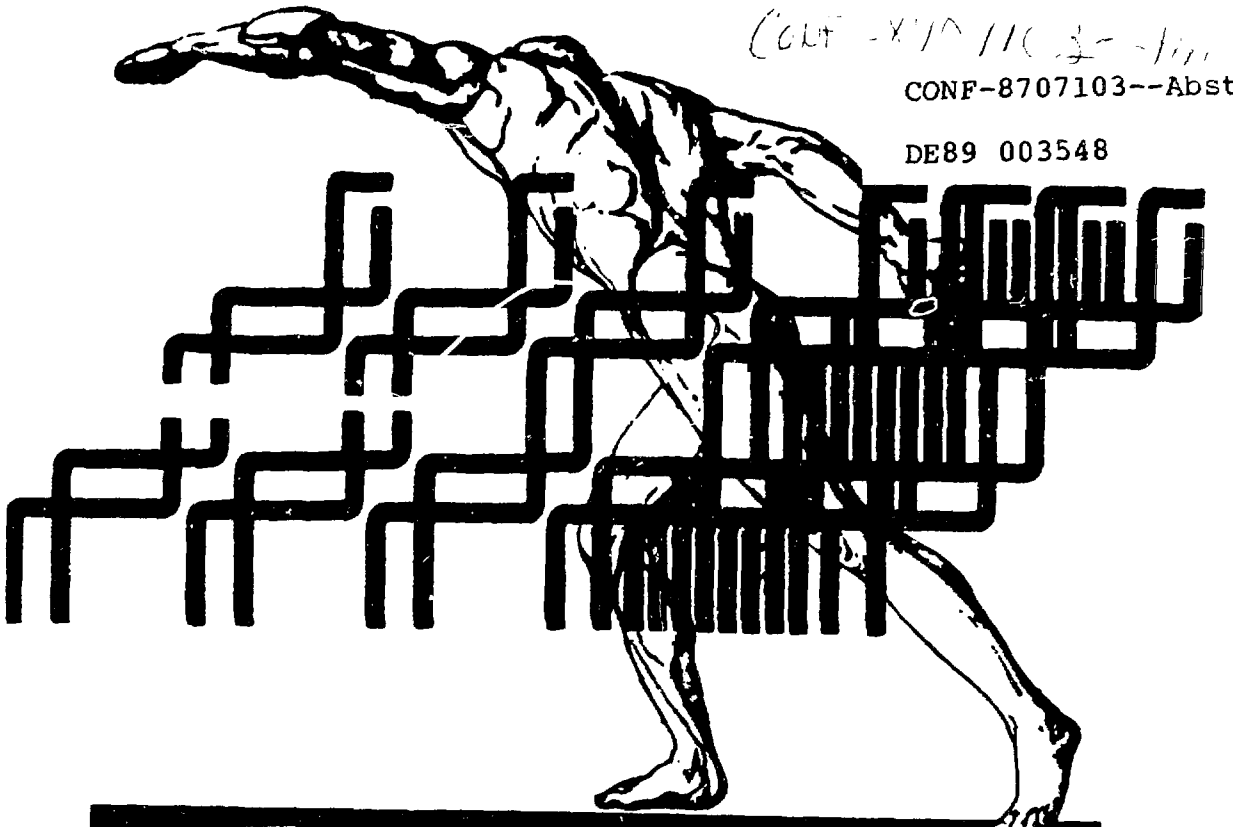

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BOOK OF ABSTRACTS

25 YEARS OF DNA REPAIR

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The crucial experiments identifying pyrimidine dimers as lesions in DNA were done in 1962, but the history begins in 1935 and covers many subjects and people up to the present. My personal view of the history is summarized as follows:

1935	Recovery of UV-irradiated bacteria
1939	The wavelengths effective in mutagenesis are those absorbed by nucleic acids
1944	Bacterial transforming principle is DNA
1949	Photoreactivation (PR)
1949	Liquid holding recovery (LHR)
1952	The infectious material of bacterial viruses is DNA
1953	Watson-Crick double helix
1955	Host cell reactivation
1958	*E. coli* B_{s-1}
1959	PR works enzymatically on DNA
1961	v-gene reactivation
1961	Thymine dimers
1962	Dimers inactivate DNA
1962	PR works on dimers
1963	"Repair Replication"
1964	Nucleotide excision repair of DNA in *E. coli*
1964	LHR overlaps PR
1966	Strand break repair in *E. coli*
1968	Excision repair in mammalian cells
1968	Xeroderma pigmentosum: Humans defective in repair of UV-damage
1974	Alkylation repair and carcinogenesis
1975	Ataxia telangiectasia: Humans sensitive to ionizing radiation
1977	Adaptive response to alkylation
1983	Molecular understanding of bacterial repair systems Transfer of human repair genes to repair-deficient mammalian cells
1984	Excess internal cancers in xeroderma pigmentosum
1986	Heterogeneity in DNA repair among genes in mammalian cells

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THE DYNAMICS OF THE ESCHERICHIA COLI UVR NUCLEOTIDE EXCISION REPAIR SYSTEM.

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Having cloned, mapped, sequenced and amplified the E. coli *uvrA*, *uvrB* and *uvrC* genes made available to us reagent quantities of reagent quality Uvr proteins for in-depth mechanistic studies. The experimental results to date have provided insights into the four-step nucleotide excision reactions (incision, excision, resynthesis and ligation) which, as indicated below, lead to insights into the highly plastic nature of the Uvr proteins as well as the DNA substrate-effector relationship. These separate pathways will be discussed in detail:

I - **PREINCISION REACTIONS**

step 1 - UvrA dimerization:

$$ATP + 2\ UvrA \rightarrow (UvrA)_2$$

step 2 - Nucleoprotein formation:

$$(UvrA)_2 + DNA \rightarrow (UvrA)_2{\cdot}DNA$$

step 3 - UvrAB nucleoprotein formation:

$$(UvrA)_2{\cdot}DNA + UvrB \rightarrow UvrA_2B{\cdot}DNA$$

step 4 - Topological unwinding:

$$UvrA_2B{\cdot}DNA + ATP \rightarrow ATP_n\text{-}UvrA_2B{\cdot}{}_{tu}DNA$$

[tu=topologically unwound]

step 5 - Translocation:

$$ATP_n\text{-}UvrA_2B{\cdot}{}_{tu}DNA \rightarrow ADP + P_i + UvrA_2B{\cdot}{}_{tu}DNA^{\bullet}$$

Damaged site primed for incision by UvrC

II - **INCISION REACTIONS**

step 6 - Dual endonucleolytic events:

$$UvrA_2B{\cdot}tuDNA^{\bullet} + UvrC \rightarrow UvrA_2BC{\cdot}tuDNA^{\bullet} \rightarrow UvrA_2BC{\cdot}inDNA^{\bullet}$$

[inDNA = incised damaged DNA]

III - **EXCISION REACTIONS**

step 7 - UvrD:DNA polymerase I interactions:

$$UvrA_2BC{\cdot}inDNA^{\bullet} + UvrD + pol\ I + 4\ dNTPs \rightarrow 2\ UvrA + UvrB + UvrC + res,nicked{\cdot}DNA$$

IV - **LIGATION REACTION**

step 8 - Polynucleotide ligase reaction:

$$res,nicked\ DNA + ligase + ATP \rightarrow repDNA + AMP + ligase$$

GENES AND PROTEINS REQUIRED FOR NUCLEOTIDE EXCISION REPAIR IN THE YEAST S.cerevisiae

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In the yeast Saccharomyces cerevisiae five unlinked genes are required for the incision of UV irradiated DNA during nucleotide excision repair in vivo. All five genes (RAD1, RAD2, RAD3, RAD4 and RAD10) have been isolated by molecular cloning. The nucleotide sequences of the coding regions predict that these genes encode polypeptides of calculated M_r = 126.2, 117.7, 89.7, 79.1 and 24.3 kDa respectively. All except RAD4 have been tailored into expression vectors for overexpression of protein in yeast and in E.coli, and the proteins expressed in E.coli have been used as immunogens to raise polyclonal antibodies in rabbits. In all cases antibodies react specifically with the appropriate Rad proteins expressed in yeast. Native Rad3 protein as well as a Rad3/β-galactosidase fusion protein have been partially purified following their overexpression in yeast. Both fractions exhibit DNA-dependent ATPase activity which is inhibitable by affinity-purified Rad3 antibodies. Additionally, the RAD3 gene is required for yeast ATPase III activity, an ATPase distinct from Rad3 protein.

ALL five RAD genes are weakly expressed; however, the steady-state level of RAD2 mRNA is increased in cells exposed to a variety of DNA-damaging agents. Deletion mapping of RAD2 upstream sequences suggests that induction of this gene by DNA damage is positively regulated and specific sequences required for induction have been identified. The RAD10 gene has been tailored into a mammalian expression vector and transfected into excision-defective CHO cells from genetic complementation group 2. Cells containing amplified copies of RAD10 express RAD10 mRNA and Rad10 protein. Stable transfectants containing amplified or non-amplified copies of RAD10 show enhanced sensitivity to UV radiation or mitomycin C, relative to control cells without the gene.

ISOLATION AND CHARACTERIZATION OF GENES INVOLVED IN MAMMALIAN EXCISION REPAIR.

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The biochemical and genetic complexity of mammalian DNA repair processes is amply illustrated by the extensive genetic heterogeneity displayed by human repair syndromes and laboratory induced rodent mutants. The understanding of the mechanism of repair systems and the relationship between cancer and ineffective repair is dependent of the availability of the genes and gene products involved.

One of the most straightforward approaches for the isolation of repair genes relies on the correction of repair deficient cells by genomic DNA transfection. This strategy has been successful using various Chinese hamster ovary (CHO) mutants but has failed thusfar for human cells. We have found that the poor transfection properties of many human cells (particularly, the limited amounts of exogenous sequences incorporated by these cells) are -at least in part- responsible for this failure.

Using a UV and mitomycin-C sensitive CHO cell line belonging to complementation group 2 we have cloned the human excision repair gene ERCC-1. The cloning of other excision repair genes using CHO mutants are recipients for DNA transfection is in progress. Gene transfer experiments have shown that ERCC-1 is not defective in XP-complementation groups A,C, D,E and F. Molecular analysis has revealed that the gene spans a region of 15-17 kb on chromosome 19q1.3.2-1.3.3 and consists of 10 exons of which exon 8 (72 bp) is alternatively spliced, generating two main RNA species of 1.0 and 1.1 kb. Minor longer ERCC-1 transcripts arise by the use of alternative polyadenylation sites. The two types of ERCC-1 mRNAs encode largely identical proteins of 273 and 297 amino acids (aa). Both polypeptides harbor a putative nuclear location signal and DNA binding domain. Only the larger protein which is, sufficient for correction of the mutant, contains in addition a potential ADP monoribosylation site. Mutation analysis suggests that the N-terminal part of ERCC-1 protein is less essential for its function. This is supported by aa-sequence comparison of human and mouse ERCC-1, which shows that the N-terminal region displays the lowest level of sequence conservation. Computer analysis revealed significant aa-homology between ERCC-1 and the yeast excision repair protein RAD10 and with parts of the E.coli uvrA and C proteins. This strong evolutionary conservation prompted us to investigate whether other repair genes are conserved as well and whether they can be isolated on the basis of nucleotide sequence homology starting from cloned yeast repair genes. Preliminary results obtained with the yeast RAD1 gene will be presented (In collaboration with Dr. S. Prakash, Rochester).

SYNTHETIC DNA PROBES TO STUDY REPAIR AND MUTAGENESIS OF ALKYLATED DNA BASES AND *cis*-PLATINUM ADDUCTS

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This presentation will focus on a system for examining the after-effects of DNA damage. The approach described involves building defined DNA adducts of chemical carcinogens, chemotherapeutic agents, and radiation into a genome at a known site and then introducing the modified genome into cells, or exposing it to purified repair proteins *in vitro*. The goal of these investigations is to probe the biochemical mechanisms by which DNA lesions exert their lethal and mutagenic effects.

An example of this approach is our studies on O^6-methylguanine, one of the adducts formed by DNA alkylating agents. A tetradeoxynucleotide containing this modified base, 5'-d(TpG^{O6Me}pCpA)-3', was chemically synthesized and then ligated into a four-base gap situated in the genome of M13mp8, an *Escherichia coli* virus. The site-specifically modified genome was used to transfect *E. coli* to produce progeny phage. When the transfecting genome was single-stranded, the mutation frequency of O^6-methylguanine was 0.4%. The mutation frequency of the adduct in double-stranded DNA was approximately 20-fold lower, a result that probably reflects the known preference of the O^6-methylguanine repair protein for a duplex substrate. DNA sequencing of the mutants showed that O^6-methylguanine induced exclusively targeted guanine-to-adenine transitions. In further studies, treatment with N-methyl-N'-nitrosoguanidine was used to deplete cellular stores of the repair protein for O^6-methylguanine. We found that the level of mutagenesis due to the adduct increased linearly with the dose of MNNG, from the value of 0.4% indicated above, to approximately 20%.

Recently, we have extended these studies to include investigation of mutagenesis and repair of DNA adducts located in the chromosomes of mammalian cells. An SV40-based shuttle vector was constructed, into which an oligonucleotide containing O^6-methylguanine was inserted. The vector was introduced by transfection into chinese hamster ovary cells, where the shuttle vector integrated into the hamster chromosome. After about fifteen cell divisions, the shuttle vector was excised from the host chromosome by COS fusion, amplified in bacteria, and the DNA sequence was determined in area of the vector genome that originally contained the O^6-methylguanine residue. The result was obtained that 8-15% of the replicated shuttle vectors contained a mutant adenine at the site originally occupied by the modified guanine base. In further studies it was shown that the mutation frequency of the adduct was strongly modulated by the level of the O^6-methylguanine-DNA methyltransferase in the host.

Similar studies will be described on extension of this technology to study biochemical processing of the adducts of the chemical chemotherapeutic agent, cis-diamminedichloroplatinum(II).

ANTINEOPLASTIC AGENTS INHIBITORS OF TOPOISOMERASE II

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Treatment of mammalian cells with antitumor DNA intercalators (Amsacrine, Adriamycin, Ellipticines) and demethylepipodophyllotoxins (Etoposide, Teniposide) produce protein-linked DNA single- and double-strand breaks. Alkaline elutions show that the breaks are associated with equal frequency of DNA-protein crosslinks and that all the DNA fragments are covalently bound to proteins. These proteins have been purified and shown to be DNA topoisomerase II molecules.

DNA topoisomerases II (topo II) reversibly relax DNA supercoiling and decatenate DNA molecules in the presence of ATP and Mg^{2+}. DNA supercoiling activity has been found only for prokaryotic topo II (DNA gyrase). Mammalian topo II is bound to the nuclear scaffold at the base of chromatin loops where it is involved in DNA replication, transcription, and perhaps repair.

Antitumor drugs inhibit topo II by stabilizing covalent DNA-topo II complexes (cleavable complexes). The molecular interactions between DNA, topo II, and drugs can be studied with purified Mouse Leukemia L1210 topo II and SV40 DNA. Native SV40 DNA is negatively supercoiled (Form I). Drug-induced DNA single- and double-strand breaks can be detected easily by the appearence of nicked circular (Form II) and linear DNA molecules (Form III). Inhibition of topo II catalytic activity can be seen as a reduction of DNA relaxation or catenation. The DNA sequence selectivity of drug action can be investigated by mapping the location of topo II cleavage sites in [^{32}P]-end labeled DNA. Antitumor drugs appear only to enhance DNA cleavage at sites that were already in existence by the virtue of the topoisomerase II activity. Each class of drug produces a specific DNA cleavage pattern.

PREFERENTIAL DNA REPAIR IN THE TRANSCRIBED STRAND OF AN ACTIVE GENE IN MAMMALIAN CELLS

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A quantitative approach to investigate the fine structure of excision-repair in the mammalian genome has recently been developed in our laboratory by Bohr et al. (Cell 40:359 (1985). Repair of UV-induced pyrimidine dimers in the active dihydrofolate reductase (DHFR) gene in Chinese hamster ovary cells was shown to be much more efficient than that in silent upstream sequences or in the genome overall. The preferential repair of vital sequences such as DHFR may account for the high UV resistance of rodent cells in spite of low overall repair efficiencies. In mouse cells Madhani et al. have shown that sequences in the active c-ael protooncogene are repaired much more efficiently than sequences containing the inactive c-mos protooncogene (Cell 45:417, 1986). In human cells we have found that the rate of repair in the DHFR gene is greater than that in the overall genome or in the nontranscribed alpha DNA sequences. (PNAS 83:8070, 1986). Resistance of human cells to UV correlates with repair in an essential gene rather than with overall genomic repair (PNAS 83:3830, 1986). Vos and Hanawalt (Cell 50 Aug 14, 1987 issue) have measured repair of psoralen photoadducts in the human DHFR gene and have shown that most of the interstrand crosslinking but only half of the monoadducts are removed within 24 hours. Replicative bypass of the monoadducts but not the crosslinks was also demonstrated. It is likely that most bulky lesions in DNA pose no insurmountable problems for replication but they must be removed from essential transcribed sequences to maintain cellular viability.

In the above studies we measured repair in specific DNA sequences by using a nick translated DNA probe which hybridizes to both DNA strands. We have now constructed plasmids that allow us to independently examine repair in the transcribed and nontranscribed strands using strand-specific RNA probes. We find a dramatic difference in the efficiency of removal of pyrimidine dimers from the transcribed and nontranscribed DNA strands of the DHFR gene in hamster and human cells. In hamster cells 80% of the dimers have been removed from the transcribed strand in 4 hours but little repair has occurred in the nontranscribed strand in 24 hours. In human cells, repair is significantly faster in the transcribed strand of the DHFR gene. Furthermore, in the 3' flanking region of the human DHFR gene, selective rapid repair is found in the opposite DNA strand relative to the transcribed strand of the DHFR gene. This strand is thought to serve as a template for transcription of a divergent transcript. We hypothesize that an excision-repair complex may be coupled to the transcription machinery to ensure removal of transcription-blocking lesions. Additional freely-diffusing repair-complexes may account for the repair in nontranscribed DNA strands and in silent regions of the genome. Our findings have important implications for mechanisms of excision repair and mutagenesis in mammalian cells. (Supported by grants from the National Cancer Institute and the American Cancer Society)

RELEVANCE OF DNA REPAIR IN MALIGNANT TRANSFORMATION BY CHEMICAL CARCINOGENS

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Structural modifications of genomic DNA are primary events in the process of malignant transformation by chemical carcinogens. Alkylating N-nitroso compounds, such as the highly carcinogenic N-alkyl-N-nitrosoureas, are now well characterized in terms of the nature of their reaction products initially formed on oxygen and nitrogen atoms in DNA. Recent methodological progress in molecular genetics and the development of ultrasensitive detection methods (e. g., anti-alkyldeoxynucleoside monoclonal antibodies with affinity constants $\geq 10^{11}$ l/mol) allow us to determine the sites and distribution of specific DNA alkylation products in defined DNA sequences. Considerable advances have also been made regarding the characterization of the varying capacity of different types of mammalian cells for enzymatic removal from DNA of specific alkylation products (e. g., of O^6-alkylguanine, which may cause GC-AT transition mutations if persisting unrepaired through DNA replication in proliferation-competent target cells). We have attempted to evaluate the importance for the risk of malignant conversion, of the specific enzymatic elimination of O^6-ethylguanine (O^6-EtGua) from the DNA of cells exposed to N-ethyl-N-nitrosourea (EtNU) in vitro. O^6-EtGua repair-proficient (R^+) variants were isolated from cloned O^6-EtGua repair-deficient (R^-) 208F rat cells. Contrary to the R^- cells, the R^+ variants exhibited O^6-methylguanine-DNA methyltransferase activity. R^- and R^+ cells were briefly treated with EtNU, and the frequencies of piled-up foci and of colonies formed in 0.15 % agar medium were determined as well as the tumorigenicity of cells derived from agar colonies. After exposure to 500 µg of EtNU/ml, the frequency values for the R^+ variants were up to 20-fold (piled-up foci) and 50-fold (agar colonies) lower than those obtained for the R^- wild type. In contrast to cells derived from R^+ variant agar colonies (0/8), the cells from 4/6 R^- agar colonies were highly tumorigenic in syngeneic animals. After exposure to activated benzo(a)pyrene (BPDE I), no significant differences were found between R^- cells and R^+ cells with respect to the frequencies of piled-up foci. The specific enzymatic removal of O^6-EtGua from the DNA of cells exposed to N-nitroso carcinogens thus appears to provide substantial protection regarding the risk of malignant transformation.

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CHROMATIN CONFORMATIONAL CHANGES: RELATIONSHIPS TO DNA FRAGMENTATION.

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It has been shown that sedimentation at neutral pH of DNA anchored to proteins of the nuclear matrix (nucleoids) is not only sensitive to DNA fragmentation but also to changes in chromatin conformation (P.D. Lipetz et al., Adv. Cancer Res., 36:165-210, 1982). Following this indication we have tried to adapt the DNA neutral elution technique (M.O. Bradley and K.W. Kohn, Nucleic Acids Res., 7: 793-804, 1979) to the detection not only of double stranded breaks but also of changes in chromatin conformation. Nuclei were lysed in two different ways: a) high ionic strength and little detergent (2M NaCl + 0.2% N-lauroylsarcosine sodium salt + 20 mM disodium EDTA, pH 10); b) high detergent concentration (2% SDS + 20 mM disodium EDTA, pH 10).

Analyzing for proteins of the nuclear matrix remained attached to DNA with the two types of lysis, large quantities of them were found with the first type of incubation (the preparation appeared similar to nucleoid preparations), and very little with the second type. We have now several experimental evidences (for instance: P. Russo et al., Cancer Res., 47: 2866-2874, 1987) suggesting that a neutral elution performed in the presence of nucleoid like structures is sensitive both to breaks and changes in chromatin conformation.

The second type of lysis leaves a DNA practically completely naked, sensitive only to double stranded breaks.

We propose this - double differential elution - as a method for discriminating changes in chromatin conformation from real DNA fragmentations. Changes in chromatin conformation could be relevant for modulations of cell differentation and also during the cell cycle.

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DNAase I-HYPERSENSITIVE SITES OF THE c-Ha-ras-1 PROTO-ONCOGENE AS TARGETS FOR RAPID BENZO[a]PYRENE BINDING AND REPAIR

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General correlations between covalent binding of carcinogens to total cellular DNA and actual carcinogenic activity are insufficient to explain the selectivity and specificity of carcinogenic responses and the rarity of neoplastic events. We investigated selective conditions capable of revealing preferential binding of carcinogens to certain regions of DNA, namely to DNAase I-hypersensitive (HS) regions, where chromatin structure allows rapid DNA digestion with low doses of DNAase I (0.1-1.0 Kunitz units for 3 min). We examined the binding of benzo[a]pyrene (BP) in vivo to hamster liver DNA after a single intraportal injection. At selected times after BP, liver nuclei were isolated and then treated with DNAase I (at 3 dose levels). Without DNAase digestion, total BP binding was maximal at 30 min and 86% lower by 60 min. At 30 min, 80% of the bound BP was removed by DNAase I treatment; at 60 min, DNAase I had no effect on residual bound BP. Thus, most BP appears to be bound initially at DNAase I-HS sites, where rapid repair occurs, whereas the fraction remaining after repair (14% at 60 min) appears to be bound outside the HS sites.

The c-Ha-ras-1 proto-oncogene was examined in the same experimental system. DNA, isolated from nuclei treated as above, was digested with restriction endonucleases (Eco RI or Bam HI), transferred by Southern blotting and hybridized with a 0.5 kb v-Ha-ras probe. Treatment with 3 dose levels of DNAase I without BP showed progressive digestion of the original restriction fragments and appearance of shorter fragments. Treatment with BP alone (no DNAase) showed formation of a fragment of a size also produced by DNAase I. When BP treatment was followed by digestion with DNAase I, the HS bands were produced at lower DNAase doses than without BP; at 60 min after BP treatment, evidence of repair was seen. Thus the Ha-ras gene was found to have DNAase I-HS sites, selectively affected by BP.

Mapping of the DNAase I-HS sites in the human c-Ha-ras-1 gene was studied by DNAase I treatment of nuclei from normal human liver. Preliminary results suggest that the DNAase I-HS sites are localized in the promoter region and possibly in the VTR (enhancer) region. This localization suggests that, if carcinogens damage these sites preferentially, they may act by altering gene expression.

INHIBITING DNA REPAIR: MODELS, MANIPULATIONS AND MISCONCEPTIONS

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Although there is great interest at present in exploring the genetics of DNA repair, the biochemical approach should not be neglected. In fact, the two approaches are complementary; biochemical assays are essential for characterising the altered phenotypes in mutant cell lines and in cells transfected with foreign DNA.

DNA synthesis inhibitors are particularly useful for dissecting the biochemistry of DNA repair. Incubating UV-irradiated cells with hydroxyurea and either cytosine arabinoside or aphidicolin seriously impedes DNA polymerisation, causing the quantitative accumulation of incomplete repair sites, and it is thus possible to analyse the kinetics of incision, the rate-limiting step of nucleotide excision repair. On removal of inhibitors, ligation of the accumulated breaks can also be studied.

The myth persists that hydroxyurea and cytosine arabinoside do not affect repair DNA synthesis, for unscheduled DNA synthesis is still detected in proliferating cells in the presence of the inhibitors. In all probability, a much reduced rate of polymerisation is balanced by a much increased period of synthesis, since repair sites remain open for far longer than normal.

Another common misconception is that DNA polymerase β is the main repair synthesis enzyme. However, the potency of specific polymerase α inhibitors (such as aphidicolin) at blocking repair argues strongly against this.

Hydroxyurea acts on repair via a depletion of the pool of DNA precursors. These pools can also be manipulated in a CHO-derived purine auxotroph cell line, Ade^{-}C, by transferring the cells to medium lacking hypoxanthine. DNA replication quickly comes to a halt, but an abortive form of repair continues. Repair synthesis and incision are detected, but damage is not removed. This abortive repair is associated with increased cell killing and with an elevated incidence of mutation.

Since the majority of cells in an adult organism are in a state of quiescence and have very small DNA precursor pools, the precursor-starved state obtained with hydroxyurea or with purine auxotrophs may provide a model system for evaluating effects of DNA damage *in vivo*.

ENZYME DEFICIENCIES IN INHERITED HUMAN SYNDROMES WITH DEFECTIVE DNA REPAIR OR REPLICATION

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We are attempting to establish functional assays with cell-free systems that distinguish between cells from normal individuals vs. cells from human inherited syndromes associated with chromosome instability. Access to EBV-immortalized lymphoblastoid cell lines representative of different syndromes is important to these studies, because large amounts of cells grown in suspension cultures are required for the biochemical experiments. These investigations may facilitate the molecular cloning and characterization of the defective genes in diseases such as Bloom's syndrome, xeroderma pigmentosum, ataxia-telangiectasia and Fanconi's anaemia.

As part of these ongoing studies we have measured the levels of DNA ligase activities in size-fractionated cell extracts from several different lines representative of Bloom's syndrome. DNA ligase I, which is believed to be active in DNA replication, is consistently altered in such cells, whereas ligase II is normal. Two different types of structural alteration in ligase I have been observed. Our data support a model in which Bloom's syndrome is due to a leaky point mutation occurring at one of at least two alternative sites in the structural gene for DNA ligase I.

FANCONI ANEMIA : Genetics and correction of the defect

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Fanconi's anemia (FA), an inherited autosomal recessive disorder in man, belongs to the class of diseases characterized by chromosomal instability, predisposition to cancer and possibly linked to deficiencies in DNA lesions processing. Cells from individuals with FA have, among other features, an increased sensitivity to agents that cause interstrand DNA cross-links. When we started our work 3 years ago, the number of genetic complementation groups involved in FA and their phenotypic expression were not established, the role and nature of defective DNA repair in the disease were controversial and the expression as well as regulation of the gene(s) concerned were completely unknown. The progress accomplished by us and others along these three lines will be reported. We have shown that the two genetic complementation groups reported for FA correspond to two phenotypic classes identifiable by measurements of DNA semi-conservative synthesis after a DNA cross-linking treatment. This allows a rapid classification of FA patients. On the other hand, these two genetic classes can be distinguished on the basis of clonogenic cell survival and in their ability to incise psoralen photoinduced cross-links. We have shown that the correction of the FA defect by transfection with high molecular weight DNA from normal cells can be achieved and preliminary results toward the cloning of the gene(s) involved will be presented.

REPAIR OF O^6-METHYLDEOXYGUANINE (O^6medG) AND O^4-METHYLTHYMIDINE (O^4-meT) IN HAMSTER AND RAT CELLS

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The mutagenic and carcinogenic effects of methylating agents like NMU or DMN are associated with the presence and persistence of two DNA adducts: O^6medG and O^4-meT which possess miscoding properties during DNA replication (1). In *E. Coli* both these DNA adducts are carried out by the same O^6-methylguanine DNA alkyltransferase (2), whereas in mammalian cells the data are contradictory and limited.

The aim of the experiments reported is to compare the rate of disappearance of O^4-meT from DNA extracted from hamster or rat cells after treatment *in vitro* or *in vivo* respectively with NMU or DMN. It is known that cells from these two species significantly differ in their capacity to repair O^6-medG. The level of these DNA adducts was carried out using specific antibodies against these alkylated nucleosides following their separation by HPLC.

Rat liver epithelial cells (IAR 27) and V 79Z hamster cells were treated with NMU (1mM) and the DNA adducts were then measured at various times. The results show that the mutagenic response to NMU in these two cell types (high mutagenic effect in V 79Z and low in IAR 27) parallels the low repair of O^6-medG in V 79Z versus IAR 27 cells; no such correlation is observed with repair of O^4-meT. The rate of repair of O^4-meT and O^6-medG in rat and hamster livers treated *in vivo* with DMN will be reported.

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IN VITRO CORRECTION OF CELLS FROM PATIENTS WITH MUTAGEN SENSITIVITY

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An important strategy in the study of mutated cells is based on the principle of correction. Correction of the mutation can be either genotypic (DNA-mediated) or phenotypic (RNA- or protein-mediated). Its purpose can be either characterisation of the mutated system by introduction of known factors, or identification of the non-mutated correct counterparts from the wild-type system. Correction is also the basis of the analysis of the genetic complexity of the mutated system by complementation studies. In human cells this approach was first shown to be succesful with the inherited disorder xeroderma pigmentosum (XP).

Results will be presented from complementation studies on about 30 different patients with ataxia telangiectasia (AT). The relatively radioresistant DNA synthesis, characteristic of AT cells was used as a marker. Four different complementation groups, named AB, C, D and E were discerned so far.

Six patients with a genetic disorder were identified on the basis of published clinical symptoms, i.e. microcephaly and growth delay in addition to immune deficiency, cancer proneness and chromosomal instability, but no ataxia and telangiectasia. These patients' cells were shown to be radiosensitive, having a diminished X-ray induced inhibition of DNA synthesis typical of AT. Genetic analysis revealed, that the 6 cell strains comprise two different complementation groups, called V1 and V2 and distinct from the AT groups.

In XP cells a transient correction of the defect can be accomplished by microinjection of cell-free extracts from wild-type sources, such as HeLa cells, human placenta or bovine liver and thymus. The correcting factors are able to restore normal levels of UV-induced unscheduled DNA synthesis (UV-UDS) in XP cells of most complementation groups. Some properties of the XP-A correcting factor were determined and purification of this factor from calf thymus with the correction as assay is currently in progress.

Microinjection was also used to introduce the purified yeast photoreactivating enzyme into cells. Using an immunocytochemical method an almost complete light-dependent loss of pyrimidine-dimers was demonstrated in injected cells. However, both in wild-type and XP-fibroblasts the levels of UDS were found to decrease only slowly and with different kinetics after photoenzymatic removal of the dimers. Kinetic studies suggest that the average processing time of dimer excision in normal cells is less than 1 hour and in some XP groups more than 3 hours.

THE ASSOCIATION OF XERODERMA PIGMENTOSUM (XP) WITH TRICHOTHIODYSTROPHY (TTD): A CLUE TO A BETTER UNDERSTANDING OF XP-D?

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A defective DNA repair of the UV-induced damage, similar to that present in XP, was found in four photosensitive patients affected by TTD.
The lack of complementation of the defect in TTD cells fused with XP cells belonging to complemented group D indicated that the XP-D mutation is present in the patients under study.
TTD is a rare autosomal recessive disorder in which the main clinical features are brittle hair with reduced sulfur content, ichthyosis, physical and mental retardation. Photosensitivity is reported only in some TTD patients.
Normal UV repair capacity was observed in patients affected by TTD but not showing photosensitivity. Cells from these patients were able to complement the defect of TTD UV sensitive cells as efficiently as the normal ones; this demonstrates that TTD and XP-D are independent mutations.
Since the four TTD/XP-D patients were from three apparently unrelated families, but living in the same region, we checked consanguinity and concluded that the affected individuals are carriers of a genetic defect identical by descent as a consequence of remote multiple inbreeding.
This genetic defect could consist of two mutations either at linked loci or affecting the same gene. However a chromosome rearrangement or a deletion involving XP-D and TTD loci, appear to be a more probable cousative event.
Cytogenetic analysis performed on carriers and affected individuals did not show any chromosome anomaly; also blood markers typing in the three families did reveale neither segregation anomaly nor linkage between the pathologic condition and the genetic markers analyzed.
Interestingly, DNA repair investigations on other TTD patients demonstrated that the TTD/XP-D association is not confined to the four italian cases. The same situation could hold for all the TTD photosensitive patients reported in the literature that are about half of the total TTD cases.
These data stress the interest of extending the study to new cases because the TTD/XP-D association may represent a valuable system for elucidating the genetic and molecular bases of the two syndrome.

REPAIR OF SECONDARY LESIONS INDUCED BY ALKYLATING AGENTS IN DNA.

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The biological implications of the alkylation of DNA differs according to the nature of the modified bases. 7-methylguanine is believed to be harmless and 3-alkylpurines lethal lesions in contrast to O^6-alkylguanine and O^4-alkylthymine which are mutagenic. Most of these lesions appear to be actively repaired. The former one by DNA glycosylases and the latter one by O-alkyltransferase.

However, a given lesion can evolve with time. Alkylation of the nitrogen in the position 7 of purines labilizes the glycosidic bond and favors depurination yielding an apurinic site. AP-sites are lethal or mutagenic lesions depending upon the circumstances. They are actively repaired by specific AP-endonucleases which are widely distributed. Most of them incise the DNA backbone either on the 5' or the 3' side of the AP-site.

An other consequence of the methylation of the nitrogen of the purines at the 7 position is the labilization of the imidazole ring which opens up yielding 2,6-diamino-4-oxo-5-methylformamidopyrimidine (Fapy). This lesion is a powerful block to DNA replication. It is excised by a specific DNA-glycosylase which is active when the lesion is harbored by a DNA in the B-form but inactive when the lesion is in the Z-form. The gene coding for this enzyme in E. coli has been cloned and its sequence determined.

In conclusion beside the primary lesions which occur in DNA after alkylation, secondary lesions may have important biological properties.

MECHANISMS OF ALKYLATING AGENT INDUCED CYTOTOXICITY IN E.Coli AND MAMMALIAN CELLS

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The alkylating agents, of which methylating agents offer the simplest example, exert both mutagenic and cytotoxic effects on cells. In E.coli, cellular resistance to these effects is controlled partly by constitutively expressed DNA repair enzymes and partly by the enzymes induced during the adaptive response to alkylating agents. There is considerable evidence, both genetic and biochemical to suggest that the presence of 3-methylpurines in DNA, blocks DNA replication, and represents the major cytotoxic threat to the cell. Two enzymes, one constitutive (3-methyladenine-DNA glycosylase I) and one inducible (3-methyladenine-DNA glycosylase II) repair this type of damage. In contrast, the major mutagenic threat is posed by O^6-methylguanine in DNA. Repair of this lesion is carried out by a methyltransferase induced as part of the adaptive response. Efficient action of this enzyme protects cells against the transition mutations induced following replication of O^6-methylguanine in DNA. It appears therefore, that the two processes of cell killing and mutation are to a large degree separate and result from the effects of two different types of lesion.

Mammalian cells constitutively express DNA glycosylase and methyltransferase activities analogous to those of E.coli. Although well characterised mutant mammalian cell lines defective in these activities are not yet available, the Mex-(Mer-) phenotype offers a close approximation. Cells of this phenotype are sensitive to both killing and mutagenesis by methylating agents. However, they have normal (Mex+) levels of a DNA glycosylase active on N-methylated purines and, of the DNA repair enzymes so far assayed, are deficient only in O^6-methylguanine-DNA methyltransferase. Complementation of this methyltransferase defect by expressing the bacterial gene in Mex- mammalian cells abolishes the sensitivity of these cells to mutation induction and cell killing. Thus, in contrast to the situation in E.coli, O^6-methylguanine in DNA appears to play a major role in the cytotoxicity of methylating agents in mammalian cells.

DNA REPAIR OF ALKYLATION DAMAGE AND ITS RELEVANCE TO MUTATION FIXATION IN MAMMALIAN CELLS.

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Considerable evidence suggests that O^6-alkylguanine is the premutagenic lesion responsible for mutagenicity and carcinogenicity induced by alkylating agents. The repair of O^6-alkylguanine adducts to DNA, both in bacteria and in mammalian cells, proceeds via the transfer of the alkyl group to a cysteine residue in an acceptor protein, the O^6-methylguanine-DNA methyltransferase (MT). We have analysed the relevance of repair of this lesion on cell killing, ouabain resistance (oua^r) mutations and SCEs induced by alkylating agents in MT-deficient and MT-proficient CHO cells. The MT-proficient CHO cell line was obtained by transfection of the parental MT-deficient cells with human liver DNA (Ding et al. Mol.Cell.Biol. 1985, 5:3293-3296). The advantage of comparing these two lines is that the effect of repair by MT can be examined in cells with the same genetic and biochemical background. MT-proficient cells resulted to be more resistant than MT-deficient ones to the cytotoxic and mutagenic effects of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea. Furthermore a lower number of MNNG-induced SCEs was found in MT-proficient CHO as compared to MT-deficient cells. These data clearly indicate that repair of O^6-methylguanine highly correlates with resistance to cytotoxic and mutagenic effects of methylating agents.

Similar oua^r mutation frequencies and number of SCEs were observed in the two cell lines after N-ethyl-N-nitrosourea treatment. Determination of O^6-ethylguanine levels by monoclonal antibodies showed removal of this adduct in the ENU-treated MT-proficient cells and persistance of this lesion in the parental cell line. A role of lesions other than O^6-ethylguanine in mutagenesis and SCEs induced by ethylating agents is strongly suggested.

Derivatives of MT-proficient CHO cells with enhanced resistance to the cytotoxic effects of alkylating agents have been isolated and their characterization is in progress. The analysis of these mutants may facilitate the comprehension of the mechanisms of repair and genotoxicity induced by alkylation damage.

THE RESPONSE OF HUMAN CELLS TO METHYLATION DAMAGE

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Individuals with Hodgkin's disease (HD) may be treated by a chemotherapeutic regimen which includes procarbazine, a compound metabolized to a methylating species. A small proportion of all HD patients eventually develop ANLL some years after therapy (t-ANLL). We therefore initiated a study of the O^6-methylguanine DNA methyltransferase (MT) activity in normal controls and in HD and primary and t-ANLL patients. As part of the study we also prepare lymphoblastoid lines by Epstein Barr virus (EBV) transformation of peripheral blood lymphocytes (PBL's). We express MT activity per µg of DNA. The activity in normal individuals varies from about 2.2 to 14.7 fmol/µg of DNA with a mean ± SE of 7.2 ± 0.35. HD patients before treatment have MT values of 4.0 ± 0.42 with a range of 0.7 to 8.6. HD patients on Procarbazine have a MT value of 4.0 ± 0.36; t-ANLL patients before treatment gave 4.2 ± 0.63 and six ANLL patients had MT values of 7.8 ± 1.72. There is significant variation in the MT values of normal PBL's sampled at different times. MT activity diminishes slightly with age in both normal and HD groups but this does not account for the observed difference.

We find a significant correlation between the MT activity of PBL's and of the lines derived from them, particularly when normal or untreated individuals are used as the source of the lines. There is variation in the the MT values of successive lines from the same individual but it is within a factor of about two in these experiments. Two lines each with zero MT activity have been obtained from two individuals undergoing therapy including procarbazine.

Although there is significant variation in repeated samples of PBL's from the same individual, there are differences in the MT activity of different groups. Individuals with Hodgkin's disease have significantly lower MT activity than do normal controls. In addition, the HD group includes individuals with almost no MT activity. The observation that individuals with t-ANLL have lower MT activity than either controls or ANLL patients would, if confirmed, lend credence to the hypothesis that MT levels play a role in the etiology of secondary malignancy. There is a clear relationship between the MT level in lymphocytes and in the lines derived from them. We interpret these results to mean that the MT level of a cell is a characteristic which survives the events of EBV transformation.

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MOLECULAR MECHANISMS IN REPAIR AND MUTAGENESIS INDUCED BY AROMATIC AMINES

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The conversion of DNA lesions into mutations is an active biochemical process. Due to the remarquable efficiency of the error free repair mechanisms only a very small number of DNA lesions will eventually be processed into a mutation. Under normal conditions, less than one percent of DNA lesions give rise to mutations. This makes the biochemical study of the mechanisms involved in mutagenesis very difficult. As a first molecular approach, the study of the mutationnal specificity of a given mutagen will provide important informations concerning the mechanisms that are involved. This is particularly true if the analysis of the mutational specificity can be performed in hosts having altered genotypes for repair and (or) mutagenesis. Bacteria are in this respect the organisms of choice, due to the large number of existing repair, replication and recombination mutants.

When the chemical carcinogen *N* -2-acetylaminofluorene binds to DNA *in vivo* , two major adducts are formed, both at position C-8 of the guanine residues : the acetylated (-AAF) and the deacetylated (-AF) adducts respectively. Unlike -AAF adducts, which trigger important structural changes of the DNA secondary structure (either the Insertion-Denaturation model or the induction of a Z -DNA structure, depending upon the local DNA sequence), -AF adducts do not cause a major conformational change in the *B* -DNA structure.

In a forward mutation assay, namely the inactivation of the tetracycline resistance gene located on plasmid pBR322, we found that more than 90% of mutations induced by -AAF adducts are frameshift mutations (1,2,3). These frameshift mutations depend upon the UV irradiation of the host cell and are found to be clustered within specific sequences referred to as mutation hot spots. These hot spots occur either at monotonous runs of guanine residues (*umuC* dependent) or within short stretches of alternating GC base pairs (*umuC* independent). Using the same assay we showed that -AF adducts induce primarily base substitution mutations (85%), mainly G->T transversions (4). Both -AAF and -AF induced mutations occur at GC base pairs and are therefore likely to be targeted at the site of a DNA adduct. We would like to stress that there is a strong correlation between the nature of the carcinogen-induced conformational change of the DNA structure and the corresponding mutation specificity (i.e. the highly deforming -AAF adducts induce frameshift mutations whereas the non deforming -AF adducts induce base pair substitutions).

In recent experiments, using double stranded DNA molecules carrying -AAF adducts in only one strand we observed a highly reduced mutation efficiency. Genetic labelling of the two DNA strands allowed us to demonstrate that there is a preferential loss of the damaged strand during replication (5).

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DNA STRUCTURAL ALTERATIONS INDUCED BY BULKY ADDUCTS

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Removal of bulky adducts is initiated by a multiprotein complex, the product of the uvr ABC genes. Because of its broad substrate recognition range, it has been proposed that the uvr ABC complex recognizes a structural distortion common to this class of adducts rather than the chemical nature of the adducts themselves. In order to understand the mechanism of enzyme-damage recognition we have initiated an analysis of the DNA perturbation created by bulky adducts.

The conformational changes caused by UV photodamage and by 4-NQO adducts have been investigated by analyzing the electrophoretic behaviour on agarose gel of DNA rings with different degree of supercoiling (band shift method). We observed, after irradiation at 254 nm light, an upward shift of negatively supercoiled topoisomers and a downward shift of positively supercoiled topoisomers corresponding to a topological unwinding angle per pyrimidine dimer of -14.3° and -8.5° respectively. By averaging the upward shift and the downward shift, we obtained an unwinding angle of -11.4°, a value which had to be further reduced to -8.8°, because photoreactivation experiments have shown that topoisomer shift is caused for about 20% by other kinds of photodamage. The role played by dimers in conferring a single-stranded character to UV irradiated DNA was tested by comparing the number of UV induced S1-sensitive sites before and after photoreactivation. We found that these sites are present after photoreactivation, suggesting that they are due to rare UV photoproducts.

An analogous study performed with the adducts produced by the o-acetyl derivative of the carcinogen 4-nitroquinoline-1-oxide has also revealed a discrepancy between the upward shift of the negatively supercoiled topoisomers and the downward shift of the positively supercoiled topoisomers. After a correction due to the contribution of AP sites to the variation in electrophoretic mobility of damaged molecules, we calculated an unwinding angle per adduct of -9.3° which is of the same order as that produced by dimers.

These results suggest that UV-like damage cause a local unwinding of duplex DNA that is inconsistent with the actual disruption of the hydrogen bonds at the damage level. In addition they introduce distortion of the helix axis which imposes a positive writhe in the negatively supercoiled DNA and a negative writhe in the positively supercoiled DNA.

DNA DAMAGE, REPAIR AND MUTATIONS IN XERODERMA PIGMENTOSUM CELLS MEASURED WITH PLASMID VECTORS

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We have used plasmid vectors to measure DNA repair and mutagenesis in human cells. Plasmids constructed to permit replication in bacteria and utilization of human host cell enzymes for expression or replication were used. Plasmids are damaged in vitro (permitting precise quantification of the extent of damage and possible further in vitro modification), transfected into xeroderma pigmentosum (XP) or repair proficient human cells, permitted to be expressed, repaired, mutated or replicated for 2-3 days and then harvested and the plasmids assayed. Repair is measured by determining the extent of expression of novel enzyme activity coded for by the damaged plasmid. Repair deficient cells show less activity than repair proficient cells. Similarly, transformation of indicator bacteria by the harvested plasmid is used to assess the plasmid survival, and mutation frequency and DNA sequencing can be performed on mutated plasmids recovered.

Using a non-replicating plasmid coding for the bacterial cat (chloramphenicol acetyl transferase) gene we have shown that one pyrimidine dimer inhibits expression in XP group A cells. Use of photolyase specifically to remove cyclobutane dimers revealed that non-dimer photoproducts are also blocks to replication in XP group A cells.

Using a replicating shuttle vector plasmid we found that there is a restricted spectrum of ultraviolet induced mutations found with the XP group A cells. Plasmid survival was reduced and fewer plasmids had multiple base substitution mutations or transversions. The major UV photoproduct, the TT dimer was only weakly mutagenic. The majority of mutant plasmids had G:C to A:T base substitution transition mutations. Treatment of the UV damaged plasmid with photolyase prior to transfection of the XP group A cells showed increased plasmid survival and decreased mutation frequency indicating that cyclobutane dimers contributed to a major portion of lethality and mutagenicity. Since the major class of mutations involved G:C base pairs, cytosine rather than thymine containing dimers are implicated. The remaining non-dimer photoproducts were also mutagenic and were major contributors to transversion mutations.

DNA DAMAGE AND REPAIR IN HUMAN SKIN *IN SITU*

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Sunlight can induce erythema, melanization and cancer in the skin of man. Understanding the molecular and cellular origins and progression of such processes requires delineation of damage inflicted on DNA by sunlight, as well as the ability of cells in human skin to repair, bypass or tolerate such damage. Studies on procaryotic and eucaryotic cells, including human cells in culture, have outlined the basic damages inflicted on DNA by wavelengths in sunlight, and the fundamental cellular processes for repair of such damage. Important differences betweeen skin and cells in culture indicate that measurement of DNA damage and repair in skin *in situ* is essential to understanding the effects of sunlight on man. First, absorption and reflection of impinging ultraviolet and visible radiation--both resulting from the multilayered structure of skin and from the overlying non-living strateum corneum--is complex in skin and varies as a function of incident wavelength. Second, studies from several laboratories indicate that repair in skin differs from that in human cells in culture both quantitatively (rate of repair) and qualitatively (presence or absence of repair pathways). These differences in repair rates and paths probably reflect the inadequacy of current culture media and techniques to reproduce exactly *in vivo* conditions; until such conditions can be duplicated in the laboratory, it will be difficult to assure that *in vitro* measurements are faithful measures of *in vivo* processes.

Evaluation of DNA repair capability requires knowledge of the extent of damage inflicted on the DNA of cells in human skin by exposures in the biological range--that is, those to which humans normally expose their skin in occupational or recreational activities. Ultraviolet B (290-320 nm) radiation induces pyrimidine dimers (measured as sites for the dimer specific endonuclease from *Micrococcus luteus*) at suberythemal exposures: the dimer yield depends on the sun sensitivity of the individual, with highest dimer yields in skin of the most sunsensitive individual. Although UVB is absorbed by DNA and is thus efficient in damaging DNA the principal ultraviolet component of sunlight is Ultraviolet A (320-40 nm), at which wavelengths the absorption of DNA is quite low. Broad band UVA induces dimers in human skin DNA; production of dimers even by broad band UVA filtered to remove possible UVB leakage and by narrow band radiation centered around 365 nm indicates that UVA radiation is indeed able to damage DNA of human skin. Does DNA damage in human skin result from direct absorption by DNA, or is it mediated by sensitizers present in cells of skin? Determination of an action spectrum for production of DNA damage is a principal method of determining the roles of direct and of sensitized absorption. Measurement of dimer yields in DNA of human skin as a function of photon flux at wavelengths between 289 and 436 nm, and correction for skin transmittance allows construction of an action spectrum for DNA damage in skin *in situ*: this action spectrum closely resembles a DNA absorption spectrum, and does not indicate the participation of sensitizing moieties in the production of DNA damage in the cells of human skin *in situ*.

XERODERMA PIGMENTOSUM : PRENATAL DIAGNOSIS AND ONCOGENE ACTIVATION.

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Xeroderma pigmentosum is an autosomal recessive disease characterized by hypersensitivity to ultraviolet radiations and early development of skin tumors. Nine complementation groups have been detected among classical XP patients, whose cells are deficient in excision DNA repair. All these cells are unable to carry out the incision of DNA at the lesion. Other biochemical defects may be specifically found in some complementation groups (1).

We have prenatally diagnosed XP in amniotic cells and in chorionic biopsies cultured in vitro. Fetal cells are irradiated in vitro with various doses of UV-light and unscheduled DNA synthesis (UDS) is measured in nuclei from unreplicating cells in the presence of H^3-thymidine. UDS values from the fetal sample are compared with those found with the heterozygote parental and the XP proband cells. The use of chorionic biopsies for the diagnosis allows us to know the repair state of fetal cells in less than 12 weeks after conception.

Since XP cells are hypermutable and more prone to cell transformation towards UV-mimicking agents, we determined if oncogene activation due to unrepaired DNA lesions could be involved in XP skin tumor development. In two skin tumors among six samples tested, we found N-ras activation, detected by the DNA transfer assay in 3T3 mouse cells, accompanied by the overexpression and, in one example, by the alteration of the p21 protein. In the same tumors, c-myc amplification and overexpression, and Ha-ras gene rearrangement were also detected. The presence of several oncogene alterations in the same tumor is correlated with the large number of UV-induced DNA lesions found in the exposed skin cells, due to the absence of efficient repair.

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MEASURING OXIDATIVE DNA DAMAGE IN HUMANS

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Oxidative damage to DNA is likely to be a major contributor to cancer, genetic birth defects, aging, and heart disease. DNA damage in man can result from exposure to environmental mutagens, and, in addition, can be produced by a variety of endogenous processes. Of particular importance as endogenous processes may be metabolic pathways that generate oxygen radicals and other reactive oxygen species. Oxygen radicals have been shown to produce DNA base damage and strand breaks. Three products that are formed in DNA in vitro by chemical oxidation or ionizing radiation (an oxidative mutagen) are thymine glycol and hydroxymethyluracil, both oxidation products of thymine, and 8-hydroxyguanine. Specific mammalian DNA repair systems are known to excise these thymine lesions from DNA in vitro (1). Our laboratory has recently reported the identification, in both human and rat urine, of thymine glycol, thymidine glycol, and hydroxymethyluracil (2,3). We now have considerable evidence that these products are derived from the repair of oxidized DNA. The total output of these three compounds represents the formation of about 1,000 oxidized thymine residues per cell per day in man. We now report the presence of 8-hydroxyguanine, its deoxyriboside and riboside in human and rat urine. Since these products are only four of a considerable number of types of oxidative DNA damage products described by radiobiologists, there are likely to be several thousand oxidative DNA hits per cell per day in man. Rats, which have a higher specific metabolic rate and a shorter lifespan, excrete about 15 times more thymine glycol, thymidine glycol, and hydroxymethyluracil per kilogram body weight. Results with mouse and monkey urine indicate that mice have even higher levels of these products than rats (2,3), and monkeys have more than man.

We have developed a new method for measuring lipid hydroperoxides in tissues (4). We find cholesterol ester hydroperoxide and hydrogen peroxide in normal human blood and rat tissues (4). We discuss these as endogenous agents contributing to heart disease, cancer, and aging. We also have described two major unrecognized antioxidants in man, urate and bilirubin (5-7). These non-invasive assays of DNA oxidation products may allow the direct testing of current theories that relate oxidative metabolism to the processes of cancer and aging in man.

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Killing, Stress Responses and Mutagenesis Induced in E. coli by Hydrogen Peroxide

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Killing of Escherichia coli by hydrogen peroxide preceeds via two modes: Mode-one killing appears to be due to DNA damage, has a maximum near 1-3 mM H_2O_2, requires active metabolism during exposure, and is inhibited by iron chelators. Strains lacking exonuclease III, DNA polymerase I, or NADH dehydrogenase and cells grown anaerobically are especially sensitive to this mode. Mode-two killing is due to uncharacterized damage, occurs in the absence of metabolism and exhibits a classical multiple-order dose-response curve up to at least 50 mM H_2O_2. It is not inhibited by iron chelators.

H_2O_2 induces the SOS response in proportion to the degree of killing by the mode-one pathway--i.e., induction is maximal after exposure to 1-3 mM H_2O_2. Mutant strains that cannot induce the SOS regulon are hypersensitive to peroxide and analysis of the sensitivities of mutants that are deficient in individual SOS-regulated functions suggests that the SOS-mediated protection is due to the enhanced synthesis of recA protein, which is rate-limiting for recombinational DNA repair. After exposure to H_2O_2, mutagenesis and filamentation also occur with the dose response characteristic of SOS induction and mode-one killing, but these responses are not dependent upon the lexA-regulated umuC mutagenesis or sfiA filamentation functions, respectively.

Exposure of E. coli to H_2O_2 also results in the induction of functions under control of the oxyR regulon that enhance the scavenging of active oxygen species, thereby reducing the sensitivity to H_2O_2. Catalase levels increase 10-fold during this induction, and katE katG mutants, which totally lack catalase, while not abnormally sensitive to killing by H_2O_2 in the naive state, do not exhibit the induced protective response. Protection equal to that observed during oxyR induction can be achieved by the addition of catalase to cultures of naive cells in an amount equivalent to that induced by the oxyR response. Thus the induction of catalase is necessary and sufficient for the observed oxyR-directed resistance to killing by H_2O_2. Although superoxide dismutase appears not to be involved in this enhanced protective response, sodA sodB mutants, which totally lack superoxide dismutase, are especially sensitive to mode-one killing by H_2O_2 in the naive state.

The peculiar shape of the dose-response curve for mode-one killing, mutagenesis, SOS induction, and filamentation can also be observed for the iron-mediated, H_2O_2-driven oxidation of NADH or paranitrosodimethylaniline or killing of phage lambda. We propose, therefore, that these biological phenomena are due to an aspect of "Fenton chemistry" which is analogous to a cycle proposed by Haber and Weiss for the breakdown of H_2O_2 to H_2O and O_2.

MECHANISMS OF HYDROGEN PEROXIDE CYTOTOXICITY IN MAMMALIAN AND BACTERIAL CELLS

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The toxic potential of molecular oxygen is related to one electron reduction. A number of active oxygen (AO) intermediates are generated such as superoxide anions, hydrogen peroxide and hydroxyl radicals. While cells in aerobic atmosphere are subjected to oxidative stress under conditions of normal metabolism, AO can be produced at abnormally high levels in certain disease states, during the metabolism of specific drugs and xenobiotics and under iperoxia. Cellular defenses normally suffice to protect against the insult produced by AO. However,when such species are generated at a very high rate, toxicity may occur. Therefore, it is important to elucidate the exact mechanisms responsible for cell injury and other biological effects produced by these reactive intermediates. In this paper we have focused on the mechanism by which hydrogen peroxide induces DNA damage and cell death. This oxidant reacts within the cell with ferrous ions to hydroxyl radicals which are highly cytotoxic. Furthermore, Fe^{3+} can be reduced to Fe^{2+} by superoxide ions, thereby allowing the recycling of Fe in Fenton reactions. It is known that OH radical scavengers and iron chelators prevent hydrogen peroxide induced DNA damage and cytotoxicity. In addition,a number of reports have recently indicated that H_2O_2 may produce alterations in intracellular calcium homeostasis. We have therefore studied the effect of Quin 2, an intracelluar calcium chelator, on H_2O_2-induced DNA damage and cytotoxicity and found that this agent could prevent, at micromolar concentrations, geno- and cyto-toxic actions generated by the oxidant. The extracellular calcium chelator, EGTA, was not effective in these systems. Experiments performed with partially purified DNA indicated that Quin 2 only at very high concentrations was able to significantly reduce H_2O_2-induced DNA damage. These results suggest that OH radicals produced via iron-dependent Fenton-type reactions may not directly induce DNA damage and cytotoxicity; rather, these radicals may induce calcium mobilization which elicites secondary reactions ultimately leading to DNA damage and cell death. Experiments performed in Escherichia coli have indicated that the bimodal pattern of H_2O_2 toxicity can be explained by the involvement of at least two distinct radical species. Superoxide ions seem to be of extreme importance in the production of mode one killing whereas OH radicals appear to produce lethality only in the mode two killing region.

LOW-DOSE RADIATION EFFECTS IN HUMAN CELLS

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There is considerable current interest in the nature of the radiation dose response curve for mutagenesis and carcinogenesis at low doses and low dose rates, and the role that induced DNA repair processes might play in such effects. Sheldon Wolff and his colleagues have described an adaptive response for the induction of chromosomal aberrations in human lymphocytes. We have been unable to demonstrate a similar effect for neoplastic transformation or mutations. Pretreatment of cells with x-rays or UV light under a variety of protocols has not significantly altered the effects of subsequent exposure to radiation.

Many studies of low dose effects have been carried out with rodent cells, especially transformed hamster cell lines. However, hamster cells may respond differently to radiation than human diploid cells. First, the effect of lowering the dose rate on survival seems to be reduced in human cells. Second, damage mediated by free radicals appears to differ in the two cell types; the mutagenic effect of x-rays in CHO cells was markedly reduced by incubation with DMSO, whereas DMSO had no effect on x-ray induced mutations in human cells. Rodent cells have yielded important data on neoplastic transformation in vitro for which no quantitative counterpart yet exists in human cells. Recent studies by Elkind and his colleagues, for example, have shown that while protraction of x-ray exposure may reduce its effects, protracted exposure to low doses of fission spectrum neutrons leads to a higher yield of transformants than does acute exposure.

We have studied the induction of mutations by low doses of x-rays and fast neutrons in a human diploid lymphoblastoid cell system. Cells were x-irradiated with daily doses of 1, 2, 5, or 10 rads for 5 to 30 days. Samples were taken every 5 days to determine the induced mutant fraction at two genetic loci. Cells receiving daily doses of 1-10 rads showed a mutant fraction virtually identical to that for cells receiving the same total dose of radiation in a single, acute exposure. Thus, the effects of small daily fractions were cumulative and additive, suggesting that doses as small as one rad are mutagenic in human cells. When the mutation frequency was plotted against days of irradiation, a positive linear slope was observed for all dose groups; a linear increase in mutation frequency was observed over the dose range of 1-10 rads. A similar linear dose-response curve was observed for continuous exposure to radiation from tritiated water. Interestingly, tritiated water was more mutagenic per rad than x-rays but less cytotoxic.

The mutagenic effects of continuous fast neutron (Pu-Be) exposure over periods of 5 to 20 days were compared with those of more acute irradiation. Acute exposure to neutrons was more effective than x-irradiation in inducing mutations (RBE 3.8). However, protracted exposure to neutrons was much more mutagenic than acute exposure, consistent with the results on neoplastic transformation described above. All of these results will be discussed in terms of their implications for the effects of low doses of ionizing radiation on human cells.

THE INDUCTION AND REGULATION OF RADIOGENIC TRANSFORMATION IN VITRO.

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Transformation of rodent and human cells in vitro resembles neoplastic development in vivo, as a multistage process. Transformation is initiated through a series of events associated with irreversible DNA damage. These may include mutations, amplification and activation of oncogenes. Unhindered cell replication shortly after exposure to the carcinogen is essential for the fixation of transformation as a hereditary property, and additional cell replications are required for its expression. Exposure of mammalian cells in vitro to low doses of physical or direct-acting chemical carcinogens rapidly induces pleiotropic effects which include the reduction of DNA repair systems, cellular stress responses and altered gene expression.

Transformation in vitro of genetically susceptible cloned populations is dependent on optimal permissive physiological factors which prevail in the cell and in its environment. These factors are required for fixation and expression of the transformed phenotype. They play a role in establishing the growth of transformed cells in the presence of normal cells and in determining the ability of normal and transformed cells to interact by means of cell-cell communication via permeable intracellular membrane junctions.

Cells depleted of endogenous permissive factors such as thyroid hormones became refractory to transformation by radiation, chemical carcinogens, viruses and tumour promotors. Thyroid hormones act by modifying metabolism and by inducing "specific protein" in the cell which may be associated with transformation.

Radiation and some chemicals including tumor promoters induce a cellular prooxidant state, i.e. increased stationary levels of active oxygen and organic radicals. Active oxygen has been shown to possess carcinogenic activity and antioxidants were found to be anti-carcinogenic.

Poly-ADP-ribosylation (ADPR) is a post-translational modification of nuclear proteins. it appears to play a role in DNA repair, DNA replication, cell differentiation and transformation. It is intimately related to the cellular redox state because it is formed by the polymerization of NAD residues. The promoter phorbol-12-myristate-13 acetate TPA increases poly-ADRP levels in fibroblasts in a reaction which can be prevented by antioxidants.

Inhibition of Poly(ADP) ribosylation results in the inhibition of transformation of cells by UV, x-rays and methylating agents. A suppression of TPA action in enhancing transformation is also observed. Inhibitors of Poly-ADP-ribose include benzamides and some low molecular weight protease inhibitors.

DAMAGE, REPAIR, AND DNA SYNTHESIS IN RADIATION LETHALITY OF MAMMALIAN CELLS

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The evidence is compelling that in mammalian cells genomic DNA contains the principal sites of cell killing by ionizing radiation. It also has been demonstrated that radiation produces single- and double-strand lesions in nuclear DNA. These lesions are repaired quite effectively in surviving as well as in cells which are destined to die and are not propagated to the progeny of surviving cells. Even in radiation sensitive human or rodent cells, an appreciable excess of breaks is registered relative to what is required to kill a cell. It follows from the preceding that: 1) either a small fraction of the lesions is effective because it is not repaired or is misrepaired; and/or 2) a small part of the whole genome is critically sensitive. Evidence that in radiation sensitive cells DNA damage is repaired as completely as it is in resistant cells does not support the first possibility. But if the second is the more likely, what is the part of the genome in which radiation lesions in DNA are closely coupled to lethality?

The dose dependence of the inhibition of the rate of DNA synthesis is biphasic. The initial part of the response has a sensitivity comparable to that of cell killing. This similarity, plus similarities between the single- and fractionated-dose survivals of cells exposed to x-rays compared to the single- and fractionated-dose survivals of cells pulse-labeled with BrdUrd and then exposed to near-UV light, suggest that a locus of action is downstream from the replication complex. Evidence in support of this hypothesis will be discussed as well as the kinds of lesions in DNA which may be involved.

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THE INDUCTION AND REPAIR OF DOUBLE-STRAND DNA BREAKS IN MAMMALIAN CELLS AS DETECTED BY NEUTRAL ELUTION

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One of the important types of damage induced by ionizing radiation in intracellular DNA is the double-strand break (dsb). This damage, involving disruption of both strands of the DNA-helix at the same or neighbouring sites, is produced at a much lower frequency than other lesions (single-strand breaks, nucleotide damage). One would expect, however, that double-strand breaks would be much more deleterious for the cell than lesions of the latter type as these probably are repaired more easily.

The neutral filter elution technique has proven to be a sensitive means of detecting DNA dsb after exposure to low doses of ionizing radiation (Bradley and Kohn, 1979). However, the results obtained with it reported in literature are in some cases controversial as well as in disagreement with results of other methods. For example, according to data of Radford (1985) and Radford and Hodgson (1985), the induction of dsb by 250 kV X-rays does not show a simple linear dose-effect relationship, whereas others (Ross and Bradley, 1981, Van der Schans et al. 1982, and Woods et al. 1982) found data consistent with a linear relationship. Also Blöcher (1982) observed a linear relationship in his sedimentation st dies.

Another discrepancy arose when repair of dsb was studi ' with the elution technique; half-lifes of about 10 min were found, in ' trast to the earlier values of 1-2 h obtained in sedimentation experimen ' It has been suggested that the fast-repair component seen in filter 'ution studies might represent repair of DNA single-strand breaks (ssb). T s is unlikely since Bradley and Kohn (1979) have found that the diffe nt dsb/ssb ratios determined with filter elution for various agents are comparable with those found with other methods. The fact that most of the filter elutions are carried out at rather high pH, could be ruled out as a possible reason for this discrepancy, since we found that elution at neutral pH leads to the same results.

In this lecture the reliability of the different methods for the detection of dsb induced by ionizing radiation and other agents will be discussed.

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X-RAYS INDUCED DOUBLE STRAND BREAKS: DAMAGE DISTRIBUTION AND MEASUREMENTS.

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Experimental evidences have been produced that at least a portion of the mammalian cell genome, due to its complex structure, may be less or not at all accessible to chemicals and enzymes. So far, for convenience, it has been assume that the damage introduced in the genetic material by ionizing radiation (IR) is more or less randomly distributed throughtout cellular DNA. However, in the last few years, some results have been pubblished showing that different DNA regions may have different radiosensitivity. In gamma irradiated cells ssb production and repair are greater in actively transcribing genes than in total nuclear DNA and the newly replicated DNA is hypersensitive to the induction of thymine base damage. Moreover, in irradiated trinucleosomes, the dsb are not randomly distributed along the DNA molecule but localized mainly in the linker regions. These results toghether with others obtained using isolated high molecular weight chromatin, seem to indicate the influence of chromosomal proteins in modifying radiation damage to DNA. It must be emphasised that the mechanisms for the induction of damage in DNA of isolated chromatin appear to be comparable to those operating in the intact cell.

Two different techniques are available to detect double strand breaks in mammalian cells: the neutral elution (NE) and the sedimentation on neutral sucrose gradient at low centrifugation speed (NG). The differences in the dose-range used for the two techniques are not large enough (10-60 Gray for NE and 20-100 Gray for NG) to justify the differences detected in both the initial yield of dsb induced by IR and the repair kinetics. While for the NG the measurement of dsb is straight forward, the analysis of the real number of dsb measured by NE is more complex. The DNA eluted is double stranded but the kinetic of elution is not linear. Lysis conditions and the pH of the elution buffer can modify the fraction of DNA retained on the filter. In any case even with very strong lysis solutions at least 10 % of DNA is not free from other nuclear components. Such results can be explained in several, not mutually exclusive, ways: (i) the NE is measuring two or more types of damage, (ii) DNA lysed on top of filter is not completely free and (iii) a different distribution of damage along the DNA molecule is introduced by IR.

UV MUTAGENESIS IN MAMMALIAN CELLS DETECTED BY A SV40-BASED SHUTTLE VIRUS

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We have constructed a SV40-based shuttle vector that can be packaged as pseudovirions and thus can be introduced into mammalian cells by infection : the shuttle virus (1). These vectors replicate efficiently in monkey COS7 cells and extrachromosomal DNA from infected cells can be rescued in E. coli, so that DNA alterations can be easily screened. The lacO sequence present in the genome of the shuttle virus provides a target to study induced mutagenesis.

The use of infectious virus has many advantages when compared to other "classical" SV40-based shuttle vectors. The latter have to be transfected as naked DNA into the cells, what makes this molecule unprotected in the mammalian environment. Moreover, DNA transfection is inefficient and toxic to some cells and certainly alters cell metabolism. Instead, the genome of our shuttle virus is introduced into the cells as a virus chromatin structure and infection is a natural biological process. By using a shuttle virus which presents a low spontaneous mutation frequency, we are studying induced mutagenesis in UV-irradiated virus. We have found an increased mutation frequency in the lacO target sequence after this treatment and DNA sequence of the mutants are being determined.

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SV40-base Escherichia coli shuttle vectors infectious for monkey cells.
Gene 53 (1987) 21-29

C.F.M. Menck and M.R. James have post-doctoral fellowships from Association pour le Développement de la Recherche sur le Cancer (France) and Ligue Nationale contre le Cancer respectively.

UV-STIMULATED REPAIR REPLICATION IN MAMMALIAN CELL EXTRACTS

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Methods are being developed to study nucleotide excision repair *in vitro* using soluble, cell-free extracts from cultured human cells. Repair replication of ultraviolet light (UV)-irradiated plasmid DNA can be observed in such extracts. All steps of repair take place in the extract: incision, gap filling, ligation, and supercoiling of the closed circular product. In the absence of ATP, the UV-dependence of repair is lost.

Mammalian cells contain a glycosylase/endonuclease which can act on hydrated pyrimidine UV photoproducts. To avoid detecting repair initiated by this enzyme, plasmid DNA is irradiated to produce about 20 cyclobutyl pyrimidine dimers per circle, and then treated with endo III from *E.coli* to nick circles containing hydrated pyrimidines. Unnicked circles are re-purified and then used as the substrate for *in vitro* repair. Therefore, the observed UV-induced repair is not initiated by incision at hydrated pyrimidines. The introduction of a few apurinic sites per circle, or a few DNase I-induced nicks, causes only modest repair replication, much less than that seen after UV irradiation.

Extracts from cells of some lines derived from xeroderma pigmentosum patients are deficient in this *in vitro* repair replication, compared to extracts from normal cells. Repair activity can be restored to these extracts by addition of UV - DNA endonuclease/glycosylase from *Micrococcus luteus*.

Using the repair replication as an assay, we are attempting to isolate factors from proficient cell extracts which can complement deficient xeroderma pigmentosum extracts. Preliminary results indicate that a protein fraction from normal cells can complement deficient extracts derived from a xeroderma pigmentosum group C cell line.

DAMAGE BY TOXIC OXYGEN SPECIES AND ITS REVERSAL BY AN INHIBITOR OF POLY(ADP-RIBOSE) SYNTHETASE IN LYMPHOCYTES FROM AGED PEOPLE AND PATIENTS WITH DOWN SYNDROME (DS)

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An intriguing relationship appear to exist between aging and defects of DNA repair. Previous studies from our laboratories suggested that peripheral blood lymphocytes (PBL) from aged people and young subjects with DS, which ranks at the top among human "segmental progeroid syndromes", have a defective ability to repair damages to DNA by ionizing radiations (Licastro et al.: Carcinogenesis, 2: 45, 1982; Franceschi et al.: "Immunoregulation in Aging", Eurage, 1986, pp. 77-83). In particular, in DS an increased production of oxygen radicals, due to the increased activity of superoxide dismutase (SOD-1), a gene located in the chromosomal band 21q22, could contribute to the genomic instability characteristic of this syndrome. The purpose of the present investigation was to ascertain whether cells from normal aged subjects (>80 years old) or patients with DS show an abnormal sensitivity to the toxic effect of oxygen radicals, which are continuously produced either in normal condition or during chronic inflammatory states.

PBL suspended in nicotinamide-free RPMI-1640 medium were exposed in vitro (1 hr, at 37 C) to an oxygen radical producing system, i.e. xantine oxydase (100 mU ml) plus hypoxantine, washed, stimulated with phytoemagglutinin, cultured for 96 hrs and pulsed with 3H-TdR for the last 6 hrs of culture. The amount of oxygen radical species produced in the system was graded by adding different amounts of hypoxantine (1-100 uM). In some cases, 3-aminobenzamide (3-ABA) was added during the damaging period. Preliminary results suggest that the sensitivity of PBL to toxic oxygen radicals showed an high degree of variability among different subjects. However, PBL from aged or DS subjects appeared to be more sensitive to the damage produced by toxic oxygen species, as demonstrated by the fact that 3H-TdR incorporation was still markedly decreased using low concentrations of hypoxantine (5-25 uM). It is interesting to note that in spite of their higher sensitivity, PBL from aged and DS subjects showed a nearly complete recovery of 3H-TdR incorporation when the damage was produced in presence of 3-ABA (5 mM). Taking into account that cells exposed to such an enzymatic oxidizing system show DNA strand breaks which trigger the rapid synthesis of poly(ADP-ribose), and a rapid fall of NAD^+ and ATP pools, these data suggest that the higher sensitivity to toxic oxygen species of PBL from aged and DS subjects may be due to a decreased energy charge of the cells.

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SENSITIVITY TO IONIZING RADIATION AND HEAT SHOCK IN LYMPHOCYTES FROM PATIENTS WITH MULTIPLE SCLEROSIS

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An increase of spontaneous sister chomatid exchange (1) and of gamma radiation-induced chromosome aberrations (2) have been reported in peripheral blood lymphocytes (PBL) from multiple sclerosis (MS) patients. In particular, studies of phytoemagglutinin (PHA)-stimulated T lymphocytes, B lymphoblastoid cell lines, and fibroblasts in MS patients and among their unaffected first-degree relatives suggested an autosomal dominance inheritance of the cellular radiosensitivity (2). These data suggested that a genetically inherited reduced ability to cope with DNA damages could predispose to MS (2).

We tested this hypothesis in freshly isolated PBL from 15 patients affected by definite MS and 15 age- and sex-matched healthy control subjects, by the following methods :
a) evaluation of DNA repair capability (unscheduled DNA synthesis) after high doses of gamma rays (100 grays) in PHA-stimulated PBL, and b) ability to undergo cell proliferation of PBL irradiated with graded low doses of gamma-rays (2-12 grays) and then stimulated with PHA, according to a method we previously described (3); c) PHA-induced proliferation of PBL previously exposed to heat shock (2 hours at 39 or 41 or 43 or 45 C).

No significant difference as far as unscheduled DNA repair synthesis and survival after gamma radiation or exposure to high temperature was found between PBL from MS patients and controls.

Our data are in agreement with those of Robbins (4), who studied post-X rays viability in MS lymphoblastoid lines and do not support the hypothesis that a DNA defect is present in PBL from MS patients, as suggested by Gipps and Kidson (2).

The hypothesis may be put forward that the previously reported genomic instability in MS is likely of viral origin and not due to a genetic DNA repair defect.

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SISTER CHROMATID EXCHANGE AND DNA REPAIR IN PERIPHERAL BLOOD LYMPHOCYTES OF SANITARY WORKERS EXPOSED TO LOW LEVELS OF ETHYLENE OXIDE (EtO)

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Determination of EtO in the working environment and induction of sister chromatid exchange (SCE) and DNA repair synthesis (Unscheduled DNA Synthesis, UDS) in peripheral blood lymphocytes of 10 exposed sanitary workers and 10 control subjects matched for sex, age and smoking habits are reported.

The relationship between the external dose of EtO and the frequency of SCE was determined in the above groups and in a group of 41 sanitary workers previously studied.

The 10 newly examined workers were exposed to EtO concentrations (1.84 p.p.m. as Time Weighted Average, TWA) intermediate between the high (10.7 p.p.m.) and low (0.35 p.p.m.) levels of exposure of the two previously examined groups (19 and 22 workers, respectively).

A statistically significant ($p < 0.002$) increase of SCE frequency was observed between the present control and the exposed groups. The inducibility of UDS by gamma rays was lower in the lymphocytes of the exposed workers than in controls, but the difference was statistically not significant. A significant relationship between the frequency of SCE and the level of EtO exposure for the three exposed groups was demonstrated by two different statistical methods.

It is suggested that the present Italian threshold limit value for EtO (3 p.p.m.) might not protect the exposed workers against possible genotoxic effects and that even a chronic exposure to 1 p.p.m. might not be devoid of genotoxic risk.

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CONFORMATIONAL CHANGES INDUCED BY THE O-ACETYL DERIVATIVE OF 4-NITROQUINOLINE-1-OXIDE IN SUPERCOILED DNA

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Double helix structural changes induced by adducts of Mono-Acetyl-4-Hydroxyaminoquinoline-1-oxide (Mono-Ac-4-HAQO) on pAT153 plasmid DNA were studied by the "band shift method". This method takes advantage of the properties of supercoiled DNA molecules. In a closed circular DNA molecule the helix geometry is tightly coupled to the degree of supercoiling, therefore alteration in the helical structure caused by DNA damage can be detected as changes in the overall DNA conformation. Since the shape of a closed circular DNA molecule dictates its hydrodinamic properties, we can ultimately monitor variations in DNA helical structures as changes in the electrophoretic mobility on agarose gel of single topological DNA species.

Different forms of plasmid molecules were treated at increasing molar ratios of Mono-Ac-4-HAQO. For RFI, partially relaxed negatively supercoiled (p.r.n.s.)and partially relaxed positively supercoiled (p.r.p.s.) forms, single topoisomers were resolved in 1% agarose gel and the variation in the electrophoretic mobility of each band was quantitatively measured. From the number of adducts necessary to unwind one molecule of 360°, the unwinding angle per adduct was calculated. Unwinding angles of 5.45°, 6.23°, and 16.66° were calculated for RFI, p.r.p.s. and p.r.n.s., respectively.

HPLC analysis of the three main 4-NQO adducts showed that N2-guanyl, C8-guanyl and N6-adenyl adducts accounted for 50%, 25% and 10% of total DNA binding, respectively, when RFI, RFII, linear native and p.r.p.s. forms were treated. A different distribution of the three adducts was found for the p.r.n.s. form (80%, 15% and 5%, respectively).

The reactivity of Mono-Ac-4-HA(2-^{3}H)QO depended on the conformational state of target DNA (RFI>p.r.n.s.>linear denaturated>RFII>p.r.p.s.>linear native).

The presence of AP-endo sensitive sites was also investigated. The amount of AP-endo sensitive sites per pAT153 molecule was a function of the conformational state of target DNA and of the number of adducts per pAT153 molecule. We suggest that in vitro tretment of pAT153 with the ultimate carcinogen of 4-NQO induces labile adducts able to generate AP-sites. We assume, also, that SSBs occurring in the chemically modified DNA during electrophoresis are due to the breakage of DNA containing AP-sites.

EARLY STEPS IN THYMINE_DIMER REPAIR IN PATIENTS AFFECTED BY MELANOMA AND DYSPLASTIC NEVUS SYNDROME.

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A monoclonal antibody specific for UV-induced thymine-thymine dimers in single stranded DNA has been used in an enzyme immunoassay to investigate the loss of antigenicity associated with the repair of this lesion in the first hour following an UV-irradiation of 10 Joule/m^2 (254nm). Biopsies of 30 melanom affected patients and of 20 Patients suffering from Dysplastic Nevus Syndrome were cultivated and UV-irradiated. The extracted DNA of each patients was tested for antibody binding capacity directly after the irradiation and after different recovering time intervals (10, 30, 60 and 90 min. after UV-exposure). In comparison to healthy controls (n=30) the percentage of antigenicity was determined.

The melanoma patients showed a much higher variability than it was observed among the healthy controls. Comparing the means of the antigenicity of both groups there was a significant difference between healthy controls and the melanoma patients ($p=0.01$). But there was no unique response for the melanoma affected individuals. Some of them did not show any difference form the healthy controls, others showed the same response as observed in Xeroderma pigmentosum patients.

Different to melanoma patients the probands affected by the Dysplastic Nevus Syndrome supriesingly showed a unique reduction in the loss of antigenicity during the first two hours after UV-irradiation. Reaching the levels of normal controls latestly after 120 min. after UV-treatment. We also observed an individual response for the time point reaching the normal levels in these patient group, varying between 60 - 120 min post irradition.

While the antibody is specific for the conformational changes induced by UV-light, without repair, we assume that the loss of antigenicity is the result of one of the initial enzymatic reactions starting the excision repair process in human fibroblasts.

BETEL INDUCED VARIATIONS IN NUCLEAR DNA IN MOUSE GASTRIC MUCOSA

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Experimental studies on carcinogenicity and mutagenicity of the betel quid ingredients (Piper betle L. leaf, Areca catechu L. nut, Nicotiana tabacum L. leaf and lime) had produced variable results. We have recently reported the capacity to induce repair DNA synthesis in the gastric mucosa of Swiss albino mice to assay the genotoxicity of orally administered chewing mixtures.

In the present study, 17 different betel chewing habits, currently practiced in India, were simulated and administered orally to inbred Swiss albino mice for prolonged periods (1-10 months). Nuclear DNA content of the gastric crypt cells, measured in situ by Feulgen cytophotometry, revealed significant variation ($P < 0.01$ to $P < 0.001$) in mean DNA content. This was associated with time dependent production of aneuploid cells and disturbed crypt kinetics. Tobacco in any combination greatly augmented these effects.

Variation in ploidy, as in the present report, occur early during malignant transformation and may be taken as a reliable indicator of development of apparent cancer at any site. In the light of a proposed relationship between betel chewing and cancer of the mouth, our results may exemplify the way in which the quid or its individual ingredients, which are ingested daily in rather large quantities, could affect carcinogenesis in habitual chewers in a country where oral cancer exists in alarmingly high numbers.

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A SPECIFIC MISMATCH REPAIR EVENT PROTECTS MAMMALIAN CELLS FROM LOSS OF 5-METHYLCYTOSINE.

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5-Methylcytosine spontaneously deaminates to form thymine, generating thus G/T mispairs in DNA. We investigated how these lesions are addressed in mammalian cells by introducing specific G/T mispairs into the genome of SV40 and determining the fate of the mismatched DNA in simian cells. Mispairs were incorporated in 12 bp duplexes ligated into SV40 DNA between the BstXI and TaqI restriction sites. Sequences were chosen so that correction of the G/T mispair to a G/C or an A/T pair would create different restriction sites. Analysis of 347 plaques obtained after transfection of this modified DNA indicated that mispairs were corrected in 343 cases (99%) revealing 314 repair events in favor of guanine (90%) and 29 in favor of thymine (8%). Correction favored guanine regardless of the orientation of the mispair in DNA and regardless of whether the mispair was in the commonly methylated CpG dinucleotide. These results attest to a specific mismatch repair pathway that restores G/C pairs lost through deamination of 5-methylcytosine residues.

EXPRESSION OF E. coli DNA ALKYLATION REPAIR GENES IN HUMAN CELLS

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We recently adopted a new approach to the generation of isogenic variant human cell lines that differ in their ability to repair DNA alkylation damage. Our experiments indicate that one may be able to apply the information gained through studying bacterial DNA alkylation repair to the elucidation of the mechanisms of alkylation toxicity in human cells. We have demonstrated that E. coli DNA alkylation repair enzymes can be stably expressed in human cells and that their activity can suppress the sensitivity of Mer$^-$ human cells to the induction of cell killing, mutation and chromosome damage. Thus, in principle, we have the means to endow human cells with the ability to repair one or two specific DNA lesions more efficiently, by engineering the over-expression of particular bacterial enzyme species. In other words, we can generate variant cell lines of a predetermined genotype.

The ada-alkB operon protects E. coli against the effects of many alkylating agents. We have subcloned it into the pSV2 mammalian expression vector to yield pSV2ada-alkB, and this plasmid has been introduced into Mer$^-$ HeLa S3 cells which are extremely sensitive to killing, mutation and SCE induction by alkylating agents. One transformant (the S3-9 cell line) has several integrated copies of pSV2ada-alkB and it was found to express a very high level of the ada gene product, the 39kDa O^6-methyl-guanine-DNA methyltransferase. Although we could detect alkB mRNA in S3-9 cells, we could not determine whether the AlkB protein was expressed because the function of this protein is not yet known and because we do not yet have AlkB antiserum. S3-9 cells were found to have become extremely resistant to killing, mutation and SCE induction by two alkylating agents, N-methyl-N'-nitro-N-1-nitrosoguanidine (MNNG), and 1,3-bis(2-chloro-ethyl)-1-nitrosourea (BCNU). Thus, bacterial DNA alkylation repair genes are able to complement alkylation repair defects in human cells. One of our current goals is to determine the relative contributions of the ada and alkB gene products to the extreme alkylation resistance of S3-9 cells. We have therefore subcloned the ada gene and the alkB gene separately into mammalian expression vectors and are currently measuring the effects of the expression of each of these genes upon the alkylation sensitivity of Mer$^-$ human cells. The depletion of DNA methyltransferase activity in S3-9 cells (by growth in the presence of O6MeG free base) sensitizes the cells to the induction of SCEs by MNNG, but does not sensitize the cells to killing by MNNG. This is consistent with the notion that O6MeG does not cause cell death in HeLa cells.

HUMAN DNA-REPAIR SYNDROMES: CONSTITUTION OF A CELL REPOSITORY

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We are working with various Institutions for the cytogenetic study of patients with DNA-repair syndromes, especially Ataxia-Telangiectasia (AT). During the last two years we have examined nine families in which the gene(s) for AT was suspected to segregate. Clinical, cytogenetic and laboratory data confirmed the diagnosis in probands from seven out of the nine families. Furthermore, we studied one patient with Bloom syndrome (BS) and her family, one patient with Xeroderma Pigmentosum (XP) and one with Fanconi anemia (FA).
Peripheral blood lymphocytes from the patients and, when possible, their family members were in part directly cultivated for cytogenetic analysis and in part transformed and immortalized by cocultivation with Epstein-Barr virus. The transformations were successfull in all but one AT family In one AT patient the treatment needed to be repeated twice because of a severe immunodeficiency at the time of first sampling. Fibroblastoid lines were generally established directly from the referring Institution except for the XP patient, in which was done by ourselves.
At this moment our collection is made up by the lymphoblastoid cell lines of seven AT patients, twelve AT heterozygotes, four subjects at risk of being AT heterozygotes; one BS patient, two BS heterozygotes, one individual at risk of being BS heterozygote; one XP patient; one FA patient
The aim of the constitution of this repository is the availibility of a great amount of cells from subjects with rare mendelian disorders and their relatives in view of forthcoming tests and as exchange material with other laboratories studying DNA-repair syndromes.

ITALIAN REGISTRY FOR ATAXIA-TELANGIECTASIA: PURPOSE FOR A COLLABORATIVE STUDY

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The aim of the Italian Registry for Ataxia-Telangiectasia (AT) is to determine wether the incidence of any cancer is increased in homozygotes and heterozygotes for AT gene(s) with respect to general population.
AT homozygotes die generally before the end of second decade of life because of recurrent sinopulmonar infections; nevertheless, in about 10% of them the cause of death is a lymphoreticular malignancy.
The AT heterozygote frequency in white populations probably fall between 0.68% and 7.7% with 2.8% being the most likely estimate (Swift et al., 1986). They have a two/three-fold risk to develop any cancer and a six-fold risk for breast cancer (Swift et al., 1987).
At this moment, the only reliable way to identify an AT heterozygote is by relation to an affected individual. Natural parents of AT patients are heterozygotes, while natural siblings, when healthy, have a 67% chance of being heterozygotes; relatives are at risk of heterozygosity.
On the basis of the excess risk for AT heterozygotes to develop cancer all the families with an AT affected individual would have a number of subjects at risk for malignancy. The Italian Collaborative Study for Ataxia-Telangiectasia will constitute with this Registry a basis for a prevention.

The incidence of the disease is estimated to be 1/40.000 births. The prevalence, calculated on the basis of births/year in Italy (about 600.000) and the mean lifetime of AT patients, will be about 200 affected individuals. The clinical heterogeneity of the disease may lead to an underestimation of the patients; we hope to insert into the Registry 100-150 prevalent cases of Ataxia-Telangiectasia. Patients will be ascertained by direct contact with all the clinicians (geneticists, pediatricians, neurologists) working in neurogenetics and DNA-repair syndromes and with regional hospitals and medical universities. Furthermore we are collaborating with the Italian Registry for Immunodeficiencies and the Childhood Cancer Registry of the Province of Torino.

- Swift M. et al.: Am. J. Hum. Genet. 39, 573-583, 1986
- Swift M. et al.: N. Engl. J. Med. 1987, in press

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Photoproducts in bacterial spores, UV (200-300 nm)-irradiated in vacuo

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The examination of survival (colony formation) of B. subtilis has demonstrated the high resistance of its spores to u.v. radiation. Cell killing in the u.v. range (200 - 300 nm) is known to be the consequence of a direct effect on cellular DNA.

The formation of thymine photoproducts of the nucleic acids as "critical molecules" was examined at different environmental conditions. Radiolabelled bacterial spores were irradiated in suspension, in the dry state at atmosphere and in vacuum (10^{-8} mbar) with various fluences of u.v. (254 nm)-light. A total formic acid hydrolysate of the spores was examined for photoproduct formation by thin layer chromatography.

The photoproduct formation in spores irradiated at vacuum conditions is compared to the photoproduct formation in spores irradiated in suspension and in the dry state at atmosphere. The major photoproduct that was detected in spores irradiated in suspension and in the dry state at atmospheric pressure was the spore specific photoproduct (5-thyminyl-5,6-dihydrothymine) and -in minor quantities- the (t,s)thymine dimer. After irradiation in vacuum, in addition, the (c,s)thymine dimer was produced.

The action spectra (200 - 300 nm) for the formation of spore photoproduct and (c,s)thymine dimer in suspension and in vacuum are compared. The spectral efficiency for lethality and formation of photoproducts is compared. The different yield of photoproducts at the various environmental conditions proposes different mechanisms of u.v. radiation action. Variations in the microenvironment of DNA in dehydrated spores may give rise to the formation of less repairable photoproducts.

BIMODAL PATTERN OF ESCHERICHIA COLI KILLING BY HYDROGEN PEROXIDE CAN BE EXPLAINED BY THE INVOLVEMENT OF AT LEAST TWO DISTINCT RADICAL SPECIES: SUPEROXIDE IONS AND HYDROXYL RADICALS

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The cytotoxicity of hydrogen peroxide in prokaryotic cells is well documented. Although the critical lesion produced by the oxidant has not been as yet identified, it is known that iron facilitates the bactericidal action by reacting with H_2O_2 to form a highly toxic radical species, the hydroxyl radical (OH·). Over the last few years various studies on E. coli have indicated that mutations in recA, polA or xth strains confer hypersensitivity to H_2O_2, suggesting an important role for the DNA repair system in the protection of the cell against the oxidative insult. Therefore, DNA seems to be the site of the H_2O_2-induced lethal damage. Recently, two modes of killing of E. coli by hydrogen peroxide were described, the first occurring at concentrations of H_2O_2 below 2.5-5 mM and the second at concentrations higher than 10 mM. These modes of lethality differed in that unlike mode one, mode two killing did not require active metabolism and was not dramatically enhanced in strains with DNA repair defects.
In the current work we have investigated the role of the superoxide ion and OH· in mode one and mode two killing of E. coli by hydrogen peroxide. We have found that OH· are not involved in the induction of mode one lethality. In fact, the OH radical scavengers, thiourea and dimethyl sulfoxide, and the iron chelator, desferrioxamine, did not affect the survival of cells exposed to 2.5 mM H_2O_2. In addition, cell vulnerability to the same H_2O_2 concentration was independent from intracellular iron content. In contrast, mode two lethality was markedly reduced by OH· scavengers and desferrioxamine and was augmented by increasing the intracellular iron content. It is concluded that OH· are required for mode two but not for mode one killing of E. coli by hydrogen peroxide.
In other experiments the wild type strain and mutants of E. coli lacking the expression of the manganese or iron superoxide dismutase genes were compared for their sensitivity to the H_2O_2 insult. A number of different experimental conditions were used such as drug exposure in M9 salts vs a rich medium or treatment of cells previously grown anoxically. The results obtained have suggested that a) superoxide ions may be involved in the production of mode one killing, b) superoxide ions mediate mode two killing by H_2O_2 by reducing trivalent iron to the divalent form which results in the production of OH· by reacting with the oxidant and c) the intervening zone of partial resistance, observed by increasing the H_2O_2 concentration from 2.5 to 10 mM, may be a consequence of MnSOD induction.

THE RELEVANCE OF TRANSFECTED PLASMID DNA TO THE REPAIR OF Pt-DNA ADDUCTS IN MAMMALIAN CELLS

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The Walker 256 carcinoma cell (WS) is inherently sensitive only to difunctional agents such as Cisplatin, Melphalan or Sulphur Mustard, all of which can produce DNA interstrand crosslinks, and not to a wide range of other cytotoxic agents, that we have examined, that cannot. In contrast a resistant Walker cell line (WR), derived from the original WS cell line by continuous exposure to chlorambucil, shows sensitivity towards difunctional agents comparable to that of conventional cell lines such as HeLa or Chinese hamster V79. However both the sensitive and resistant lines have the same specific binding for Cisplatin, the same ability to remove DNA bound platinum adducts, including interstrand crosslinks and to circumvent DNA adducts during replication. Further no differences can be detected between the response of these cell lines when transfected with plasmid probes containing specific, defined, damage or platinum lesions. These observations are consistent with there being a deficiency in a late step in the repair of a rare lesion on DNA, such as an interstrand crosslink, in WS cells.

Genetic and biochemical studies in bacteria indicate that recombination events, as well as excision repair, are required for the repair of DNA interstrand crosslinks. The plasmid pDR1 contains two truncated (and therefore nonfunctional) *non* tandem, but overlapping, segments of the neo gene separated by a functional transcription unit coding for the gpt gene. This plasmid was transfected into the Walker cells (using the gpt gene to select transfectants) and any subsequent recombination of the integrated defective neo gene segments can be assayed by the appearance of G418 resistant cells and confirmed by Southern blot analysis.

U.V. INDUCED DNA REPAIR IN MOUSE SPERMATOGENESIS

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Spermatogenesis can be used as a model system for the study of the enzyme mechanisms of different types of DNA synthesis: DNA replication which specifically occurs in replicating premeiotic cells, DNA repair during crossing over in pachytene spermatocytes and DNA repair induced by chemical or physical agents in spermatocytes and spermatids. Studies of DNA replication and repair shows that DNA polymerase α and δ are required for chromosomal DNA replication, that DNA polymerase γ is needed in mitochondrial DNA synthesis, while it is controversial the role of DNA polymerase α and β in the reactions of DNA repair. We have used premeiotic cells obtained from immature mice and pachytene spermatocytes from adult animals to study with different approaches the role of DNA polymerase α and β in DNA replication and repair in this model system. In in vivo experiments germ cells were labelled in culture with ^{3}H-dTR in the presence or absence of aphidicolin, a specific inhibitor of DNA polymerase α, and incorporation was evaluated by autoradiography. Aphidicolin inhibited DNA replicative synthesis in premeiotic cells, while it was ineffective on DNA repair synthesis U.V. induced in pachytene spermatocytes thus indicating that different DNA polymerase forms are active in the two events. Experiments in vitro demonstrated in crude extracts of premeiotic cells the presence of both aphidicolin sensitive (possibly α) and dideoxythymidine,5'-triphosphate (ddTTP, specific inhibitor of DNA polymerase β) sensitive DNA polymerase activityes. The same experiments in crude extracts of meiotic germ cells demonstrated the presence of ddTTP sensitive DNA polymerase activity alone (DNA polymerase β only). Furthermore DEAE chromatography and velocity sedimentation analyses showed that DNA polymerase α-DNA primase complex is present only in premeiotic cells, while DNA polymerase β activity is present in both premeiotic and meiotic cells. The results of the experiments reported indicate that DNA repair synthesis in pachytene spermatocytes, during meiotic crossing-over and after U.V. irradiation, requires DNA polymerase β alone.

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MUTAGENIC ACTIVITY OF UVA LIGHT ON PLASMID DNA

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UVA light is able to produce several kinds of lesions on DNA, such as pyrimidine dimers and single and double strand breaks, leading to lethal and, probably, mutagenic effects in different biological systems. Plasmids, which can be modified *in vitro*, are useful to approach the study of the bacterial repair mechanisms involved in the removal of DNA lesions. We previously studied the effects of UVA light using *E. coli* strains differing on their repair capacities (wild type, *uvrA*, *recA13*, *recA430*, *uvrArecA13* and *umuC*) as recipients of UVA irradiated plasmids. The UVA dose ranged from 0 to 388 KJ/m^2. Results on plasmid survival showed the lethal effect of irradiation on plasmid DNA, although lethality was attenuated by DNA repair processes. The main process involved in repairing plasmid lesions appeared to be the excision pathway, although the *recA*-dependent pathway also seemed to participate, mainly through a recombinational-like process. The SOS response did not seem to be involved in the removal of plasmid lesions, although the only evidence for this suggestion was the similar plasmid survival observed in wild type, *umuC* and *recA430* strains. In order to study the possible involvement of the SOS repair in removing plasmid lesions we have performed a mutagenic assay: the mentioned *E.coli* strains were transformed with irradiated plasmids (233 KJ/m^2) and plated onto ampicillin-containing plates. Colonies with $Ap^r Tc^s$ phenotype were scored by replica-plating onto ampicillin plus tetracycline containing plates, and mutation frequencies on the plasmid tetracycline resistance gene were determined. The mutation frequencies observed for the irradiated plasmids and controls (untreated samples) were similar. This result, rather than allow think of the lack of mutagenicity of the UVA damages, would suggest that the UVA lesions present in plasmid DNA are unable by themselves to induce the SOS response. In order to confirm this last possibility we preinduced the SOS functions in the host cells by irradiation with far UV light and determined plasmid survival and mutagenesis. We observed a great increase in mutagenesis in *uvrA* and wild-type strains (mutation frequencies in *umuC* and *recA* cells were negligible) which was significantly higher in UVA irradiated plasmids than in controls. We also found a considerable plasmid recovery in *uvrA*, *umuC* and wild type strains (no changes were observed in *recA* strains). These results suggest that UVA-lesions on plasmid DNA are susceptible of repair by the SOS process and confirm that the presence of those damaged plasmids does not suffice to induce the SOS functions in the host cells. The increase in plasmid survival was higher in the wild type and *umuC* strains than in *uvrA* cells while the mutation frequency in *uvrA* was two- fold the one observed in wild type strain. This fact would indicate that the induced SOS repair acting on plasmid molecules are mainly error free, and could probably be adscribed to excision pathway.

INTRASPECIFIC COMPLEMENTATION ANALYSIS BETWEEN UV SENSITIVE CHINESE HAMSTER MUTANTS

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Complementation studies were carried out on four UV sensitive (UV^S) clones isolated from CHO-K1 $prol^-$ cell line and characterized by different levels of reduction in the ability to perform UV-induced DNA repair synthesis (UDS).
The mutant cells were fused with wild-type or UV^S cell lines representative of the six Chinese hamster complementation groups (c.g.) so far identified, and survival after UV irradiation was analyzed in the hybrids. Wild-type cells did complement the defect conferring UV sensitivity indicating that the mutation in all the clones behaves as a recessive character.
Complementation analysis after fusion with the six Chinese hamster c.g. indicated that three clones (CHO43RO, 423PV, 30PV) belong to c.g. 2. Despite their homology at the genetic level, these three clones show different degree of mutagen sensitivity, UDS and chromosomal fragility, suggesting that defects in the same gene may result in a different degree of phenotypic alterations.
The fourth mutant analyzed (CHO7PV) shows complementation after fusion with cells belonging to any of the six Chinese hamster c.g. suggesting that it represents a new c.g.
In order to localize on human chromosomes the gene defective in this mutant, hybrids between CHO7PV cells and human lymphocytes have been isolated. Characterization of the hybrids for human chromosome content and UV sensitivity is in progress.

CHROMOSOME INSTABILITY IN SKIN FIBROBLAST CULTURES FROM A PATIENT AFFECTED BY XERODERMA PIGMENTOSUM

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We report an unexpected result of a cytogenetic analysis on fibroblasts from an individual affected by xeroderma pigmentosum (XP).
The 30 years old patient presented the cutaneous alterations typical of the disorder with several precancerous and cancerous lesions. At cellular level marked hypersensitivity and reduced unscheduled DNA synthesis after UV irradiation was demonstrated. Cell fusion experiments allowed to attribute the patient to complementation group C.
Cytogenetic analysis on fibroblasts at passages 4th and 5th of the culture, revealed the presence of pseudodiploid metaphases with multiple variable structural rearrangements. The frequency of abnormal mitoses ranged from 8.6 to 36.7% in independent cell samples. Identical structural changes, namely t(2;13)(p23;q12 or 13) and t(13;15)(q12 or 13;q13) were observed in different mitoses, indicating a clonal expansion of chromosomally abnormal cells. A similar cytogenetic condition, termed variegated translocation mosaicism (VTM) is distinctive of Werner syndrome, a rare hereditary disorder with progeroid features and increased frequency of neoplasia. XP cells usually do not exhibit chromosome aberrations, the frequency of chromosome breakage being increased only after UV irradiation.
To decide whether the occurence of VTM in a XP-C case is fortuitus or is a consequence of a DNA repair defect, further investigations on XP cells from different complementation groups are required.

GENOTOXIC EFFECT OF PHOTOCHEMOTHERAPY

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Various cellular parameters were analyzed on lymphocytes from psoriatic patients treated with 8-methoxypsoralen and long-wave ultraviolet radiation (PUVA) in order to establish whether photochemotherapy induces genotoxic damage.
The efficiency of the mechanisms involved in DNA replication and repair, the chromosomal fragility and the mutability were assessed in parallel on blood samples from 22 patients and 22 healthy donors.
We found that photochemotherapy does not significantly affect the sensitivity to mutagens which induce different kinds of damage in DNA molecule nor does it influence the mutation frequency for 6-thioguanine resistance. Compared to cells from normal individuals, a reduced responsiveness to mitogens was observed in cells from PUVA treated patients. Cytogenetic analysis revealed normal frequency of sister chromatid exchanges whereas the rate of the chromosomal breakage was significantly higher in patients under PUVA treatment compared to healthy individuals.
These observations on long-term consequences of PUVA treatment to circulating lymphocytes, indicate that patients under photochemotherapy are at risk for genotoxic damage.

CHARACTERIZATION OF AN SOS-LIKE SYSTEM IN *BACILLUS THURINGIENSIS*

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Bacillus thuringiensis is shown to have an inducible error-prone repair system triggered by genotoxic agents as UV irradiation (UV), mitomycin C (MC),Aflatoxin B_1 (AFB_1) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), as found in *Escherichia coli* and *Bacillus subtilis*. In this bacterium exposure to these agents which damage DNA or inhibit DNA replication elicits several coordinately induced phenomena :

1. Cellular filamentation : treated cells show a temporary loss of normal septation formation resulting in the induction of greatly elongated filamentous cells. These bacteria appeared to lack septa and show a dispersed nuclear apparatus coupled with hypertrophic mesosome-like structures.

2. Mutagenesis of the bacterial chromosome : *B. thuringiensis* was mutated at the rifampicin (Rif) resistance marker by UV light and to a lesser extent by MNNG. One mutant (YA 200) selected for its greater sensitivity to UV expressed a higher freqency of mutagenesis after UV treatment and appeared to be defective in an excision-repair pathway.

3. Weigle-reactivation (WR) and Weigle-mutagenesis (WM) of UV-damaged bacteriophage : accompanying the WR of phage Ø8 we observed a weak WM. The presence of chloramphenicol before and after irradiation abolish these phenomena. The kinetics of these responses differs significantly from those described in *E. coli* and *B. subtilis*.

4. Increased expression of a Rec A-like protein : preliminary analysis of total cellular proteins by gel electrophoresis indicated amplification of a major protein of 42000 Da. This is similar in size of the Rec E protein of *B. subtilis*, an analogue of the *E. coli* Rec A protein. This enhanced synthesis is accompanied by an increase in ATP concentration of about two to three times the initial ATP cellular pool.

5. Discriminated induction of prophage and bacteriocin : in lysogenic strains, DNA damages lead to the induction of resident prophage; cell lysis begins after 1.5 to 4 h. In the bacteriocinogenic strains there was no obvious increase of thuricin (bacteriocins produced by this species)production.

These lines of experimental evidence support the model that an SOS-like repair system exists in *B. thuringiensis*.

ANALYSIS OF THE FATE OF FUROCOUMARINS PLUS UVA INDUCED LESIONS IN NORMAL AND FANCONI'S ANEMIA FIBROBLASTS

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Fanconi's anemia (FA) cells have an increased cellular and chromosomal sensitivity to DNA cross-linking agents. It has been suggested that the defect of FA cells is related to a deficiency in the repair of DNA-interstrand cross-links (CL). Up to now, however, conflicting results were reported.

Using two bifunctional furocoumarins, 8-methoxypsoralen (8-MOP) and 4,5',8-trimethylpsoralen (TMP), we performed experiments with genetically well defined FA fibroblasts, belonging to recently described complementation group "A" and "B", and normal and heterozygote human cell lines. The fate of DNA interstrand cross-links (CL) was followed by alkaline elution analysis in parallel to cell sensitivity as determined by clonogenic survival assays.

In comparison to normal and heterozygote cells, FA cells from group "A" and "B" showed a reduced capacity for CL incision after treatment with 8-MOP and UVA. At 24 hr of post-treatment incubation, the extent of incision was about half in FA group "A" cells whereas in group "B" cells it approached that of normal and heterozygote cells. Using a re-irradiation protocol with the more photoreactive psoralen TMP, FA cells were found not only more sensitive to mixtures of CL and monoadducts (MA) but also to MA alone. The reduced capacity of FA cells which was observed for psoralen induced lesions appeared to be correlated with their differential sensitivity.

RADIATION SENSITIVITY OF ADULT HUMAN PARENCHYMAL HEPATOCYTES.

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The radiosensitivity of rat and human parenchymal hepatocytes to DNA single strand breaks and their repair kinetics were compared using the alkaline elution technique. Rat hepatocytes were isolated from F344 female rats using the 2-step *in situ* collagenase technique. Discarded viable human liver was obtained from the surgical pathology laboratory, and the cells were enzymatically isolated via a modification of the perfusion technique used for the rat. The isolated hepatocytes were cultured with MEM media (10% FBS), insulin (10^{-7}) and gentamycin (50 ug/ml) for 24 hr, removed from the plates with collagenase and irradiated in sunspension at 0° C with different doses of ^{60}Co-gamma rays (1 Gy/min). A linear dose response curve was obtained when the strand scission factor was plotted versus radiation dose and the slopes for the rat (n=12) and human (n=5) hepatocytes were 0.018 ± 0.003 Gy^{-1} and 0.014 ± 0.002 Gy^{-1}, respectively (p=0.5). The repair kinetics were determined in hepatocytes irradiated with 20 Gy ^{60}Co and incubated at 37° C for different times. The half-time for the fast and slow repair were 13.9 ± 6.1 min and 121 ± 31 min in rat hepatocytes (n=7) and 17.8 ± 4.4 and 253 ± 67 min in human hepatocytes (n=5). They were not significantly different (p>0.1). After 3 hr of repair 15% and 25% of DNA damage was still present in rat and human hepatocytes, respectively. This study shows that rat and human hepatocytes are equally sensitive to the induction of DNA single strand breaks by ionizing radiation, and repair these lesions at a similar rate. The low capability of parenchymal hepatocytes to repair DNA damage implies that the liver would be relatively sensitive to fractionated radiation treatment. (Supported by NCI grants CA40172 and CA25951).

DEVELOPMENT OF ANTIBODIES AGAINST N^7-METHYLDEOXYGNANOSINE (N^7-MEDG) TO DETERMINE EXPOSURE TO ALKYLATING AGENTS

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The exposure of organisms to alkylating agents results in alkylation at some 12 sites of the DNA bases and these were associated with the toxic, mutagenic and carcinoginic effect of these chemicals.

The main target of methylating agents is the position N^7 of guanine that accounts for 70-80% of total DNA methylation. This DNA adduct persist for a long time in cellular DNA (Lawley et al., (1981) Proc. Natl. Acad. Sci. 2, 154-157) since it is repaired enzymatically with low efficiency (Laval et al., Singer et al., Margison et al., (1981) Proc. Natl. Acad. Sci. 78, 852-855, 856-860, 861-865). These properties indicate that determination of this adduct in human tissue DNA would be informative in assessing past exposure to alkylating agents with a sensitivity and specificity applicable to epidemiological studies. Presence of O^6-methyldeoxyguanosine (O^6-medG) that occurs at 10 times lower level than N^7-medG, has been detected in human tissues (Umbenhauer et al., (1985) Int. J. Cancer, 36, 661-665).

Polyclonal antibodies against N^7-medG are raised in rabbits by immunization with two antigens: N^7-medG imidazole ring open form (i.r.o.) conjugate either to Bovine Serum Albumine (B.S.A.) or hemocyanine (H.C.). Sera obtained were purified by affinity chromatography and the ELISA assay permits the detection of 1.25 pmol of N^7-medG i.r.o. for 50% inhibition, with 1 mg of DNA; this corresponds to detection of one DNA adduct in 10^7 deoxygquanosines. The antibodies recognised both the i.r.o. and the close-form of N^7-medG but with sensitivity 200 times lower for the close form. The amount of other DNA adducts or normal nucleosides required to produce 50% inhibition was: N^7-meG $1.25.10^4$ pmol; dG $1.1.10^5$ pmol; dA, dT and dC > $1\ 10^6$ pmol. Chromatography procedures were also developed that permit the separation of a single hydrolysed DNA sample of N^7-medG, O^6-medG, O^6-etdG and O^4-medT with subsequent detection by ELISA or RIA assays.

Validation of immunoassay measurements were carried out in comparison with radiochemical determinations. Experiemnts in vivo show that N^7-medG can be detected in peripheral blood cells DNA after treatment of rats with dimethylnitrosamine.

ENHANCEMENT OF THE DNA REPAIR CAPACITY OF MAMMALIAN CELLS BY CARCINOGEN TREATMENT.

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To determine whether DNA excision repair is enhanced in mammalian cells in response to DNA damage, as it is in bacteria as part of the SOS response, we used an expression vector-host cell reactivation assay to measure cellular DNA repair capacity. When UV-damaged chloramphenicol acetyltransferase (CAT) vector DNA was introduced into monkey CV1 cells, the level of CAT activity was inversely related to the UV fluence due to inhibition of cat gene expression by UV photoproducts (Protic-Sabljic and Kraemer, PNAS USA 82:6622-6626, 1985). When CV1 cells were treated with either UV radiation (10 J/M2) or mitomycin C (1 ug/ml, 2.5 hours) 24 hours before transfection, CAT expression from the UV-irradiated plasmid was increased. This increase did not occur in repair-deficient human xeroderma pigmentosum group A cells. We confirmed that this increase in CAT expression was due to repair, and not to production of damage-free templates by recombination; the frequency of generation of neo+ recombinants after transfection with vectors carrying partially deleted neomycin genes did not significantly increase in carcinogen-treated CV1 cells.

From these results we conclude that treatment with certain carcinogens, like UV-light or mitomycin C, enhances the excision-repair capacity of normal mammalian cells.

PURIFICATION OF 3 METHYLADENINE DNA GLYCOSYLASE II FROM *ESCHERICHIA COLI*

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We have purified 3-methyladenine DNA glycosylase II from *Escherichia coli* to apparent physical homogeneity by a modification of a procedure originally designed for the purification of 3-methyladenine DNA glycosylase I (Bjelland, S. and Seeberg, E. (1987) *Nucl. Acids Res.* 15, 2787-2801). Cell extract was made by sucrose plasmolysis/lysozyme treatment and the enzyme purified by gelfiltration on Ultrogel AcA 54, cation exchange chromatography on Mono S HR 5/5 and anion exchange chromatography on Mono Q HR 5/5. We used as an enzyme source a strain carrying the *alkA* gene on a multicopy plasmid, where the bacterial production of glycosylase was induced by the addition of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of the various fractions obtained during purification indicated that the enzyme was essentially homogeneous after the Mono S fractionation where it eluted at about 0.24 M NaCl at pH 6. The enzyme was excluded from the Mono Q column at pH 8.5. We are presently investigating the biochemical properties and possible sequence specificities of 3-methyladenine excision by 3-methyladenine DNA glycosylase I and II to elucidate the different roles of these enzymes in DNA alkylation repair.

THE USE OF DOUBLE RADIOLABELLING IN DNA REJOINING EXPERIMENTS.

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Two procedures based on DNA unwinding in alkali followed by hydroxylapatite chromatography for determining differences in the number of DNA breaks between two cell populations after irradiation have been developed.

The first of these assays measures the number of breaks in irradiated cells that are treated with a DNA repair inhibitor in comparison to equally irradiated, not drug-treated, control cells. Two cell populations, in which DNA was labelled with different radionuclides, were used. One cell population was labelled with [^{3}H]thymidine and the other with [^{14}C]thymidine. They were both irradiated but only one was treated with a drug affecting DNA repair. After different repair periods the analysis of DNA strand breaks was performed after mixing of the two cell populations, which is the important advantage with this procedure, since it allows the simultaneous DNA unwinding and hydroxylapatite chromatography of both drug-treated and reference cell populations. This procedure gives directly the difference between DNA rejoining in cells with and without inhibitor, which improves the accuracy in determining the influence of the inhibitor compared to indirect measurements, i.e. measuring the difference between two independently determined curves.

The second assay can be used to determine the kinetics of DNA rejoining with great sensitivity. Two cell populations of different radiolabelling were again used. They were both γ-irradiated on ice and for rejoining injected into 37°C medium with a fixed time interval between the differently labelled cell populations. The kinetics of the rejoining was then followed by withdrawing samples at different times followed by DNA unwinding and hydroxylapatite chromatography. The result was expressed as the difference in DNA breaks between the beginning and end of the fixed time interval at different times of rejoining.

A BACTERIAL MODEL SUGGESTS THE INVOLVEMENT OF APURINIC SITES IN THE SYNERGISTIC ACTION OF ANTITUMOR AGENTS AND INTERCALATING DRUGS.

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The intercalating compound, 9-NH_2-ellipticine (9AE), and derivatives, were previously shown to break DNA at apurinic sites with specificity. We have shown that a related compound, Isopropyl-oxazolopyridocarbazole (Ipr-OPC), interacts with apurinic sites without inducing breakage. The inhibitory effect of 9AE or Ipr-OPC with non toxic doses of the alkylating agent Dimethylsulfate (DMS) on the growth of E.coli is additive. The same result has been observed with a exonuclease III mutant which has only 10 % of the AP-endonuclease activity. However 9AE or Ipr-OPC display a synergistic toxic effect with a DMS concentration which allows 20 % of E.coli survival. This synergy is increased for 9AE in the AP-endonuclease mutant when compared to the wild-type strain. Under the same conditions 9AE and Ipr-OPC have no synergistic effect on a mutant deficient in the enzymes which generate AP-sites. This is not caused by a difference in DNA accessibility. The direct interaction of 9AE and Ipr-OPC with AP-sites in E.coli is therefore likely responsible for the inhibition of their repair and enhancement of DMS toxic action. Synergy between some antitumor intercalating drugs and antitumor agents which induce AP-sites (X-rays alkylating agents) might be caused by a similar process.

It is remarkable that the antitumor agent 2-methyl-9-hydroxy-ellipticine displays a synergistic effect with alkylating agents on L1210 leukemia in vitro and in vivo. This compound interacts with apurinic sites without inducing breakage.

REPAIR OF ALKYLATED BASES IS INCREASED IN MAMMALIAN CELLS AFTER VARIOUS PRETREATMENTS.

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We have shown that the O^6-methylguanine-DNA-methyltransferase (transferase) activity was increased in a rat hepatoma cell line after treatment with various agents : UV-light, X-rays, cis-platinum, ellipticines... This increase is observed 48 hours after a single treatment with a low dose of these agents. This is at variance with the adaptive response which is induced after repeated pretreatments.

Similar results were obtained with different cell lines, among them a human liver cell line (LICH cells): the transferase activity is increased for instance about 4-fold when these cells are pretreated with cis-platinum II. In each cell line, the enhanced activity is due to a protein having the same properties than the constitutive one, as judged by gel electrophoresis characterization.

The influence of the pretreatments on the repair of the lethal lesion 3-methyladenine was investigated. The removal of 3-methyladenine residues from the DNA of ^3H - MNU treated cells is faster in pretreated compared to control cultures. The 3-methyladenine glycosylase activity measured in cellular extracts, using as substrate ^3H - DMS treated DNA, is also increased by the pretreatments.

These enhanced proteins are biologically active, as the lethal and mutagenic effects of MNNG are lower in pretreated cells.

As these enhanced activities are observed after a single pretreatment with agents which induce different types of DNA damage, our results suggest that the repair of alkylated bases can be increased in mammalian cells by a process which is different from the adaptive response.

INTRACHROMOSOMAL PROBES FOR INVESTIGATING MUTAGENESIS BY CHEMICAL CARCINOGEN-DNA ADDUCTS IN MAMMALIAN CELLS

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An SV 40-derived shuttle vector was modified to contain a single DNA adduct formed by certain carcinogenic alkylating agents, O^6-methyl guanine (O^6-MeGua), and then introduced into mammalian cells to investigate the mutagenic processing of this defined DNA lesion. The shuttle vector pJPvu I was constructed to contain the pML2 and SV40 origins of replication, the neomycin resistance (neo^r) gene under the control of both bacterial and SV40 promoters, and a unique Pvu I site located in a position unnecessary for vector viability. A single O^6-MeGua residue was inserted into pJPvu I by constructing a duplex genome with a 4-base gap in the Pvu I site, into which a chemically synthesized tetranucleotide, 5'-pTpCpm^{6}GpA-3', was ligated. The adduct-containing pJPvu I was transfected into CHO cells where it randomly integrated into the host chromosome and where mutations were fixed. Progeny vector genomes were rescued by fusion of CHO cells possessing the neo^r-marker to COS cells, followed by amplification in Escherichia coli. O^6MeGua-induced mutant genomes were selected by virtue of their insensitivity to Pvu I digestion. To study the influence of repair processes on the final yield of mutations, two isogenic CHO cell lines either proficient (mex^+) or deficient (mex^-) in the removal of O^6MeGua were used. A mutation frequency at the Pvu I site of 8-15% was observed when the O^6MeGua-adducted shuttle vector was replicated in the repair-deficient cell line. The frequency of mutations was background (~2%) when the vector was replicated in repair-competent CHO cells. The DNA sequences of 19 mutant genomes revealed that the genetic change arising after replication of O^6MeGua in mammalian cells was exclusively the G to A transition. The feasibility of determining the mutation frequency at the Pvu I site directly in the mammalian cell genome by Southern blot analysis is under investigation. The system is currently being used to determine the mutagenesis caused by O^6-ethylguanine in mammalian cells and to investigate the role of the mammalian O^6MeGua-methyl transferase in modulating its mutagenesis.

CONSTRUCTION OF A SITE-SPECIFICALLY MODIFIED VIRAL GENOME CONTAINING THE MAJOR DNA ADDUCT OF THE ANTITUMOR DRUG cis-DIAMMINEDICHLOROPLATINUM

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Platinum antitumor drugs, the prototype of which is cis-diammine dichloroplatinum (II), exert their biological effects by binding to DNA in the tumor cell. In order to study the consequences of cis-DDP on biological processes such as replication and mutagenesis, a bacteriophage M13 genome has been constructed containing the major adduct formed between this drug and DNA, a cis-[$Pt(NH_3)_2$-(d(GpG)] intrastrand crosslink, at a unique site. The dodecanucleotide d(TCTAGGCCTTCT) was synthesized by the solid phase phosphotriester method and was allowed to react with 1 equivalent of cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$. The resultant platinated product, cis-$[Pt(NH_3)_2$(d(TCTAGGCCTTCT)$)]$, was purified by reversed phase HPLC. An NMR pH titration of the non-exchangeable base protons of the unplatinated and platinated dodecanucleotides identified the platinum adduct as an intrastrand crosslink between the N(7) atoms of the adjacent guanosine residues. A 12 base pair insertion mutant of M13mp18 was constructed by ligating the duplex dodecanucleotide d(TCTAGGCCTTCT)-d(AGAAGGCCTAGA) into the unique Hinc II restriction site, thereby destroying the Hinc II site and generating a unique Stu I site. A duplex M13 genome containing a 12-base gap in the minus strand was created by hybridizing the circular plus (viral) strand genome of the insertion mutant with Hinc II-cleaved M13mp18 duplex DNA. The platinated dodecamer, cis-$[Pt(NH_3)_2$(d(TCTAGGCCTTCT)$)]$, was ligated into the minus strand gap with high efficiency to create a biologically viable site-specifically adducted genome. Modification of this general procedure has permitted the construction of duplex and single-stranded genomes with the cis-$[Pt(NH_3)_2$(d(GpG)$)]$ crosslink in the plus strand as well. These procedures are general ones and can be modified to accommodate other DNA adducts.

The presence of the cis-$[Pt(NH_3)_2$(d(GpG)$)]$ crosslink in the Stu I site conferred Stu I resistance to the duplex genome, whereas restriction at other nearby sites was unaffected. Removal of the platinum by treatment with sodium cyanide quantitatively restored Stu I sensitivity. Gradient denaturing gel electrophoresis was used to investigate the stability of a 300 bp fragment containing the unique cis-$[Pt(NH_3)_2$(d(GpG)$)]$ crosslink. The platinated fragment melted out at a lower concentration of denaturant than the unmodified parent fragment; localized melting of the DNA duplex around the cis-$[Pt(NH_3)_2$(d(GpG)$)]$ crosslink could provide a nucleation site for the cooperative melting transition that was not observed in the unplatinated fragment. Data on the survival of these site-specifically adducted vectors in bacterial cell lines with various genetic backgrounds will be presented.

CLONING OF *M. LUTEUS* 3-METHYLADINE-DNA GLYCOSYLASE IN *ESCHERICHIA COLI*

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3-methyladine is formed in DNA after treatment with alkylating agents. This lesion is repaired by 3-methyladine-DNA glycosylase. In *Escherichia coli* the repair of 3-methyladenine depends on the products of the genes *tagA* and *alkA*, which code for 3-methyladenine-DNA glycosylases I and II, respectively. The tagA$^-$ and alkA$^-$ mutants are sensitive to alkylating agents, the double mutant much more so.

We have cloned two genes of *Micrococcus luteus* that partially restore the resistance of alkylating agents to the tagA$^-$ alkA$^-$ double mutant of *E. coli*. The two hybrid plasmids contain *M. luteus* DNA which code for two separate 3-methyladenine-DNA glycosylases which only excise 3-methyladenine from the DNA. These two glycosylases both have molecular weight of 21 kD and are not inhibited by 3-methyladenine. Although there is an adaptative response in *Micrococcus luteus* neither of the recombinant DNA glycosylases is inducible under adaptative treatment in different mutants of *E. coli*. The restriction map of the two clones are totally different and the DNA of the two genes do not cross hydridize. Moreover, these genes do not hybridize to the tagA or alkA genes in *E. coli*. Sequencing of the two recombinant DNAs is in progress.

Molecular cloning of the *fpg*$^+$ gene of *Escherichia coli*, coding for the 2-6 diamino-4 hydroxy-5N-methylformamidopyrimidine-DNA glycosylase (FAPY-DNA glycosylase).

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Guanine methylated at the N7 position is by far the main product in DNA after chemical methylation. N7 methylguanine alone does not seem to be harmful for the cell. However, N7 alkylation favors the cleavage of the imidazole ring yielding 2-6 diamino-4-hydroxy-5N-methylformamidopyrimidine (FAPY). Observations suggest that FAPY residues might play an important role in cellular processes leading to mutagenesis and/or carcinogenesis by alkylating agents, (i) *in vitro*, FAPY inhibits DNA chain elongation by *Escherichia coli* DNA polymerase I, (ii) *in vivo*, FAPY is specifically excised by a DNA glycosylase in both prokaryots and eukaryots. To assess the biological significance of FAPY, we have cloned the FAPY-DNA glycosylase gene of *Escherichia coli*.

High molecular weight DNA was purified from the HB1100 wild type strain and submitted to a limited digestion by the Sau3A restriction enzyme. Then 10-15 Kb fragments were purified on a neutral sucrose gradient, and ligated at the BamHI site of pBR322. Recombinant molecules were transformed into the HB101 recA$^-$ strain. Over 800 clones were isolated and the FAPY-DNA glycosylase activity was measured in crude lysates. Among them, one clone overproduced the glycosylase by a factor of 10. A 15 Kb plasmid was isolated, characterized and subcloned. Finally a 1.8 Kb insert containing the *Escherichia coli* *fpg*$^+$ gene was cloned into pUC18 and 19. The resulting recombinant plasmids were introduced into HB101. These strains overproduced the FAPY-DNA glycosylase by a factor of 50. The only product encoded for by these plasmids is a 31KD protein as shown by the maxicell method. The FAPY-DNA glycosylase purified from the overproducer shows a single protein at 31 KD. The sequence of the *fpg*$^+$ gene was also determined. The construction of a FAPY-DNA glycosylase deficient strain of *Escherichia coli* is in progress.

IDENTIFICATION OF TWO RAT LIVER LIGASE SPECIES WITH DIFFERENT SUBSTRATE SPECIFICITY : DO THEY CORRESPOND TO TWO DIFFERENT ENZYMES ?

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Although only one DNA ligase gene product exists in procaryotes and in yeast, in mammals, in some laboratories two DNA ligases have been reported which differ in their chromatographic properties, molecular mass and ability to ligate oligo(dT) annealed to poly(rA) : DNA ligase I, a high molecular mass (130 kDa) species, is not active with a poly(rA):oligo(dT) substrate, whereas DNA ligase II, a smaller enzyme (60-80 kDa), is able to ligate oligo(dT) annealed to poly(rA). However, in other laboratories, only one DNA ligase has been purified and this corresponds to DNA ligase I.

We have previously described the partial purification of a single ligase species from both normal and regenerating rat liver which, after activity gel analysis, is composed of a single 130 kDa polypeptide (Eur. J. Biochem., 1987, 165, 325-332). Recently we have identified a second, minor, species of DNA ligase which is active with a poly(rA):oligo(dT) substrate, unlike the major species. Following separation of the two ligase species by phosphocellulose chromatography, further purification was carried out by hydroxylapatite and ds-DNA cellulose chromatographies and gel filtration. The purified fractions also display different Kms for ATP and pH optima. Since the activity of the major ligase species in normal rat liver increases markedly after partial hepatectomy, reaching the maximum level after 44h, while that of the minor species remains at a constant low level during the period of regeneration, we conclude that these species are equivalent to the DNA ligase I and II previously described for this tissue.

However, when these fractions were analysed on activity gels, the ligase activity for both fractions appears to be correlated to polypeptides with the same high molecular mass (200, 150 and 130 kDa) and no smaller molecular mass polypeptides, analogous to the previously reported DNA ligase II species, were detected. In addition, when the ligase-AMP intermediate complex was analysed after polyacrylamide gel electrophoresis, again only one 130 kDa polypeptide has been revealed for both ligase fractions.

This indicates that ATP-dependent DNA ligase fractions purified from rat liver, whether active with a poly(rA):oligo(dT) substrate or not, are composed of high molecular mass polypeptides.

DNA DAMAGE INDUCED IN MAMMALIAN CELLS UPON BIOREDUCTIVE ACTIVATION OF THE NITROIMIDAZOLE, RSU 1069.

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RSU-1069 , 1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol, is, on a concentration basis, 30-100 fold more toxic towards hypoxic relative to aerobic cells in vitro. The induction of DNA single and double strand breakage by RSU-1069 and misonidazole in V-79 mammalian cells, as assessed using sedimentation techniques, after incubation for 2 hr at 37C under both hypoxic and aerobic conditions have been investigated using concentrations of the agents comparable to those used in the cytotoxicity studies. The time dependence of strand breakage in cellular DNA has also been investigated at given concentrations of the agents.

Double strand breaks are induced predominantly by RSU-1069 under hypoxic conditions. Preliminarly investigations are being undertaken to assess the time-scale of repair of such DNA damage in comparison with that produced by ionising radiation.

From the dependence of yield of DNA strand breaks upon both time and concentration of the agents, the following points will be emphasised: 1)in air, RSU 1069 is more effective than misonidazole at causing strand breakage, 2)under hypoxic conditions RSU-1069 is 10. -fold more effective than misonidazole at inducing DNA breaks and 3) the percentage of rejoining of drug-induced DNA damage is less than that witnessed for radiation-induced DNA damage. These findings are consistent with the proposed molecular mechanisms whereby the greatly increased efficiency of the nitroimidazoles under hypoxic conditions is due to bioreductive activation of the compounds to form reactive metabolites. In the case of RSU-1069, bioactivation results in the metabolite having bifunctional character.

STRUCTURAL DAMAGE AND MUTATIONS IN LAMBDA PHAGE AFTER GAMMA-IRRADIATION

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Lambda phage DNA was gamma-irradiated in aqueous solution and the amount of some radiation-induced lesions, i.e; breaks and alkali-labile sites, was determined by analytical sedimentation. This DNA exhibited twice as much minor structural damage (single strand breaks and alkali-labile sites) than DNA from irradiated phage originating from an induced lysogen. The irradiated DNA was then repackaged in-vitro into infectious particles. Using SOS-induced host cells, the induction of mutations in either of the cI- and cII-cistron was scored. Simultaneously the loss of infectivity was analyzed with respect to the extent of structural damage. An increase in the number of mutation was found to be mainely by minor lesions, i.e; single strand breaks and alkali-labile sites and unidentified base damage. These lesions also interfered with biological activity. Major lesions, i.e; double strand breaks and S1-nuclease-sensitive sites, were found to contribute to the loss of infectivity rather than to mutagenesis.

PSORALEN-DNA CROSSLINK REPAIR ANALYZED BY ELECTRON MICROSCOPY : COMPARISON BETWEEN NORMAL AND FANCONI ANEMIA FIBROBLASTS.

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Fanconi anemia (FA) is an autosomal recessive disease characterized by a high cellular sensitivity to bifunctional genotoxic agents. Two complementation groups, A and B, have been genetically distinguished. The capability of normal and FA cells to repair the DNA interstrand crosslinks photoinduced by the bifunctional furocoumarin 8-methoxypsoralen (8-MOP) was evaluated by a quantitative electron microscopy analysis.

Cell cultures were treated by 8-MOP and UVA (365 nm). DNA was extracted either immediately or after different time intervals of post-exposure incubation. DNA was restricted with PvuII in order to reduce the fragment length and allow proper numeration of crosslinks. After total denaturation and spreading of DNA according to Davis, DNA interstrand crosslinks were visualized by a transmission electron microscope Zeiss 902. DNA fragment length measurements were performed with a computer assisted digitizer.

It was observed that the number of 8-MOP-photoinduced crosslinks is similar in normal and FA cells for a same UVA dose in a same experiment. A dose relationship is found between the UVA dose and the number of crosslinks. In all the cell lines studied, the number of crosslinks decreases as a function of the repair incubation time. After a 24 h period, the removal of DNA crosslinks is comparable in normal and FA cells of complementation group B ; it seems slightly reduced in FA cells from complementation group A. Similar results are obtained using either confluent cell cultures or rapidly growing cells. On the other hand, the number of crosslink fragments as well as the number of crosslink per fragment is observed to diminish after repair.

In conclusion, this electron microscopy analysis indicates that : 1) normal human fibroblasts are able to incise interstrand crosslinks from their DNA. 2) FA cells, as well, appear to accomplish at least the first steps of the repair process, i. e. recognition and incision. However, FA cells belonging to complementation group A seems to be somewhat affected in the efficiency of such a process.

DUPLEX-DUPLEX HOMOLOGOUS RECOMBINATION CATALYSED BY HUMAN NUCLEAR EXTRACTS

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Using as substrates, 1 : the replicative form (RF) of phage M13 mp8 in which the reading frame of the lac Z' gene was disrupted by insertion of an octonucleotide, and 2 : a restriction fragment one kb long, containing the functional lac Z' gene (isolated from wild type M13 mp8), we show that nuclear extracts from human cells (3 lines tested) promote the targeted replacement of the altered sequence by the functional one. Following incubation with the extracts, the DNA's were introduced in JM 109 bacteria (rec A$^-$ and lac Z'$^-$) which were grown in presence of a colorimetric indicator of β-galactosidase activity. Homologous recombination gives rise to the genotypical modification : lac Z'$^+$ instead of lac Z'$^-$ in the bacteriophage DNA. This is revealed by phenotypical expression of the lac Z' gene product in replicating bacteriophage, i. e. the formation of blue instead of white plaques. The frequency of recombination (blue/total plaques) is increased by a factor of 50-80 as a function of protein concentration and of incubation time. The maximal frequency observed is 5×10^{-5}. There is no increase over the background when extracts are boiled.

Electrophoresis and electron microscopy of DNA's incubated with the extracts show the formation of recombination intermediates with reciprocal exchange. Restriction analysis of recombined DNA confirms that the process corresponds to targeted sequence exchange. These data allow to propose three steps for homologous recombination between two duplex DNA's : i) unpairing of the two duplexes ; ii) single-strand exchange and synaptic pairing ; iii) resolution of the cross-junctions. The three steps correspond to those predicted by the gene conversion model of Holliday.

Sequencing of the DNA from 20 recombined clones was performed over 400 nucleotides from the primer annealing site. The analysis encompasses a non expressed gene (lac I'). No modification was detected in the 20 DNA sequences. This result appears to demonstrate that the strand exchange is error-free.

PROMOTION OF DOUBLE-STRAND BREAK REPAIR BY HUMAN NUCLEAR EXTRACTS MAINLY INVOLVES RECOMBINATION WITH INTACT HOMOLOGOUS DNA

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Parameters of DNA double-strand break (dsb) repair catalysed by human nuclear extract were analysed using as substrate, the replication form (RF) of M13 mp8 in which a single double-strand break (dsb) was introduced by restriction. After incubation with the extract, the dsb repair was estimated by the ability of the incubated RF to produce plaques following transfection into JM 109 (RecA$^-$) bacteria. The possibility of recombination with a purified fragment from M13 mp8 RF enhances up to 20 times the plaquing ability of the RF.

The repair by recombination occurs under several conditions : i) the break in the RF must be located in the region of homology with the fragment. ii) the fragment has to be intact in the region corresponding to the break in the RF. iii) a minimal length of homology between the region surrounding the dsb in the RF, and the fragment is required. The *in vitro* reaction is ATP dependent and dNTP's partially dependent. Dephosphorylation of the free ends in the RF decreases the repair by ligation but is without effect on the recombination. However the efficiency of repair by recombination is dependent on the structure of the DNA ends at the cleavage site.

REPAIR OF DNA PHOTOADDUCTS INDUCED BY 3-CARBETHOXYPSORALEN (3-CPs) IN EUKARYOTIC CELLS, FOLLOWED BY A CHEMICAL METHOD.

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In recent years, one of the goals of studies on psoralens has been to characterize the chemical and stereochemical nature of psoralen-nucleoside photoadducts formed in vivo by quantitative and specific methods. A technique developped for the detection of the two thymidine< >3-carbethoxypsoralen diastereoisomers formed in DNA (Moysan et al., Biochimie, 1986) has been applied to study DNA photoadducts formed in the DNA of eukaryotic cells after treatment with 3-CPs and UV-A. This method based on the purification and enzymatic hydrolysis of DNA extracted from treated cells and its analysis by HPLC using fluorescence detection has permitted us to identify and follow quantitatively the two diastereoisomers d Thd< >3-CPs formed in Saccharomyces cerevisiae and Chinese hamster cells. At doses leaving the same survival level, the induction of these two types of adducts depended on the cells used.

The removal of each diastereoisomer has been determined in several cell lines during post-treatment incubation. The kinetics of the removal of these specific types of 3-CPs plus UVA induced DNA monoadducts obtained by this technique have been compared to results obtained with classical techniques and radioactive 3-CPs.

N-OXIDATION AND HYDROXYL RADICAL OXIDATION OF DNA BY HYDROGEN PEROXIDE

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Hydrogen peroxide may be considered as a metabolite emerging from normal and pathological biochemical pathways.

Whatever its origin (biological or by-product of gamma or UV-irradiated water), it is believed to react mostly with DNA through the intermediacy of a very reactive species : the hydroxyl radical. The transformation of hydrogen peroxide into this radical, known as the Fenton reaction is catalyzed by transition metals :

$$H_2O_2 + Fe^{2+} \xrightarrow{\text{(or } Cu^{2+}\text{, etc...)}} OH^{\cdot} + OH^{-} + Fe^{3+}$$

Using experimental conditions which minimize the Fenton reaction, essentially by lowering the amounts of transition metals, we were able to detect three main DNA damages : thymine glycols, 8-hydroxy deoxy 2' - guanosine and adenine N^1-oxide, using different and sensitive experimental approaches, combining tritium-labelled DNA, acidic or enzymatic hydrolytic digestions with electrochemical or postlabeling techniques.

For this data the following conclusions can be drawn :

1) Besides the predominant hydroxyl radical oxidation, the N-oxidation cannot be neglected as it can be observed noticeable amounts of Adenine N^1 oxide when DNA reacted with hydrogen peroxide.

2) Thymine glycols are also identified even with a medium in which the Fenton reaction is minimized. The best yield was obtained with isolated thymidine rather than with DNA.

3) 8-hydroxy 2'-deoxyguanosine was measured with a remarkable sensivity thanks to electrochemical detection ($\ll$ 1ng).

4) Particular care must be taken into account when quantitative determination of a specific damage is requested with enzymatic procedures: the worst digestion being obtained with the highest modified DNA.

Detection, quantification and computer-assisted analysis of DNA modifications in single cells

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Modification of DNA may result in mutagenesis, carcinogenesis, cell death and inhibition of cellular proliferation. Important parameters are the pattern and extent of DNA modification and the cell's capacity to repair DNA damage. The immunocytochemical peroxidase assay, introduced by our group (Heyting et al., Cancer Res.43 (1983) 2935), offers a specific, sensitive, and quantitative method for the analysis of a wide range of DNA modifications. The following DNA adducts can now be visualized and quantified in individual cells (the numbers indicate the detection limits in adducts per diploid genome): O^6-MeGua and O^6-EtGua (10^4; in collaboration with Dr.J. van Benthem, Amsterdam), 7-MeGua (10^5; with Drs.R. Montesano and P. Degan, IARC, Lyon), Gua-N^2-(A)AF ($4x10^3$; with Dr.E. Kriek, Amsterdam), Gua-N^2-BP ($5x10^3$; with Dr.F.J. van Schooten, Amsterdam), Gua-N7-aflatoxin B_1 ($2x10^3$; with Dr.R. Montesano, IARC, Lyon), and DNA adducts induced by the cytostatic platinum drugs cisplatin and carboplatin (with Dr.P. Terheggen, Amsterdam). Adducts are visualized by a yellow-brown precipitate, a peroxidation product of diaminobenzidine. Quantification has been achieved in the Sylvius Laboratories by a light microscope transmission measurement, evaluated by a program for automated scanning and plotting of transmission data (HIDACSYS).

We have found that a striking heterogeneity in the formation and repair of DNA adducts within rodent tissues is the rule rather than the exception. In case of the hamster pancreas carcinogen bis(oxypropyl)nitrosamine, the subpopulation of ductular cells capable of enzymatic activation amounts to only a few percent of all pancreatic cells.

Immunocytochemistry of DNA adducts also allows the study of small target organs or tissues. An example is the rat oesophageal carcinogen methylbenzylnitrosamine (MBN) which, incidentally, also gives tumours of the tongue. Using an antibody against O^6-MeGua, we observed an intense nuclear staining of both the oesophageal and the tongue epithelium after MBN. Interestingly, MBN also resulted in DNA methylation in epithelial cells in some other tissues (trachea and lung).

Clinical application of quantitative immunocytochemistry is one of the goals of the present investigations. DNA adducts induced by pharmacologically relevant doses of cisplatin have been visualized in mouse tissues, including solid transplantable tumours. A striking heterogeneity in adduct distribution was observed in the kidney with high levels of adducts in the proximal tubules. A substantial part of cisplatin-DNA adducts in mouse tissues, in particular in the kidney, proved to be of a highly persistent nature. Preliminary experiments suggest the feasibility of cisplatin-DNA adduct visualization in (buccal) cells from cisplatin-treated cancer patients.

RADIATION PROTECTION AND SENSITIZATION: A CHARACTERISTIC OF MODIFICATION OF DNA REPAIR

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The need for protective measures against the harmful effects of radiation and particularly the possibility of protection from potential carcinogenicity of low level ionizing radiation, or of solar radiation, is becoming increasingly important. We have recently demonstrated that some of the classical radioprotective drugs show improved effectiveness when acting not only as radical scavengers but rather as enhancers of DNA repair, as shown by increased DNA repair synthesis and strand break rejoining in mammalian cells.
A methodical and logical approach to obtaining increased proection from radiation is provided by the use of modifiers which are an integral part of a repair pathway, as is the case with nicotinamide. Nicotinamide (NA) is a precursor for NAD+, which is the substrate for production of poly-(ADP-ribose) by the enzyme PADPR-transferase, known to be induced by DNA strand breaks. It has been suggested by several authors that NA acts as an inhibitor to the production of the polymer and is therefore a radiosensitizer. It was also argued that the observed increased UDS following exposure in the presence of NA or 3-aminobenzamide is not a true increase in repair but the result of nonspecific endonuclease activity which cause more DNA breaks and thus more UDS. We have insisted that NA acts differently from other inhibitors of PADPRT and that at low concentrations its presence results in increased survival, and repair.

Mammalian and human cells with different repair capabilities were exposed to UV light or to gamma radiation. DNA repair synthesis and levels of NAD+ were measured following exposure. In the repair deficient XP cells and in K-1735 mouse melanoma C1-11 cells the level of NAD+ did not drop, indicating no production of PADPR, and DNA repair capacity remained zero or low respectively after exposure to UV-C light, but a marked drop in NAD+ and a good level of DNA repair synthesis was observed after exposure to gamma radiation. The presence of 3 mM NA moderated the drop in NAD+ level and caused an increase in DNA repair capacity. In the well-repairing HEp-2 cells the drop in NAD+ and the increased repair synthesis was observed following both UV and gamma radiations, and NA increased the level of NAD+ and of the repair synthesis, indicating that a higher, optimal level of NAD+ is required for continuous production of the polymer during repair synthesis. Higher concentrations of NA caused reduced repair and reduced survival. Nicotinamide, acting through the poly(ADP-ribose) system is therefore a sensitizer at high concentrations and a protector at low concentrations, and acts in cells in which there is an effective DNA repair machinary.

PATHWAYS FOR REPAIR OF THYMINE GLYCOLS, UREA RESIDUES AND AP SITES

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Thymine glycols, urea residues and AP sites are models for DNA base and sugar damages produced by ionizing radiation and oxidative stress. These damages may be selectively introduced into viral transfecting DNA and easily quantitated. Escherichia coli endonuclease III, exonuclease III and endonuclease IV are enzymes presumed to be involved in the repair of these damages on the basis of their in vitro specificities. Endonuclease III is a combined DNA glycosylase and class I AP endonuclease which recognizes a variety of thymine radiolysis products including thymine glycols. Exonuclease III and endonuclease IV are class II AP endonucleases which also incise DNA containing urea residues. Paradoxically, mutants in genes encoding endonuclease III (nth) and exonuclease III (xth) or endonuclease IV (nfo) are not X-ray sensitive. However, xth nfo double mutants are.

We have found that ϕX RF transfecting DNA containing thymine glycols is inactivated at a 2.5 fold greater rate in nth mutants than wild type. In addition, ϕX RF DNA containing thymine glycols, urea residues or AP sites is inactivated at a 2.5 fold greater rate in xth nfo double mutants than wild type or either single mutant. These results suggest that endonuclease III and either exonuclease III or endonuclease IV are involved in the repair of thymine glycols in vivo. Furthermore, exonuclease III and endonuclease IV are involved in the repair of urea residues and AP sites in vivo and are capable of substituting for each other. These data are in agreement with the in vitro substrate specificities of these three enzymes.

SOS repair of thymine glycols, urea residues and AP sites was also examined. Thymine glycols are blocks to DNA replication, but they are not mutagenic and retain base pairing properties. Urea residues and AP sites are also blocks to DNA replication, and are mutagenic and noninstructive. ϕX RF transfecting DNA containing thymine glycols was efficiently W-reactivated in SOS-induced cells whereas ϕX RF DNA containing either urea residues or AP sites was not. These results suggest that the ability of the SOS response to reactivate DNA damage is dependent on the nature of the lesion.

The genetic requirements for W-reactivation of thymine glycols were also examined. Reactivation of thymine glycols was dependent on UV-irradiation of the host or the presence of the recA730 allele in lexA(Def) hosts which constitutively express SOS genes. These results suggest that activated RecA is required directly in the reactivation of thymine glycols. It was also found that thymine glycols were reactivated in SOS-induced umuC insertion mutants. These data suggest that activated RecA can mediate reactivation of thymine glycols and that this process is partially umuC+ independent.

CLASTOGENICITY OF A DACARBAZINE ANALOGUE (MTBA) IN DRUG SENSITIVE OR RESISTANT HUMAN LYMPHOBLASTOID CELL LINES

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Dacarbazine (DTIC), a triazeno compound currently used in cancer chemiotherapy, was shown to be able of mediating "chemical xenogenization" (cx, i.e. induction of novel antigens) of cancer cells (Bonmassar et al.Proc.Nat.Ac.Sci.66,1089,1970). A DTIC analogue, the 1-p-methyltriazeno benzoic acid potassium salt (MTBA), less immunotoxic than DTIC and equally effective in inducing cx, was recently found to be clastogenic for normal human lymphocytes (Vernole et al.Mut.Res. in press). It has been demonstrated that triazene derivatives alkylate DNA (Preussmann and van Hodenberg Biochem.Pharmacol.19,1505,1970), but they might also imply other mechanisms as also shown by our data on clastogenicity of the drug in G2 phase.

Clastogenic studies were then extended to an in vitro cultured E. B.V. line (X 303) and to its MTBA-resistant subline (M 503) obtained following 5 in vitro exposures to 100 ug/ml of MTBA. The karyotypes of both lines were normal diploid.

X 303 and M 503 cells were treated in G2 phase with different concentrations of MTBA (2,20,100,200,500 ug/ml) for 1 hour at 37°C. After 30 minutes of incubation with fresh medium, colchicine was added and cultures were harvested after further 90 minutes. Chromosome breaks were counted in 100 metaphases per experimental points, experiments were repeated twice. The percentage of damaged cells and the mean number of breaks per cell ($p<0.05$, according to paired "t" test) showed that M 503 cells were less sensitive to the clastogenic effects than the parental X 303 cells. Differences between the 2 sublines were highest at the concentration of 200 ug/ml (X 303 0.19 breaks per cell, 15 % damaged cells and M 503 0.045 breaks per cell, 3.5 % Damaged cells). Moreover normal lymphocytes showed a sensitivity similar to that of X 303.

The present results suggest that mechanisms causing cytotoxicity and clastogenicity of MTBA are related.

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EFFECT OF PRIOR BLEOMYCIN TREATMENT ON UV-LIGHT INDUCED SCEs IN NORMAL AND AT LYMPHOBLASTOID CELLS

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Agents, like Ultraviolet light (UV), that inhibit DNA synthesis mainly by blocking chain elongation, are known to be strongly effective in inducing sister chromatid exchanges (SCEs) (Wolff,1974). The Painter's replication model for SCEs formation postulates that exchanges occur at junctions between adjacent replicated and partially replicated clusters during DNA semiconservative duplication (Painter, 1980). Confirming this model, it has been demonstrated in CHO (Painter, 1984) and frog cells (Chao, 1984) that pretreatment with X-radiation, by blocking the initiation of whole clusters, reduces the frequency of SCEs induced by UV.

Ataxia-telangiectasia (AT) is an autosomal recessive phenotype characterized by progressive neuronal degeneration, immunodeficiency, cancer proneness and extreme sensitivity to ionizing radiation. Following ionizing radiation or bleomycin treatments, AT lines do not show the DNA synthesis delay typical of normal cells: in these cells, both replicon initiation and chain elongation are radiation and bleomycin resistant (McKinnon, 1987).

In this preliminary work, one normal and one AT lymphoblastoid line have been treated sequentially with bleomycin (BLM, 5 and 30ug/ml) and UV radiation (6erg/mm^2/sec. x 10sec.) 48h before culture harvesting. The slides have been stained by a modification of the fluorescenze-plus-Giemsa method to evaluate SCEs frequencies, cell cycle progression and M.I.

The results show that both in normal and AT lines the total number of SCEs observed in cells treated with BLM before UV irradiation (40 M2 scored for each experimental point) is lower than expected for additivity of the two treatments:

	Untr.	BLM 5ug	BLM 30ug	UV	BLM5 + UV	Expec.	X^2	BLM30 + UV	Expec.	X^2
NORMAL	181	203	185	333	284	355	14.2	250	337	22.4
AT	135	177	149	314	264	356	23.7	267	328	11.3

The data discussed here demonstrate an inhibitory effect of a prior BLM-treatment on UV-induced SCEs formation in human lymphoblastoid cells. There are no great differences between the two lines, thought in AT lymphoblast the reduction of SCEs frequencies seems to be not related to BLM dose.

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"IN VITRO" AND "IN VIVO" DNA INTERACTION WITH N-ALKYLNITROSOUREAS LABELED IN THE CARBONYL GROUP

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The most widely accepted theory in chemical carcinogenesis by N-nitroso compounds and alkylating agents is that a causal relationship exists between DNA alkylation and tumorigenesis. Starting from 1960, various alkylated products like 7-alkyl-, O^6-alkyl- and 1,7-dialkylguanosines, and O^4- and O^2-alkylthymines have been implicated as critical factors in carcinogenesis. On the other hand, it should be noted that no definitive evidence has been found in favor of a causal carcinogenic role of any of these alkylated compounds.

In the present study it has been found that the carbonyl group of (m)ethylnitrosoureas interacts "in vivo" and "in vitro" with DNA. The brief time of "in vivo" exposure (1.5-3 h) and the absence of radioactivity in the bases excluded metabolic incorporation of the label; likewise a double protein digestion excluded a possible protein contamination. Moreover, the radioactivity was extremely labile to the normal hydrolytic procedures usually employed for DNA analysis.

These findings indicate: 1. another kind of DNA damage can occur in DNA-(M)ENU reactions besides alkylation; 2. this damage cannot be exactly quantified and chemically identified because of the extremely high index od lability.

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O^6-METHYLGUANINE REPAIR IN HUMAN CELLS CONTAINING A REGULATABLE O^6-METHYLGUANINE DNA METHYLTRANSFERASE GENE

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To assess the biological role of O^6-methylguanine (O^6MeG) in human DNA, cell lines were developed that contain a regulatable O^6-MeG DNA methyltransferase (MT) gene. MT-deficient HeLa SMR cells were stably transformed with a mammalian expression vector in which the E. coli ada gene was put under the control of the mmtv promoter switched on by glucocorticoids. 6 - 8 hours after addition of dexamethasone the induced MT activity reached its final level that exceeded 3 - 4 fold the low constitutive MT level expressed in uninduced cells. Addition of MNNG depleted the pre-existing activity in a dose-dependent manner. If reincubated the cells rapidly restored the lost activity. MNNG had no effect on the rate of resynthesis but declined in a dose-dependent fashion the final MT level accumulated by the cells. The tumor promoter TPA greatly enhanced the mmtv promoted synthesis whereas the gyrase inhibitor teniposide (V-16) considerably impaired the synthesis.

Cell survival was greatly enhanced not only by addition of dexamethasone and TPA before the treatment of MNNG but also if applied up to 24 hours after the MNNG treatment. These data confirm that O^6MeG are lethal to the cell and they suggest that their repair may be considerably delayed in time.

UV-INDUCED REPAIR REPLICATION BY MAMMALIAN CELL EXTRACTS

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Methods are being developed to study nucleotide excision repair in vitro using soluble, cell-free extracts from human lymphoid cell cultures. Repair replication of ultraviolet light (UV) irradiated plasmid DNA can be observed in such extracts. All steps of repair take place in the extract. Incision, gap filling, ligation, and supercoiling of the closed circular product. In the absence of ATP, the UV-dependence of repair is lost.

Mammalian cells contain a glycosylase/endonuclease which can act on hydrated pyrimidine UV photoproducts (principally cytosine hydrates). To avoid detecting repair initiated by this enzyme, plasmid DNA is irradiated to produce about 20 cyclobutyl pyrimidine dimers per circle, and then treated with endo III from E. coli to nick circles containing hydrated pyrimidines. Unnicked circles are re-purified and used as the substrate for in vitro repair. Therefore, the observed UV-induced repair is not initiated by incision at hydrated pyrimidines. The introduction of a few apurinic sites per circle, or a few DNase I-induced nicks, causes only modest repair replication, much less than that seen after UV irradiation.

Extracts from cells of some lines derived from xeroderma pigmentosum patients are deficient in this in vitro repair replication, compared to extracts from normal cells. Repair can be restored to these extracts by addition of UV DNA endonuclease/glycosylase from Micrococcus luteus.

Using the repair replication as an assay, we are attempting to isolate factors from proficient cell extracts which can complement deficient xeroderma pigmentosum extracts. Preliminary results indicate that a protein fraction from normal cells can complement deficient extracts derived from a xeroderma pigmentosum group C cell line.

Mutants of Escherichia coli hyper efficient in their DNA repair ability.

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By using selective techniques two mutants of Escherichia coli were isolated; one, designated SA236, was selected as cells more resistant to Far UV and the other, designated SA270, was selected as cells more resistant to Near UV + 8-methoxypsoralen (PUVA) than their respective parent strains (1 and 2).

Further studies with SA236 showed that it has simultaneously become more resistant than its parent strain to NUV, H_2O_2, NUV + H_2O_2, mytomycin C, nalidixic acid, novobiocin, fluorouracil and thymineless death. It remains as sensitive as its parent strain for PUVA. Biochemical analysis of this strain showed that the DNA polymerase I and the endonuclease I are synthesised at higher concentrations. However, introduction of a recA$^-$ mutation or a multicopy plasmid carrying the "SOS box" into SA236 leads the cells to become as sensitive to UV as a typical recA$^-$ mutant of E.coli. Genetic analysis of the UV-resistant phenotype showed that the mutation, designated polR, is located at 90 min. position on the linkage map of E.coli. Studies with the UV induction of λ in a lysogenic derivative of SA236 showed that the induction of this phage takes place with the same efficiency as its parental lysogenic strain.

Analysis of the polA regulatory region suggested that it contains two promoters and two operator regions in the order P1-O1-dnaA box-P2-O2-polA (3). From these studies a hypothesis for the hyper DNA repair ability in SA236 is proposed. It is suggested that the polR allele in the parental wild type strain of E.coli synthesises a repressor protein regulating the synthesis of DNA polymerase I. This protein also acts as a co-repressor of the LexA product enhancing its efficiency to repress the SOS inducible operons. Due to a mutation in the polR in SA236 this strain not only can synthesise polymerase I constitutively but also the "SOS operons" are derepressed. Hence the cells show enhanced resistance for a number of DNA damaging agents. It is furthermore proposed that for an effective DNA repair it is necessary that the cell must synthesise a certain minimum concentration of the lexA protein. If the endogenous concentration of this protein goes below the minimum required level (eg. by introducing the SOS boxes on multiple copy plasmids), the cell becomes sensitive to UV.

SDS-PAGE analysis of the total lysate from the PUVA-r strain, SA270, shows that it is synthesising a protein of ~Kd at higher concentration than its parental strain. Analysis of a typical recA$^-$ mutant of E.coli showed that it becomes more sensitive to PUVA than its parental recA$^+$ strain. When a recA$^-$ derivative of SA270 was analysed for PUVA sensitivity it was found that the double mutant is as sensitive to PUVA a typical recA$^-$ mutant suggesting the important role of the recA product in the repair of PUVA damaged DNA. It is suggested that the strain SA270 synthesises an endonuclease, the ~Kd protein, specific for psoralen/DNA adducts. In a wild type E.coli the repressed level synthesis of the protein limits the ability of the cells to repair the relevant DNA damage.

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CHARACTERISATION OF CHO CELL LINES HYPERSENSITIVE TO DNA DAMAGING AGENTS

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We have isolated 13 mutants of CHO-K1 cells which are hypersensitive to either mitomycin C (designated MMC-1 to -5), bleomycin (BLM-1 and -2) or MMS (MMS-1 to -6). Genetic analysis shows that the mitomycin C and bleomycin sensitive lines are all phenotypically recessive and represent six different complementation groups. Mutants BLM-1 and BLM-2 are also genetically distinct from the x-ray and bleomycin sensitive xrs-6 mutant isolated by Jeggo and Kemp (Mutat. Res. 112, 313, 1983).

Mutant MMC-2 is sensitive to UV light and mitomycin C, and is defective in UV-specific incision and in DNA cross-link removal. MMC-1, -3, -4 and -5 are proficient in cross-link repair (as judged by alkaline elution), although MMC-4 and MMC-5 appear to accumulate higher levels of damage following drug exposure than does the parental line.

Mutant BLM-2 is defective in the repair of both single and double strand breaks induced by bleomycin. DNA ligase I and II activities are currently being measured in this mutant. BLM-1 shows normal strand break repair kinetics.

Spontaneous mutation rates to thioguanine resistance are elevated in some of the mitomycin C and MMS sensitive lines. We are currently determining whether this correlates with a defect in AP endonuclease activity.

PROTECTIVE EFFECT OF CAROTENOIDS AND *ROSMARINUS OFFICINALIS* EXTRACT ON OXIDATIVE DNA DAMAGES IN DIFFERENT TA *SALMONELLA TYPHIMURIUM* STRAINS.

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Carotenoids were found to exert a protective effect against photomutagenesis induced by 8 methoxypsoralen (8-MOP) in *Salmonella typhimurium* TA 102 independently of pro-vitamin A activity. This protection was most probably due to quenching of $^1O_2$ and scavenging of oxyradicals generated by the 8-MOP-DNA photoadducts under UVA irradiation (1). Carotenoids were also active in preventing mutagenesis induced by benzo(a)pyrene (BP) in presence of S9 fraction in S. *typhimurium* TA 98. Thus, the carotenoids protective effect against mice skin and breast cancers induced by photodynamic action of BP and 8-MOP, respectively, was primarly consistent with the blockage of the ultimate oxidative carcinogens at the initiation stage of the carcinogenic process. Carotenoids were inactive in preventing mutagenesis induced in S. *typhimurium* TA 100 by the direct mutagens NaN_3 and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Nevertheless, carotenoids were found to be active in preventing the gastric carcinogenesis by MNNG, working, apparently, at the late progression phase, when oxygen radical species are still involved (1). This data stimulated further investigation on other natural antioxidants as those contained in the *Rosmarinus officinalis* extract obtained by high pressure CO_2 procedure. Actually, the series of experiments carried out with the above *Rosmarinus officinalis* extract showed its antimutagenic effect against indirect mutagenesis induced by 8-MOP and BP in S. *typhimurium* TA 102 and TA 98, but at an extent somehow less than that exerted by BC. The results of the tests carried out on S. typhimurium TA 100 proved that *Rosmarinus officinalis* extract did not affect the kinetics of the direct mutagenicity by NaN_3. The protective effect of *Rosmarinus officinalis* extract toward indirect oxidative mutagenesis is most probably dependent on the antioxidant properties of definite components such as carnosol, rosmarine and carnosic acids (2). The interest of all these data obtained with the above reported experimental models suggest that the latter can be successfully applied whenever the antimutagenic properties of natural antioxidant molecules should be tested.

1) Santamaria et al. Supplemental Carotenoids prevent skin cancer by Benzo(a)pyrene, Breast Cancer by PUVA, and Gastric Cancer by MNNG. Relevance in human chemoprevention. "Vitamins and Cancer" Ed. by F.L. Meyskens and K.N. Prasad, Humana Press, pp.139-159, (1985). (2) idem, J. Nutr. Growth & Cancer, 3, 73, 1987.

UMUC DEPENDENCY OF MMS MUTAGENESIS.

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It is generally accepted that MMS-induced mutagenesis in E.coli cells is SOS and umuCD-dependent. However, the percentage of umuCD-dependent mutations varies and is dependent on the marker tested. For example, we have found that the frequency of $Arg^- \rightarrow Arg^+$ and $Rif^s \rightarrow Rif^r$ in EC2401 (as AB2497 but umuC122::Tn5) is 30% and 50%, respectively, of that in AB2497. This may suggest that the umuC-dependent mutations result from different premutagenic lessions and/or arise at different sites in DNA then the umuC-independent mutations.

Arg^+ revertants of AB2497 strain can be divided, according to their requirement for histidine and/or threonine, into four phenotypic classes with a different mutational pathway for each class. It has been found that umuC-dependent mutations belong exclusively to class II, which can arise by transversion of AT->TA at the supG locus. This class of mutants dramatically decreases in EC2401. umuC--independent mutations belong to class I (AT transitions or transversions at the argE structural gene) and occur with the same frequency in both strains.

The premutagenic lessions and the mechanism of umuC--dependent mutagenesis will be discussed.

MISMATCH REPAIR OF MMS-INDUCED PREMUTAGENIC LESSIONS IN DNA.

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Most of the premutagenic lesions induced in E.coli after MMS treatment are excised from DNA by the mismatch repair system. The frequency of MMS-induced mutations to Arg^+ and Rif^r is about one order of magnitude higher in M1, a mismatch repair deficient strain (as AB2497 but mutS), than in AB2497, a mismatch repair proficient strain. Arg^+ revertants of these bacteria can be divided into four phenotypic classes: each class represents a different site of mutations and a different mutational pathway.

Analysis of Arg^+ revertants in these bacterial strains has shown that their distribution among these classes differs. In AB2497 73% of Arg^+ revertants belong to class II (AT->TA transversions) and the remaining 27% to class I (transitions or transversions of the AT in the ochre codon of argE). On the other hand in the M1 strain only 12% of Arg^+ revertants belong to class II and 72% belong to class I. This indicates (i) the mismatch repair system removes efficiently only the premutagenic lessions leading to revertants of class I and (ii) the fraction of Arg^+ revertants of class II is very small and is not affected by the mismatch repair system.

It was also established that in the mismatch repair deficient strain 78% of Arg^+ and 100% of Rif^r induced by MMS are independent from the umuC gene function.

The premutagenic lessions and mutagenic pathways leading to Arg^+ revertants of class I and class II will be discussed.

MECHANISM OF UV-INDUCED MUTAGENESIS: ROLE OF DNA REPLICATION.

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We have previously shown that in excision repair deficient E.coli cells formation of mutants precedes the resumption of DNA replication inhibited by UV-irradiation. This suggests that fixation of mutation is independent of post-UV DNA replication, which is further supported by the observation that the λsusO$_8$ mutant, unable to replicate its DNA in su$^-$ cells, is efficiently UV-mutated in E.coli 594 su$^-$ host cells.

We have presently found that in su$^-$ host cells no untargeted mutagenesis is observed and only targeted mutations in λsusO$_8$ phage are induced. UV-induced mutation in λsusO$_8$ phage in su$^-$ cells is independent of excision repair, and is as efficient in uvrA mutant as in wild type cells.

In bacteria, caffeine, a known inhibitor of mutagenesis and repair, inhibits excision but not postreplication repair. This indicates that the inhibition does not affect induction of SOS function but, most probably, other step(s) in the mutagenic process. We have shown that incubation of E.coli TK 603 uvrA cells in the presence of caffeine for the initial 30 minutes after UV-irradiation reduces the frequency of mutation by about 70 percent but has no effect on DNA replication.

REPAIR ACTIVITIES IN DEFINITE STEPS OF HEPATIC CARCINOGENESIS MODEL IN RAT

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Many models for step wise analysis of carcinogenesis have been developed. In the hepatic carcinogenesis, the Solt-Farber model protocol represents a well characterized multistep system in which foci,nodules,persistent nodules are altered cell populations which precede the tumor development. Such non cancerous cell populations display a variety of changes in many metabolic pathways. These distinctive biochemical patterns could explain in part the low conversion index of nodules into hepatomas. Since in this context the rate of DNA repair could also play a significant role,the activity of three relevant DNA repair systems was studied. 3-Methyladenine-DNA glycosylase and 7-methylguanine-DNA glycosylase catalyse base excision from DNA,whereas O^6-methyl guanine-DNA methyltransferase removes O^6-alkylguanine adducts from double stranded DNA in a stoichiometric fashion,leaving an intact guanine moiety.

We have measured these repair activities in preneoplastic hepatic nodules developed in Fischer 344 rats, 4 months after the initiation treatment of Solt-Farber model and in persistent nodules,surrounding parenchima and hepatomas developed 8 months later. Liver from rats initiator-only or promoter-only treated have been investigated too.

Concurrent experiments are in progress to account how ageing phenomena could contribute to repair modulations during carcinogenesis experiments.

CHARACTERIZATION OF 8-METHOXYPSORALEN PHOTOINDUCED DNA DAMAGE REPAIR IN *Saccharomyces cerevisiae*.

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8-methoxypsoralen(8 MOP) is a potent photosensitizing agent capable of intercalating into DNA in the dark and covalently binding to DNA when irradiated with near UV light (UVA). Since 8 MOP + UVA treatments produce both mono-and bi-adducts (cross-links) to cell DNA, the repair of such a mixture of damages seems to be a delicate and complex process, during which each type of lesion is disturbing the repair of the other one. The repair of 8 MOP + UVA induced damage in yeast requires the intervention of the three main repair pathways (excision-repair, error-prone repair and recombination repair) plus the product of the PSO genes.

In the present study we investigated 8 MOP + UVA damage repair both in haploid and in diploid strains of the yeast *Saccharomyces cerevisiae* by means of the alkaline step elution technique and we tried to follow separately the fate of monoadducts and DNA cross-links during repair.

N-123 (haploid) and D7 (diploid) yeast cells were treated with 5μM 8 MOP plus UVA light 10 kJ/m^2 and postincubated in liquid growth medium at 30°C. After different times of postincubation, cells were harvested and submitted to alkaline step elution to determine the DNA average molecular weight and the cross-linked DNA. The determination of total DNA-8 MOP photoadducts was performed by treating cells with radioactively labeled 8 MOP.

The results can be summarized as follows: 1) most of the cell DNA presented cross-linkes after 8 MOP + UVA treatment; 2) DNA was rapidly cleaved during the first 15 minutes of postincubation followed by a slow rejoining of the DNA fragments produced; 3) labelled 8 MOP amount gradually decreased from acido-insoluble radioactivity; 4) cross-linked DNA amount decrease during the whole repair period while a persistence of double-stranded, cross-linked, DNA little fragments bearing labeled 8 MOP was observed in alkaline elution patterns. These tiny acido-insoluble fragments might correspond to the cross-linked DNA excision patches while mono adducts excision probably leads to acido-soluble fragments.

THE ROLE OF THE YEAST RAD GENES IN THE REPAIR OF ALKYLATION DAMAGE TO DNA

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The RAD 3 epistasis group of Saccharomyces cerevisiae is involved in the excision repair of UV-induced pyrimidine dimers in DNA.

In this study a series of rad mutants, namely rad 1, rad 2, rad 3, rad 4, rad 10, rad 14 and rad 16 were tested for their responses to various monofunctional alkylating agents. The selected chemicals display broad reaction patterns, and, in addition, the reactive alkyl species varies from methyl through to propyl, depending on whether the agent is a methylating, ethylating or propylating agent.

Four of the seven rad mutants are sensitive to the ethylating and propylating agents which react preferentially at the O^6 of guanine ie ENNG and PNNG, but none are sensitive to methylating agents. This suggests that nucleotide excision repair acts on ethylations and propylations but not on methylations. Consequently, Saccharomyces cerevisiae may possess another repair mechanism which operates on methylation. The data are summarised in the table below.

Degrees of sensitivity to alkylating agents

Mutant	MMS	MNU	MNNG	ENNG	PNNG
a rad 1-1	0	0	0	+++	+++
a rad 2-1	0	0	0	0	0
α rad 3-2	0	0	0	+++	+++
a rad 4-4	0	0	0	+++	+++
a rad 10-1	NT	NT	NT	0	0
α rad 14-2	0	0	0	+++	+++
a rad 16-1	NT	NT	NT	0	0

MMS, Methyl methanesulphonate; MNU, N-methyl-N-nitrosourea; MNNG, N-methyl-N^1-Nitro-N-Nitrosoguanidine; ENNG, N-ethyl-N^1-Nitro-N-nitrosoguanidine; PNNG, N-propyl-N^1-Nitro-N-Nitrosoguanidine.

Index

NT = Not tested
0 = Resistant
+++ = Very sensitive

MUTAGENIC ACTIVITY OF cis-$[RuCl_2(DMSO)_4]^o$ on Escherichia coli WP2

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Mutagenic activity of chemical compounds is often evaluated by incorporating given amounts of the compound to be tested and tester bacterial strains in molten soft agar, which is spread on the surface of a selective agar plate. Ames' strains of Salmonella typhimurium are usually used as tester bacteria, since they have been proved to be highly susceptible to the mutagenic activity of a large number of compounds. However, occasionally mutagens can be met, which are not especially active on Ames' Salmonella strains in an agar incorporation assay. One of such compoumds is cis- Ruthenium-dichlorotetrakisdimethylsulfoxide , cis-$[RuCl_2(DMSO)_4]^o$, which has some biological interest as a congener of the known inorganic antitumor drug cis-$PtCl_2(NH_3)_2$ We have found that the reason, why cis-$[RuCl_2(DMSO)_4]^o$ seems to be a poor mutagen in a plate incorporation assay, lies in its interaction with inorganic phosphates, which are a major component of minimal agar medium. Furthermore, mutagenic activity of cis-$[RuCl_2(DMSO)_4]^o$ is better evident on Escherichia coli WP2 strain than on Salmonella. We have, therefore, developed a modification of the usual mutagenicity test, according to which a growing culture of WP2 strain is exposed for short periods in nutrient broth to cis-$[RuCl_2(DMSO)_4]^o$. Treated bacteria are then washed and plated for counting total survivors and revertants.

In the first hour of treatment of Escherichia coli WP2 uvrA with 2mM cis-$[RuCl_2(DMSO)_4]^o$, although bacteria stop to grow and even start to die, revertants accumulate, which are evident by plating not only on semi-enriched medium, but even on plain minimal agar. Other mutagens, as methylmethanesulfonate, cis-$PtCl_2(NH_3)_2$ or ultraviolet light, do not induce, or induce much less, revertants by this treatment. However, even cis-$[RuCl_2(DMSO)_4]^o$ treatment does not induce revertants, if bacteria are kept under strictly non-growing conditions during exposure and then plated on minimal agar medium. It seems, therefore, that even in the case of cis-$[RuCl_2(DMSO)_4]^o$ revertants are produced by some repair mechanism, which requires growth of bacteria and DNA synthesis.

REPAIR OF DNA DOUBLE-STRAND BREAKS UNDER GROWTH VERSUS NONGROWTH CONDITIONS

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In yeast cells one unrepaired radiation-induced DNA double-strand break (DSB) is lethal. The kinetics of DSB rejoining under nongrowth conditions was found to be an unsaturated process (first order reaction). Since the induction of DSB is stochastic and proportional to dose, first order repair kinetics of DSB is expected to yield exponential survival curves whose slopes decrease with increasing repair of DSB. In the dose range up to 300 Gy such exponential survival curves were found when DSB repair was allowed in cells only under nongrowth conditions. For this purpose the yeast mutant rad54-3 was used which is temperature conditional with respect to DSB rejoining. Cells of rad54-3 were held after irradiation under nongrowth conditions at the permissive temperature for DSB rejoining for various periods of time and then plated for survival test on nutrient agar and incubated at the temperature restrictive for DSB rejoining.
In contrast, whenever rad54-3 cells were allowed to rejoin DSB under growth conditions, i.e. when cells were plated on nutrient agar either immediately after irradiation or after a delay under nongrowth conditions, shouldered survival curves were observed. The reason may be that interaction between DSB resulting in misrepair is favoured under growth conditions rather than that the rejoining kinetics of DSB is saturated under growth conditions as opposed to the unsaturated rejoining kinetics under nongrowth conditions.

QUANTITATION OF N-METHYLPURINES IN SPECIFIC MAMMALIAN DNA SEQUENCES

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Simple methylating agents such as dimethyl sulfate (DMS) produce numerous types of deleterious lesions in DNA. The aberrant bases 3--methyladenine and 7-methylguanine constitute approximately 95% of the total damage to DNA. These two lesions are removed by 3-methyladenine-DNA glycosylase which cleaves the glycosidic bond, releasing the methylated base and leaving an apurinic (AP) site in the DNA. Endonucleolytic incision at the AP site followed by repair replication and ligation completes the repair process. There is increasing evidence that AP sites in DNA may be promutagenic, indicating the importance of their repair to ensure the fidelity of replication. It is, therefore, of some interest to learn whether repair of these sites differs throughout the genome.

To investigate the differential repair of methylated bases, we have developed a quantitative method for examining their removal from specific genes analogous to that devised by Bohr et al. for ultraviolet damage (Cell, 40:359 (1985)). Chinese hamster ovary cells are treated with DMS, and, after various recovery periods in the presence of bromodeoxyuridine, their DNA is purified and treated with an appropriate restriction enzyme. The parental DNA is then isolated by density gradient centrifugation. Two equal portions of each sample are heated at 50°C for 6 hr to form AP sites by removing all remaining heat-labile methylated bases. During this period, one portion from each set is heated in the presence of methoxyamine, an agent which reduces AP sites and protects them from subsequent alkaline degradation. They are then treated with 0.1 M NaOH to cleave the DNA at AP sites, and the resulting hydrolysates are subjected to electrophoresis on alkaline agarose gels. The DNA is transferred to a nylon membrane, and hybridized to a labeled probe for the fragment of interest. By calculating the ratios of the band intensities of the sample not treated with methoxyamine to its methoxyamine-treated counterpart, the percentage of the restriction fragments containing no alkaline labile sites is determined. From this information and the Poisson distribution, the frequency of N-methylpurines is calculated for assessment of their removal.

Data obtained by probing the KpnI restriction fragment of the dihydrofolate reductase gene from CHO cells indicate that it is possible to monitor the repair of methylated bases and AP sites in specific genes with this method. Currently, we are investigating the removal of N-methylpurines from specific genes in order to ascertain whether differential rates of repair occur in active genes relative to those in silent domains of the genome. (Supported by a grant from the National Institute of General Medical Sciences and a NIH postdoctoral traineeship to D.A.S..)

MISMATCH REPAIR BY XENOPUS EGG EXTRACTS

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Xenopus laevis eggs contain highly elevated levels of DNA polymerase and other replication related enzymes. When DNA containing a single base mismatch in a restriction site, and thus resistant to the restriction enzyme, is incubated with egg extracts, conversion to restriction enzyme sensitivity occurs. We show that this reaction represents an excision repair activity specific for the mismatch and not simply random strand replacement synthesis. In the presence of excess homoduplex competitor DNA the extent of the reaction is unchanged. In addition, when radiolabeled dCTP is added to the reaction, elevated incorporation in the region of the mismatch is seen. Indeed, restriction fragments that have been recovered from digestions of heteroduplex molecules that have been converted to restriction enzyme sensitivity show a 40-fold increase in specific incorporation compared to homoduplex DNA. This synthesis apparently extends through a few thousand nucleotides. The reaction occurs within 20 minutes, reaching a final level of about 300 million repair events per egg.

ENHANCED REACTIVATION AND MUTAGENESIS OF ADE 2 IN HELA CELLS

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Enhanced reactivation of a UV- irradiated mammalian virus by pre-treatment of susceptible host cells with physical or chemical agents has now been found to exist for a number of different systems. In many studies with mammalian cells an increased degree of viral mutagenesis has also been found with increasing damage to the virus, but there is still disagreement as to whether this mutagenesis is further enhanced by pretreatment of the cells.

Lately a project has been undertaken aiming to investigate what happens in the Ade2-HeLa system . HeLa cells were treated with several agents (MMS, EMS, aflatoxin B_1, sodium arsenite, mitomycin-C, heat, UV etc) in order to study enhanced reactivation of UV-irradiated Ade2. An investigation in the enhanced mutagenesis was also undertaken by measuring the induced reversion frequency of a ts Ade2 mutant.

The results have shown that the enhanced reactivation of UV-irradiated Ade2 observed in HeLa cells pre-treated with these agents is not due - at least under the used experimental conditions - to the induction of an error-prone DNA repair mechanism.

Poster Abstract : International Congress on DNA damage and Repair, 1987

Correction of cellular defects in DNA damage processing by microinjection of mRNA fractions.

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Fibroblasts from patients with the genetic diseases Xeroderma pigmentosum (XP), Fanconi's anaemia (FA) and a new chromosomal breakage syndrome (CBS) have discrete deficiencies in the processing of particular DNA lesions. These defects can be measured at the single cell level by autoradiography after incorporation of ^{3}H-thymidine subsequent to appropriate mutagenic treatments.

Since in all three cases, hybrids between deficient cells and control fibroblasts display normal damage processing, the possibility of temporarily correcting the defects by microinjection of mRNA from normal cells is given. Total cytoplasmic poly(A)+ RNA was isolated from HeLa cells and fractionated on sucrose gradients. These mRNA fractions were then microinjected into FA, XP and CBS cells prior to mutagen treatment.

The results show that XP cells (complementation group A) injected with mRNA with a length of 960 (±80) nucleotides show a normalized response to UV-light treatment, measured as unscheduled DNA synthesis. When FA fibroblasts (complementation group A) are injected with a group of mRNA fractions containing mRNAs of 400 to 1200 nucleotide lengths, they show corrected semi-conservative DNA synthesis rates after a psoralen/UVA challenge. Finally, total HeLa mRNA is able to impose a normal response to bleomycin treatment, reduction in DNA synthesis rate, on CBS cells.

Thus, individual fractions complementing each of the cell-lines can be identified and used in cDNA cloning to create partial libraries containing the correcting mRNAs at a high frequency. Use of appropriate plasmid vectors and library-partition should then lead to the identification of the correcting mRNA.

O^6-METHYLGUANINE DNA METHYLTRANSFERASE IN NORMAL AND TRISOMY 21 LYMPHOCYTES

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Alkylating agents are known to produce O^6-methylguanine (O^6MeG) in DNA. This product is believed to be a mutagenic and carcinogenic lesion due to the mispair of O^6MeG with thymidine during DNA replication leading to a GC-AT transition mutation. Both, bacterial and mammalian cells are able to repair this lesion by the action of the O^6MeG-DNA methyltransferase. The protein regenerates an intact guanine in the DNA by transferring the methyl group from O^6MeG to a cystein residue of the protein.

Trisomy 21 (Down's syndrome) is associated with an increased incidence of leukemia. Cells from these patients show also a higher frequency of chromosomal aberrations produced by X- and γ rays compared to normal cells. Furthermore, treatment of trisomic cells with chemical mutagens, such as the alkylating agents Trenimon, MNU and Busulphan induced an increase in the chromosomal sensitivity to mutagenic effect shown by the increase of sister chromatid exchanges. These findings have lead to the hypothesis of a defective DNA repair in subjects affected by Down's syndrome. In particular, it might be suggested that the increased sensitivity to alkylating agents could be ascribed to an impaired capacity in removing O^6MeG lesion from DNA. In order to test this hypothesis we have undertaken a study of the O^6MeG-DNA methyltransferase in normal and trisomy 21 lymphocytes. The enzyme was assayed by measuring the radioactivity transferred to the protein after incubation of the cell extracts with exogenous labeled alkylated DNA (a generous gift of G.P. Margison, Manchester).

Preliminary data seem to show that for both, normal individuals and subjects affected by Down's syndrome, the interindividual variation of the O^6MeG-DNA methyltransferase activity is more than 2-fold and that the activities of trisomic cells are in the same range as that of normal cells. No significant difference for sex and age was found in the two groups. Furthermore, with the present data the average activity in Down subjects does not appear significantly different from that of normal subjects. Thus, these patients seem to have the same constitutive level of the protein as normal subjects. Therefore, the problem of the increased sensitivity of trisomy 21 cells to alkylating agents remains still open. In the attempt to shed some light on this question further studies are in progress.

INDUCTION OF CHROMOSOMAL ABERRATIONS AND SCE BY VP-16 IN HUMAN LYMPFOCYTES

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It has been suggested that antitumour agents, such as m-AMSA and etoposide (VP-16) produce chromosomal aberrations (CA) and sister chromatid exchanges (SCE) by interfering with the activity of the mammalian enzyme topoisomerase II. The activity of this enzyme is known to be proliferation dependent. For instance, in unstimulated human lymphocytes there is no detectable activity, and very little is found while the lymphocytes are in the G1 phase. During the S phase the activity of the enzyme increases markedly and the peak activity occurs during the G2 phase and mitosis.

We have found that in proliferating cells, VP-16 induces CA at all stages of the cell cycle, the G2 phase being particularly sensitive. When VP-16 was given as a 2 h-treatment, the concentration required to produce 50 aberrations per 100 cells was 2 μM in the G2 phase and 20 μM in the G1 phase. Both chromatid- and chromosome-type aberrations were produced in the G1 phase. At concentrations below 20 μM, VP-16 produced mainly chromatid-type aberrations, whereas at higher concentrations chromosome-type aberrations predominated. CA were induced by VP-16 also in unstimulated (G0) lymphocytes, although the concentration had to be doubled in order to obtain the same frequency of aberrations in G0 as in G1. The effect of VP-16 in the G0 phase also differed qualitatively from that in the G1 phase. Irrespectively of the concentration used, VP-16 always produced more chromatid- than chromosome-type aberrations in G0. SCEs were induced by VP-16 with about the same efficiency in G0 as during the G1 phase.

Since the production of CA by VP-16 in G0 is not easily compatible with a mechanism involving topoisomerase II, we have considered the possibility of the G0 effect being caused by drug molecules remaining in the cells after our standard washing procedure. To explore this possibility, we have kept the VP-16-treated lymphocyte cultures in F10 medium for 2-10 h at 6, 20, and 37°C (low temperature used to eliminate the possibility of repair) before stimulation with PHA. As a result of keeping the cultures in F10, the frequency of chromatid-type aberrations (the predominant type of aberration) was reduced by 90 %, whereas the frequency of chromosome-type aberrations was little, if at all affected. The decrease in the frequency of chromatid-type aberrations was paralleled by a reduction in the frequency of SCE, and neither dependent on the temperature, nor on the time period (2 or 10 h) in F10. The results suggest that the lesions resulting in chromosome-type aberrations are likely to be induced by VP-16 during the G0 treatment, whereas those giving rise to chromatid aberrations are induced subsequent to treatment. In our opinion, the possibility has to be considered that VP-16 is capable of producing CA by two different mechanisms.

ARE METALLOTHIONEIN GENES INDUCED BY IONIZING RADIATION IN HUMAN CELLS?

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The exposure of biological material to X or γ-irradiation generates a number of highly reactive, free radical ions. Amongst these short lived species, $OH^{\cdot}$ and $O_2^{\cdot-}$ radicals may be important components of the DNA damage following ionizing radiation.

Metallothioneins are a family of short, abundant polypeptides, whose high cysteine content could provide an efficient sink for these free radical products.

Human cells contain four functional metallothionein (hMT) genes. hMT expression can be induced by a variety of agents, including heavy metals, phorbol esters, glucocorticoid hormones, γ-interferon, interleukin-1 and U.V. irradiation. We have been interested in determining whether exposure to ionizing radiation or radiomimetic drugs is capable of inducing hMT genes and whether, differences in hMT gene expression can be correlated with the sensitivities to ionizing radiation of different human cell lines.

Our poster will report on work with respect to the following aspects:

1. hMT gene induction by γ-irradiation and bleomycin in human cell lines.

2. hMT gene induction by heavy metals in human cell lines.

3. Sensitivities to heavy metals in human cell lines.

CLONING OF ADP-RIBOSYL TRANSFERASE cDNA SEQUENCES

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We have screened a λgt11 human liver cDNA library with polyclonal antibodies specifically reactive to the ADPRT enzyme protein and have isolated 6 positive clones. Judged by size of inserts and restriction mapping all these clones were identical and contained an 1.6 kb insert. This clone was identified by the following immunochemical analysis: it produced a β-galactosidase fusion protein that on Western blots was identified by the antibody which was used in the initial screening and by antibodies against β-galactosidase; the protein fragment expressed by the recombinant phage reacts with polyclonal antibodies raised against different additional highly purified preparations of ADPRT. Moreover, the fusion protein was used to affinity purify antibodies specifically reacting with ADPRT. Northern blot analysis of poly(A)$^+$ RNA of an SV40 transformed human fibroblast cell line revealed that the cDNA clone hybridized mainly to an mRNA of a size sufficient to account for the 116 kd ADPRT protein. In vitro translation of the hybrid selected mRNA is being performed.

We shall also report on the isolation of near full size cDNA sequences of ADPRT and attempts to obtain expression of such transfected sequences in mammalian cells.

VARIATIONS IN CELL KINETICS, CHROMOSOMAL RADIOSENSITIVITY AND REPAIR CAPACITY AMONG MAMMALS

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Ara-C (1-β-arabinofuranosylcytosine), an inhibitor of DNA-repair resynthesis, has been used to investigate the eventual role of the rates of incision for the repair of base damages in the interspecific differences of chromosomal radiosensitivity.

Peripheral blood lymphocytes from man, rabbit and pig have been exposed to 0 or 2 Gy of X-irradiation using a Philips RT250 machine operated at 250 kV constant potential, 20 mA, 1 mm Cu filtration and a dose rate of about 1 Gy/min. Cytosine arabinoside was administered, at a final concentration of 0.05 mM, and for increasing periods of time (1, 2 or 3 h) to the control samples and, immediately after exposure, to the irradiated ones.

Within 5 min after irradiation, the blood samples were incubated at 37°C in Ham's F10 medium supplemented with bovine serum (10%) and antibiotics, either with ara-C or in its absence to allow normal repair. At the end of the treatment, the cells were washed twice with fresh medium and reincubated in Ham's F10 culture medium in the presence of an excess of deoxycytidine (0.1 mM) to reverse the inhibition. When all the different treatments were completed, phytohaemagglutinin (PHA) was added to the cultures to stimulate the lymphocytes to divide. In order to study the cell kinetics half of the cultures received a BrdU treatment. After a culture time of 39 h, the cell cultures were arrested at the metaphase stage with 1 ml of a 0.01 mM colchicine solution for 3 h. The cells were then fixed and preparations of metaphases were made according to standard procedures.

The observations on cells treated with BrdU show that at 42 h 100% of the dividing human lymphocytes are in M1 whereas 40% of the rabbit lymphocytes and 60% of the pig lymphocytes are already in M2 or in M3.

In human lymphocytes the repair requires at least three hours but is already completed within two hours for rabbit lymphocytes. The results appear rather surprising for pig lymphocytes because addition of ara-C to culture medium does not modify apparently the yield of aberrations.

Induction of DNA Double Strand Breaks as Measured by the Neutral Filter Elution in X-irradiated Synchronous, Asynchronous and Plateau Phase Cultures of CHO-Cells.

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The induction by x-rays of DNA dsb was studied in synchronous, asynchronous and plateau-phase CHO-cells using the neutral filter elution technique (pH 9.6). Synchronous populations were obtained by selective detachment of mitotic cells and subsequent progression through the cycle for various periods of time. Aphidicolin at 1 ug/ml was used in certain experiments to resynchronize cells at the G_1/S border and nocodazole at 0.04 ug/ml to prevent division of mitotic cells if necessary. Asynchronous populations of cells were obtained by growing 1.5 X 10^6 cells in a 100 mm tissue culture dish for 24 hrs, and plateau-phase cells by growing 10^5 cells in a 60 mm tissue culture dish (3ml medium) for 4-5 days. Cells were labeled with 0.02 uCi/ml ^{14}C-TdR plus 2-5 uM TdR for at least one generation. In all experiments upwards bending dose-response curves were obtained. For a comparison of the resultls obtained the radiation dose required to give a 40% elution of the DNA from the filter (2 um porosity) after 17 hr elution at 1.5 ml/hr, was used as a parameter ($D_{0.4}$). A larger elution of DNA for the same radiation dose was observed in plateau-phase ($D_{0.4}$ = 22.5 ± 0.5 Gy) than in exponentially growing cells ($D_{0.4}$ = 28.0 ± 0.5 Gy). Cells synchronized at the G_1-phase showed elution patterns identical to those of plateau phase cells. Similar elution patterns were also obtained with cells irradiated at the G_1/S border ($D_{0.4}$ = 20 ± 1 Gy) and at mitosis ($D_{0.4}$ = 23.0 ± 0.5 Gy). As cells entered S-phase a dramatic reduction in the elution rate was observed ($D_{0.4}$ = 48 ± 1 Gy). The elution rate did not change significantly during S-phase. As cells started accumulating in G_2-phase an increase in the elution rate was observed and eventually elution rate values similar to those normally measured with cells irradiated at mitosis were obtained. These results indicate that large, usually cell survival unrelated, changes in the rate of DNA elution may occur as cells progress through the cycle. This variation in response may reflect either changes in the elution properties of the DNA or, less likely, changes in the induction rate of DNA damage by x-rays throughout the cell cycle.

Quantitation ?

This work was supported in part by NCI Grant No. RO1 CA42046 and BRSG Grant No. RR5414 awarded from NIH, DHHS.

THE K-SDS PRECIPITATION ASSAY: A SIMPLE TECHNIQUE FOR DETECTING DNA DAMAGE IN MAMMALIAN CELLS.

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Several assays, including alkali-unwinding, elution and sedimentation methods are currently used to measure DNA strand breakage in mammalian cells. All of these assays provide a sensitive measure of damage to DNA, but in all cases, specialized or expensive equipment is required, and expertise in the use of each assay must be gained. In addition, most of the assays require several hours for completion, and sample handling capacity is often limited. The simple method described below requires only a bench-top centrifuge, and a sensitive dose-response curve can be generated within a few minutes.

When mammalian cells are lysed in 2% sodium dodecyl sulphate detergent followed by addition of 0.12 M potassium chloride, a precipitate forms which can be collected by low speed centrifugation. This precipitate contains the cell protein and nucleic acid in close association with protein. In the absence of DNA damage, most of the DNA becomes tangled with the protein and precipitates, but when DNA strand breaks are created by exposing cells to ionizing radiation or toxic chemicals, DNA is released from the protein and remains in the supernatant after centrifugation. The proportion of DNA remaining in the supernatant is thus a measure of the amount of DNA damage.

This technique, called the K-SDS assay, can be applied to the study of DNA single and double-strand breaks as well as crosslinks. Comparison between results obtained on the same cell population using the alkali-unwinding method and the K-SDS assay suggests that both assays have similar sensitivity to damage by ionizing radiation, MNNG and 4NQO. However, the K-SDS assay is more sensitive than the unwinding assay to damage by bifunctional alkylating agents, and is less sensitive to damage by acridine orange and the topoisomerase inhibitor VP-16. Nitrogen mustard, formaldehyde and cis-platinum produce cross-links which are detected by a reduced sensitivity of the K-SDS assay to DNA breaks caused by ionizing radiation. In addition to allowing detection of different types of DNA lesions, the sensitivity, simplicity speed and large sample handling capacity should allow wide application of this new assay to a variety of questions concerning DNA damage and repair.

SPECIFIC ENHANCEMENT OF THE ANTITUMOUR ACTIVITY OF BLEOMYCIN AND PHLEOMYCIN BY CAFFEINE AND OTHER DNA LIGANDS

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In trials using rat and mouse models carrying rat Walker carcinoma, mouse Ehrlich Ascites, Sarcoma 180 or human tumour Xenographs, bleomycin-CHP, phleomycin-CHP, phleomycin-AAG, phleomycin-PEP, phleomycin-G and Blenoxane (a commercial mixture of bleomycins A1, A2 and B2), were administered alone at various dose rates or in combination with caffeine, and a purine analogue, N,N-dimethyl-2-(5'-pyridin-2'-yl)-1',3',4'-thiazol-2'-yl thio) ethylamine (BC151).

Both compounds considerably enhanced survival of one or more of the animal models carrying tumours. Caffeine and (BC151) in combination with phleomycin-CHP was partially effective. Toxicology studies revealed no enhancement of generalised toxic effects on normal lung, kidney, heart or liver tissue.

A number of other DNA ligands including Pyronin Y and other purine analogues had similar properties.

CIS- BUT NOT TRANS-PLATIN INCREASES THE AMOUNT OF MAJOR HISTOCOMPATIBILITY CLASS I mRNA IN CULTURED HUMAN HELA CELLS

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The class I antigens of the major histocompatibility complex (MHC) play an essential role in tumor rejection. These molecules, present at the cell surface of virtually all nucleated cells, play an essential role in the recognition of tumor cells bearing foreign antigens by cytotoxic T-cells. Suppression of class I genes increases tumorigenicity whereas derepression abrogates tumorigenicity. Although this has been established with virus transformed cells, the phenomenon might be more general as in the last few years an impressive number of primary tumors have been observed to have greatly reduced levels of class I antigens.

In this communication we have investigated the immuno-modulating properties of the antitumor and DNA damaging agent cis-diamminodichloroplatinum (II) by studying the drug's effect on the expression of MHC-class I mRNA in a model system using cultured human cervix carcinoma cells (HeLa). Whereas a 5- to 10-fold increase in class I specific mRNA was registered after treatment with cis-platin no effect was found when the isomer transplatin, which has no antitumor activity, was used. Induction of class I mRNA by cis-platin can be achieved either by treatment of cultured HeLa cells with low concentrations of the drug for a long period (several months) or with high concentrations for a short time (1 hour). Induction of class I mRNA by long term exposure to low doses of cis-platin is accompanied by DNA demethylation at the MHC locus whereas other sites are unaffected. No such demethylation is observed after short term treatment with higher doses indicating that DNA demethylation is not necessary in order to induce class I transcription. Rather, demethylation at the MHC locus might be a consequence of increased expression of class I genes.

COMPLEMENTATION OF THE EXCISION REPAIR DEFECT OF XERODERMA PIGMENTOSUM HUMAN CELL LINE BY TRANSFECTION WITH cDNA CLONE LIBRARY

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The aim of the project is to clone human DNA repair genes defective in Xeroderma Pigmentosum syndrome. For this purpose we have immortalized a Xeroderma Pigmentosum primary cell line belonging to complementation group C (XP-C). We now report the complementation by gene transfer of the UV sensitivity of the XP-C established cell line. A human cDNA clone library constructed in a mammalian expression vector, and itself incorporated in a lambda phage vector, was introduced into the cells as a calcium phosphate precipitate. Following selection to G418 resistance, provided by neo, the transformants were selected for UV resistance. Twenty-one UV resistant cell clones were obtained with resistance levels typical of normal human fibroblasts. Upon further propagation in the absence of selection for G418 resistance, about half of the transformants generated by cDNA clone library transformation remained UV resistant. However, several of the remainder retained UV resistance only under G418 selection pressure; subculturing in the absence of the drug led to a very rapid loss of UV resistance. The functional linkage between the two properties in the latter strongly suggests that at least this class of XP-C transformants acquired UV resistance through gene transfer and not by reversion. All transformants contained transduced vector DNA sequences in their nuclei. The generation of the UV resistant XP-C transformants is the first step towards cloning of a cDNA which complements the excision repair defect of the most common Xeroderma Pigmentosum genetic group.