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Radiation sensitizations at DNA level by chemical and biological agents (coord.progr. on improvement of radiotherapy of cancer using modifiers of radiosensitivity of cells)

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RADIATION SENSITIZATION AT DNA-LEVEL BY CHEMICAL AND BIOLOGICAL AGENTS

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I. RADIATION SENSITIZATION BY CHEMICAL AGENTS

Radiation sensitizers have many different action mechanisms. Potentiation of radiation effectiveness may by achieved by inhibition of DNA-repair systems. Some membranespezific drugs may have greater effects on tumors, than on normal tissue. Therefore we investigated some of these drugs if they have also an influence on DNA-metabolism. Also electron affinic sensitizers acting mainly on hypoxic cells within a tumor were included in our studies. Procaine, Halothan and Metronidazole showed no significant effect on unscheduled DNA-synthesis investigated by autoradiography and no effect on rejoining of DNA single strand breaks using nucleoid sedimentation in an ultracentrifuge. Oxyphenbutazon and prednisolon reduced the replicative DNA-synthesis in vitro and in vivo (spleen and lung cells of hamsters) but did not show a significant effect on DNA-repair in in vivo experiments.

Procaine produced some DNA strand breaks in spleen cells of mice treated with 200 mg/kg bodyweight (9-month report 1977). Metronidazole and prednisolon showed a small reduction in poly(ADP-ribose)synthesis (PAR-synthesis). Another group of chemicals studied during 1980 are furocoumarines. The biological effects of this substances are

known for more than 3000 years (1). Methoxypsoralen can sensitize animals against ionizing radiation (2) but are much more used in photosensitization of skin tumors, but especially for inhibition of DNA-synthesis during psoriasis treatment.

In the first part the mutagenic effect of 8-methoxypsoralen (8-MOP) and 5-methoxypsoralen (5-MOP) was tested in combination with irradiation of near UV ($\lambda > 300$ nm) with the bacterial strains Salmonella typhimurium TA 98 and Salmonella typhimurium TA 100. Both substances were added to the cell-suspensions in the concentrations of 10, 20, 30 and 60µg/ml. To correct the amount of mutants for toxic effects survival curves were constructed. UV-irradiation alone in the used dose range had no mutagenic influence on the bacteria. 5-MOP is more toxic to both Salmonella typhimurium TA 98 and Salmonella typhimurium TA 100 and induces more mutants than 8-MOP in all concentrations used.

In the second part mutagenicity tests were performed with eukaryotic cells: CHO cells were treated with the LD_{50} in respect to MOP and UVA light. After one and after 7 days about $5x10^5$ cells were seeded into Petri dishes. Next day 0.5 mM Quabain was added. After 4 days the medium was changed and new Quabain was added. 7 days later the resistant colonies were counted. The number of living cells was determined separately. 5-MOP was more toxic and mutagenic as 8-MOP.

In the next experiments the influence of 5- and 8-methoxypsoralen (MOP) on DNA metabolism of CHO cells was tested. 8-MOP induces interstrand DNA crosslinks when applied together with 360 nm UV-light, whereas 5-MOP induces nearly no crosslinks. Nevertheless 5-MOP is the higher toxic substance and shows a higher cytostatic action than 8-MOP. Both substances show a dose range within which they depress the DNA synthesis without beeing considerably toxic (Fig. 1 - 6).

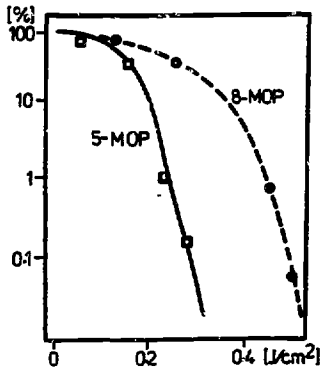


Figure 1: Survival curve: CHO cells were treated with 2µg/ml 5- or 8-MOP and irradiated with different doses UVA light. The diagram shows percent cloning efficiency versus the UV-dose.

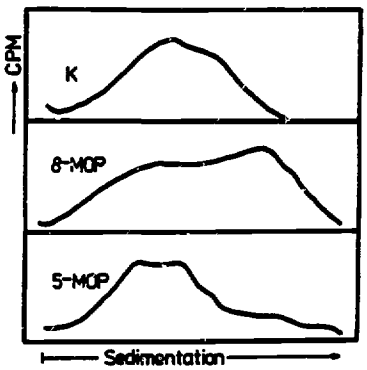


Figure 2: Sedimentation profile of DNA in the alkaline gradient after MOP treatment: Labelled CHO cells were treated with 2µg/ml 5- or 8-MOP and irradiated with 0.75 J/cm² UVA light. Then the cells were lysed and centrifuged in a 5-20% alkaline sucrose gradient. Sedimentation is from left to right.

TIME AFTER PUVA TREATMENT	MOL. WEIGHT OF DNA
0 HOURS	2.20×10^8 D
4 -"-	2.40×10^8 D
24 -"-	2.37×10^8 D

Figure 3: Repair of DNA crosslinks: Labelled CHO cells were treated with 2µg/ml of 8-MOP and irradiated with 0.75 J/cm² UVA. After definite times of repair incubation the molecular weight of the DNA was estimated by centrifugation in alkaline sucrose. The molecular weight does not decrease within 24 hours, so the DNA crosslinks are not removed.

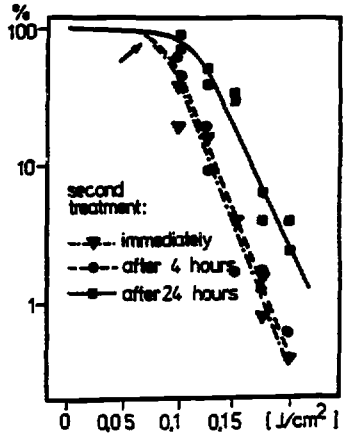


Figure 4: Repair of damage caused by 5-MOP: CHO cells were treated with 2µg/ml 5-MOP and irradiated with 0.07 J/cm² UVA (arrow). A second treatment and irradiation was done definite times later. The diagram shows the relative cloning eficiency in percent versus the total dose of the two treatments. In the case of a 24 hour interval between the two treatments the shoulder region is larger which corresponds to the repair processes within this time.

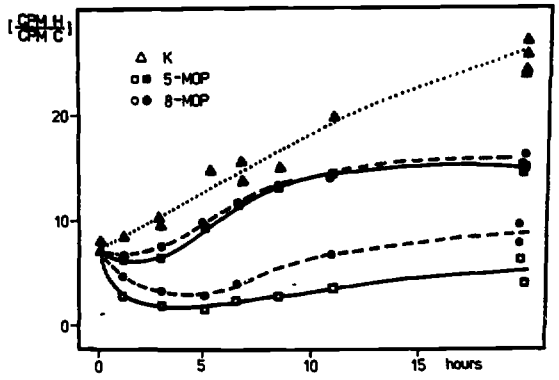


Figure 5: Incorporation of thymidine after MOP-treatment: CHO cells, prelabelled with 14-C-thymidine were treated with 2µg/ml 5- or 8-MOP and irradiated with UVA. Cells treated with 5-MOP were irradiated with 0.1 (closed symbols) or 0.56 (open symbols) J/cm². Cells treated with 8-MOP were irradiated with 0.2 (closed symbols) or 0.77 (open symbols) J/cm². After definite times the cells were labelled for 60 minutes with 3-H-thymidine, trypsinized, washed and counted for radioactivity. The diagrams shows the ratio of 3-H to 14-C-acticity versus the time after MOP-treatment.

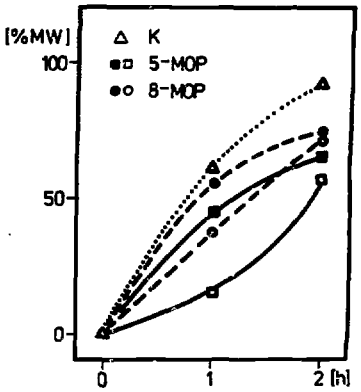


Figure 6: Incorporation of thymidine into high molecular DNA: CHO cells, prelabelled with 14-C-thymidine were treated with 2µg/ml 5- or 8-MOP and irradiated with 0.5 J/cm² UVA. Immediately after treatment (closed symbols) or after 4 hours (open symbols) cells were labelled for 30 minutes with 3-H-thymidine and then incubated with cold thymidine till the time of 1 or 2 hours was finished. Then the molecular weight of the DNA was estimated. The diagram shows the molecular weight of 3-H-DNA (newly synthesized) as percentage of the molecular weight of 14-C-DNA (high molecular DNA) versus the time of DNA-synthesis.

Both tested substances are in a dose range in which they inhibit the DNA-synthesis (the cellular growth) not markedly toxic. Interstrand crosslinks caused by them are probably not the main reason for their action because 5-MOP which induces much less crosslinks than 8-MOP is the more active one of this two substances. The repair of damage caused by MOP and UVA light is a long lasting process. At high doses nearly no repair takes place but the cells die.

It should be mentioned that all experiments were carried out with chinese hamster ovary cells. Because repair of complicated DNA damage may be slightly different in other cell types, care should be taken when comparing these results with these obtained with other cells.

II. INVESTIGATIONS ON RADIATION SENSITIZATION AT DNA-LEVEL BY BIOLOGICAL AGENTS

Mycoplasmas have been discussed as ethiologic agents in connection with various diseases. In earlier experiments we were able to demonstrate decreased repair capacity in spleen cells of rats infected with mycoplasmas compared to controls (3). The effect of mycoplasma infection on DNA excision repair of Yoshida tumor cells and the combined effects of tumor growth and mycoplasma infection on DNA repair of rat thymocytes has been studied by autoradiography and nucleoid sedimentation. Autoradiographic studies show significant inhibition of unscheduled DNA-synthesis in Yoshida tumor cells after acute mycoplama infection corresponding to the previously described inhibition in spleen cells, however there was only little effect of tumor growth on repair synthesis in thymocytes or spleen cells. γ-irradiation inducedrepair-incorpporation in Yoshida tumor cells was significantly enhanced, compared to normal tissue. Nucleoid sedimentation studies show only in the case of Yoshida tumor cells after mycoplasma infection a dramatic effect in the sedimentation behaviour Fig. 7 - 9).

III. SENSITIZATION OF CELLS BY CHANGING CHROMATIN STRUCTURE

DNA metabolism is not only influenced by enzymatic reactions, but also structurally elements can play a role in radiosensitizing DNA within the complex chromatin. Poly(ADP-ribose) polymerase is an emzyme located in the spacer region of nucleosomes which uses the ADP-ribose moiety of NAD^+ to synthesize the homopolymer poly(ADP-ribose). The activity of this enzyme increases when the DNA of cells is damaged.

It seems that ADP-ribosylation interferes at different levels with cell metabolism (Table 1 and 2).

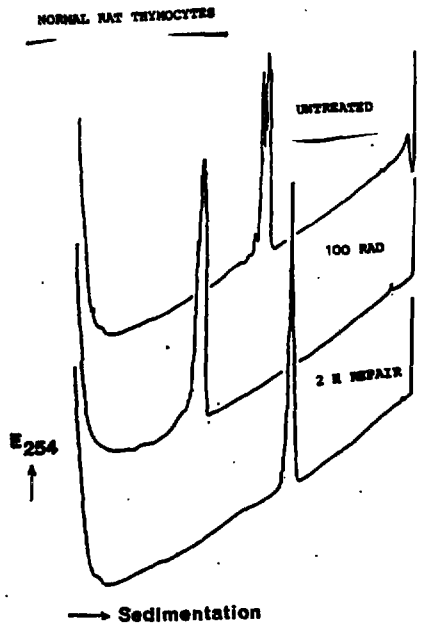


Figure 7: Sedimentation analysis of normal rat thymocytes after careful lysis of the cells at the top of gradient in a mixture of 1 M NaCl, 0.1 M EDTA, 2mM Tris and 0.5% Triton X-100. Untreated cells (a), cells after 100 rad gamma irradiation (b) and after 100 rad gamma irradiation followed by a 2 hour incubation period at 37°C (c).

Figure 8: Sedimentation analysis of rat thymocytes of an animal infected with mycoplasm and yoshida cells.
▽ indicates sedimentation of untreated normal thymocytes.

Experimental conditions as described in Fig. 7.

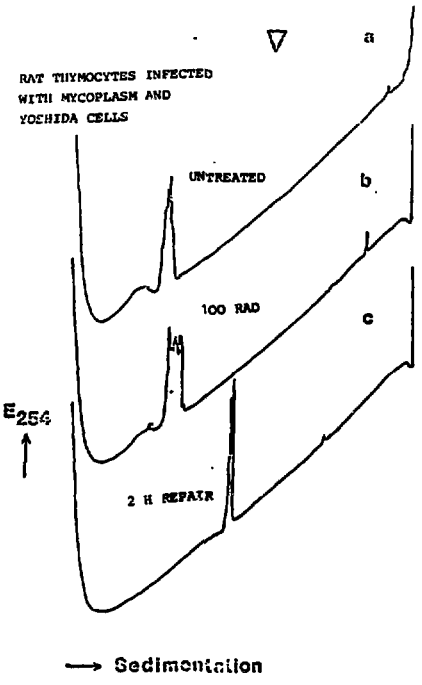


Figure 9: Sedimentation analysis of yoshida ascites cells.
▽ indicates sedimentation of untreated normal thymocytes.

experimental conditions as described in Fig. 7

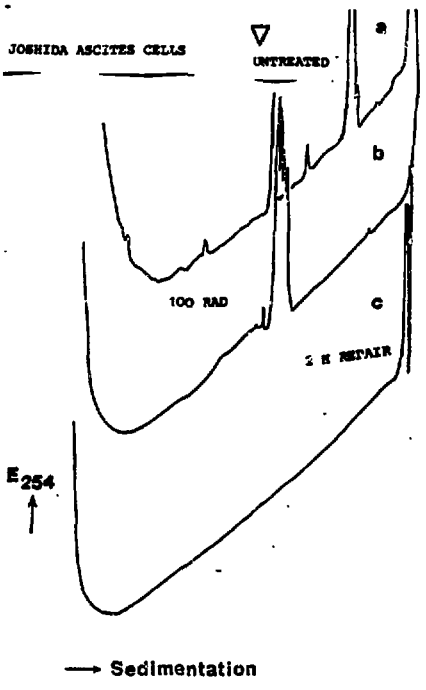


Table 1

I. INHIBITION OF ENZYMES BY ADP-RIBOSYLATION:

1. Endonuclease (Yoshihara et al., 1974, 1975) (4, 5)
2. Exonuclease (Yamada et al., 1974) (6)
3. RNA-Polymerase (Goff and Setzer, 1980) (7)
4. Elongiationfactor (Kessel and Klink, 1980) (8)
5. Protease (Inagaki et al., 1980) (9)

Table 2

II. INTERACTION OF PAR WITH HISTONES IN CHROMATIN:

1. H_2B histone (loosening of nucleosome cores) (Altmann et al., 1979) (10)
2. H_1 histone (chromatin condensation) (Tanseef et al., 1980) (11)

Inhibitors of PAR-polymerase, if administered together with these agents will inhibit the decrease in cellular NAD in a dose dependent fashion.

Immediately after γ-irradiation, biosynthesis of PAR starts and 10 min after γ-irradiation the activity has returned to a basal level (12).

The involvement of PAR-polymerase in DNA-repair is proposed not only because of its activation by DNA strand breaks, but also by the effect of inhibitors of this enzyme, on survival of cells (12). 3-Amino-benzamide, a specific inhibitor of PAR-polymerase prevents rejoining of DNA strand breaks in chromatin. Exonuclease or ligase can be inhibited in this case. Tab. 3 shows that even high concentrations of PAR-inhibitors don't reduce the molecular weight of DNA when inhibitors are not combined with a DNA damaging factor. But their is a synergistic effect in survival when PAR-inhibitors are used in combination with UV (Tab. 4). The same effect can be produced by nutritionally depleting the cells of NAD, the precursor of PAR (13).

Table 3

S U B S T A N C E	CONCENTRATION	MOL.WGHT of DNA
Benzamide	2,5 mM	202 kD
	7,4 mM	186 kD
	25,0 mM	84 kD
Caffeine	1,0 mM	194 kD
	5,1 mM	192 kD
Theophylline	1,1 mM	212 kD
	5,6 mM	196 kD
Control	- - -	200 kD

Table 4

T R E A T M E N T	% GROWING	% GROWING
NON (Control)	100	
80 erg/mm^2 UV	53	100
2,6 mM Caffeine	80	
2,8 mM Theophylline	78	
2,5 mM Benzamide	92	
UV + Caffeine	32	61
UV + Theophylline	30	56
UV + Benzamide	49	93
UV, 15 h, Caffeine	44	83
UV, 15 h, Theophyll.	42	78
UV, 15 h, Benzamide	54	101

The capacity for N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced unscheduled DNA synthesis (UDS) in NAD-depleted cells can be restored upon addition of nicotinamide which allows resumption of normal NAD levels in approximately 8 h (14).

Alkylating agents like N-methyl-N-nitrosourea in the presence of the PAR-inhibitor 5-methylnicotinamide in 2 mM concentration decreases the survival of L1210 cells,

produced a dose enhancement of the effect of the agent of some 15-fold. Theophylline in 250µmM concentration gives a similar effect, but with a dose enhancement of only 4-fold.

The following 2 figures shows the action of theophylline, caffeine and benzamide on replicative DNA-synthesis, UDS and PAR without and after irradiation (Fig. 10 and 11).

Figure 10

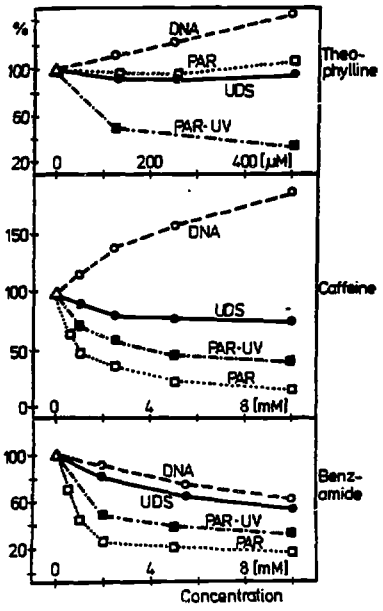
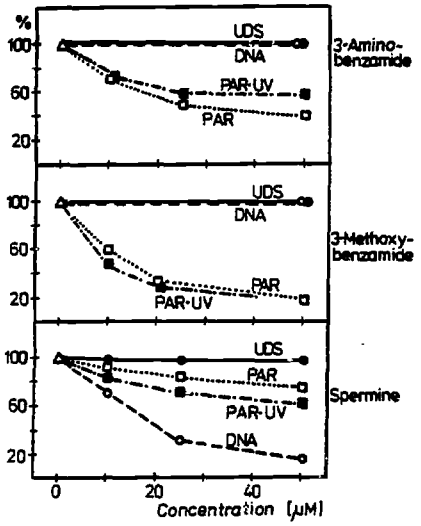


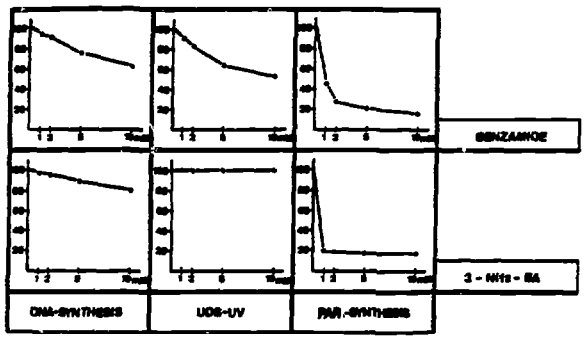
Figure 11



Replicative DNA-synthesis is a little enhanced after treatment with theophylline and caffeine, but PAR after UV-irradiation showed in all cases an inhibition. Benz-amide is a very strong inhibitor of PAR-synthesis and in

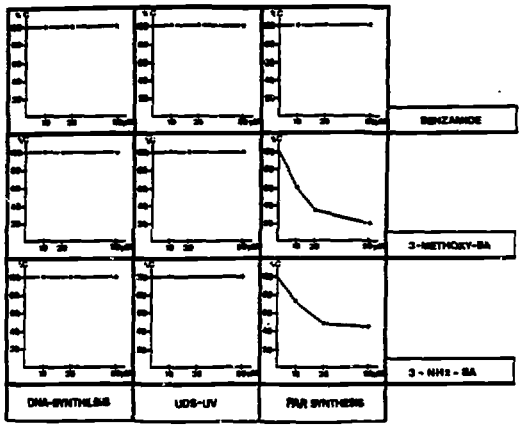
the high concentration range also UDS is decreased. The next figure shows that high concentration of benzamide but not of 3-NH_2-benzamide decreased the UDS after UV-irradiation (Figure 12).

Figure 12



And in figure 13 the effects of low concentrations of benzamide, 3-methoxybenzamide and 3-NH_2-benzamide are compared.

Figure 13



At 50µM concentrations their is a strong inhibition of PAR-synthesis by 3-methoxy- and 3-NH_2-benzamide but not by benzamide. There was no effect at this concentration on DNA-synthesis or UDS after UV-irradiation.

In conclusion we can say that incubation of CHO cells with the tested substances does not lead to strandbreaks in DNA. So they do not damage DNA itself but act on the enzymatic reactions necessary for DNA-repair. One of these reactions - in a side pathway - is the increased formation of PAR after cellular DNA has been damaged. The inhibition of these synthesis seems to have a decreasing effect on the overall DNA repair mechanism.

IV. INVESTIGATIONS ON DNA METABOLISM IN THE TUMOR MODEL USED IN THE MEDICAL INST. OF KFA JÜLICH

175.000 cells/0.3 ml of an adenocarcinoma (EO 771 in our experiments called AC) were injected i.m. to C_{57} bl.mice. After 1 week mice were killed and spleen and adenocarcinoma cells were taken for investigations and spleen cells compared with spleen from control animals.

Table 5 shows that the replicative DNA-synthesis in control spleen cells was not significant different from spleen cells of tumorbearing mice.

Table 5

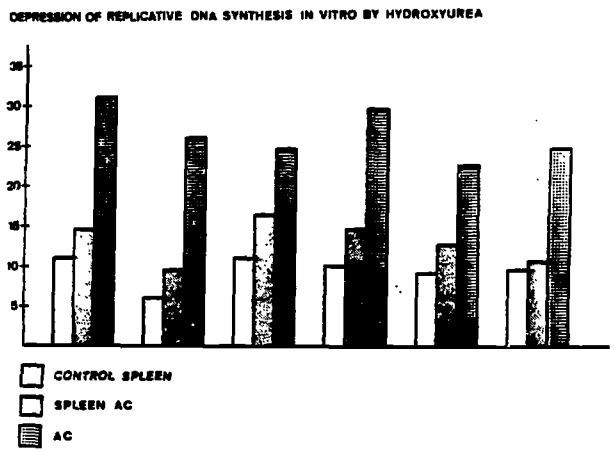
REPLICATIVE DNA-SYNTHESIS IN ADENOCARCINOMA BEARING C_{57} BL MICE

CONTROL SPLEEN CELLS		SPLEEN CELLS 1 WEEK AFTER AC INOCULATION		ADENOCARCINOMA CELLS
$\bar{x}$ 37670		$\bar{x}$ 47041		$\bar{x}$ 261298
s 16669	N.S.	s 9627	S. p<1%	s 109984
N 6		N 4		N 6

A light enhancement of DNA-synthesis of AC spleens can be due to stimulation of some lymphocytes by tumor antigens. The replicative DNA-synthesis of AC cells was significantly higher as in spleen cells.

Because hydroxyurea is also used in tumortherapy and was used in our DNA repair experiments to depress the semi-conservative DNA-synthesis we can show in the next figure that the degree of depression by 10^{-3}m HU is different in all investigated cells (Figure 14).

Figure 14



The residual DNA-synthesis after HU treatment was about 10% for control spleen cells, a little higher in AC spleen and 25-30% in Ac cells.

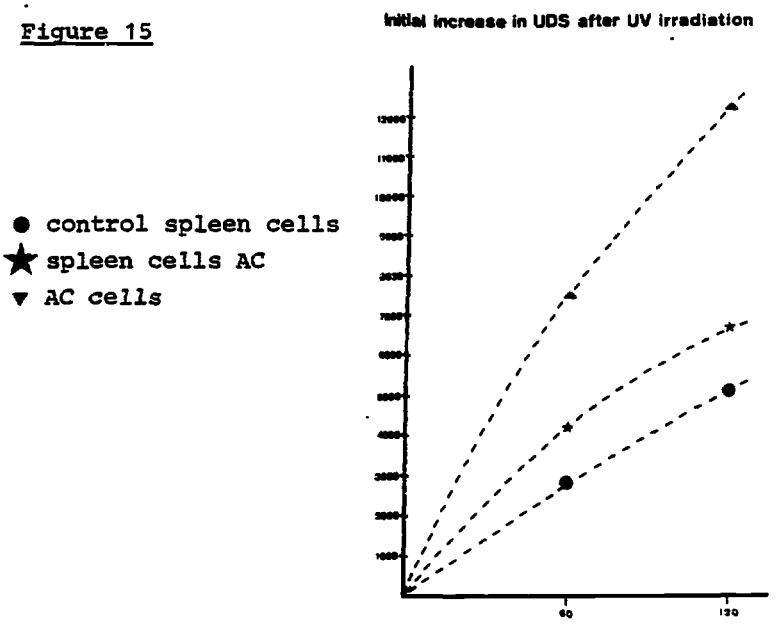
In the next figure and next table the initial increase in UDS one and two hours after UV-irradiation are shown (Table 6, Figure 15).

Table 6

UDS IN ADENOCARCINOMA BEARING C_{57} BL. MICE

	CONTROL SPLEEN CELLS		AC SPLEEN		AC CELLS
1ST HOUR					
	$\bar{x}$ 2991		$\bar{x}$ 4083		$\bar{x}$ 7673
	S 431	$\overleftrightarrow{p<1\%}$	S 768	$\overleftrightarrow{p<1\%}$	S 1691
	N 6		N 6		N 6
2ND HOUR					
	$\bar{x}$ 2061		$\bar{x}$ 2763		$\bar{x}$ 5579
	S 252	$\overleftrightarrow{p<2\%}$	S 488	$\overleftrightarrow{p<1\%}$	S 1267
	N 6		N 6		N 6

Figure 15



UDS values between the differnt cells are significantly different. Also in the case of repair incorporation the highest values shows AC cells.

The next two tables shows the distribution of H^3-thymidine activity within the nucleosomes (Table 7, 8).

Table 7 DISTRIBUTION OF H^3-THYMIDINE INCORPORATION DURING REPLICATIVE DNA-SYNTHESIS BETWEEN MICROCOCCUS NUCLEASE SENSITIVE AND RESISTANT REGION IN CHROMATIN

CONTROL SPLEEN CELLS	0,01	0,03	0,03	0,03
AC SPLEEN CELLS	0,01	0,03	0,04	0,02
AC CELLS	0,005	0,007	0,01	0,01

Table 8 DISTRIBUTION OF H^3-THYMIDINE INCORPORATION IN MICROCOCCUS NUCLEASE SENSITIVE AND RESISTANT REGION OF CHROMATIN AFTER HU-TREATMENT

	SPACER/CORE
CONTROL SPLEEN CELLS	0,45
AC SPLEEN CELLS	0,38
AC CELLS	0,40

Compared with the distribution of radioactivity during replicative DNA-synthesis, the HU treated cells showed more H^3-thymidine incorporation in the spacer region of nucleosomes.

In the next table the PAR distribution between spacer and core region after UV-irradiation is listed (Table 9).

Table 9 DISTRIBUTION OF H^3-PAR-ACTIVITY BETWEEN MN SENSITIVE TO RESISTANT REGION 1 AND 2 AFTER UV-IRRADIATION

	1ST HOUR		2ND HOUR	
	CONTROL	MBA	CONTROL	MBA
CONTROL SPLEEN	0,64	0,7	1,03	1,1
SPLEEN AC	0,99	1,06	1,30	1,21
AC CELLS	0,19	0,18	0,25	0,10

At the second hour after UV-irradiation their is a shift of PAR activity more to spacer region. Self ADP-ribosylation of PAR polymerase or, and synthesis of H1-PAR-H1 bridges could be one explanation for their effect.

The in vitro experiments have shown that 3-methoxybenzamide (3-MBA) inhibit strongly the PAR biosynthesis, but in vivo 3 hours after injection of the PAR inhibitor, their is a small increase in the PAR-radioactivity (Table 10).

Table 12

H^3-THYMIDINE REPAIR INCORPORATION AFTER UV-IRRADIATION IN SPACER AND CORE REGION OF NUCLEOSOMES

$\bar{x}$
N=6

	1ST HOUR				2ND HOUR			
	CONTROL		MBA		CONTROL		MBA	
	SPACER	CORE	SPACER	CORE	SPACER	CORE	SPACER	CORE
SPLEEN AC	903	4460	1236	4668	770	1788	3261	5871
AC CELLS	1436	4883	2134	11307	1392	3017	841	1816

In AC spleen cells no big difference can be seen between 3-MBA treated mice and controls, but their is a remarkable reduction in repair incorporation in AC cells after drug treatment. Further experiments are necessary to explain this effects because PAR synthesis is a little enhanced after 3-MBA treatment, but repair incorporation decreased. It was mentioned in the beginning that PAR is interacting at different levels in cell metabolism. Even when we did not got *in vivo* experiments the expected results it seems promising to work further with PAR-inhibitors.

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