation of /U-^{14}C/CdR-originated radioactivity incorporated into C and 5MeC. As shows Table 2, during the replicative DNA synthesis /unalkylated cells/ 1 per about 15 cytosines was methylated, which value is of the same order as in other mammalian cells, including various forms of human white cells /22/. In contrast, in DNA synthetized in alkylated cells, no more than 1 per 50-60 cytosines was modified. To explain such unusually low level of 5MeC formation in damaged cells at least two alternatives could be taken into consideration: 1. undermethylation of repair-inserted DNA sequences which in intact cells are more methylated, 2. preferential repair or/ and damage of DNA sequences which in intact cells are also poorly methylated.

Summarizing, the different features of DNA synthesis observed by us between intact and damaged lymphocytes provide some further evidence that repair DNA synthesis is not a random event but a process influenced, at least in some degree, by a higher level of chromatin organization, presumably connected with the accessibility of the selective DNA regions to appropriate enzymes.

The presence or absence of HU during the course of DNA synthesis in alkylated cells does not seem to affect any parameters studied by us.

Acknowledgments. This work was supported by the Polish National Cancer Programme PR-6, grant No 1317/13.

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