L.1. MECHANISMS of REPAIR of ENDogenous DNA DAMAGE

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DNA lesions are continuously generated intracellularly by hydrolysis, oxidative damage, and reactive co-enzymes and metabolites. Such damage is most commonly removed by base excision-repair (BER) to attain a very low steady-state level of endogenously produced DNA base alterations. The main BER pathways have been reconstituted in vitro using purified proteins, and substrates containing base lesions in either naked DNA or chromatin. In order to evaluate physiological roles of BER enzymes in mammalian cells, several gene knockout mice have been constructed or are in progress, including animals deficient in either of the two major uracil-excising DNA glycosylases, UNG and SMUG1. Our recent work on the AlkB protein, which counteracts cytotoxic alkylation damage to DNA, has shown that the enzyme acts by a mechanism unprecedented in DNA repair, employing iron-dependent free-radical chemistry to oxidatively demethylate lesions generated in single-stranded DNA.
DNA repair enzymes efficiently excise oxidatively damaged bases from DNA, preserving the integrity of genomic DNA and attenuating the mutagenic and cytotoxic potential of such lesions. As the frequency of oxidatively damaged bases is low (1 in $10^7$ - $10^8$ bases), DNA repair enzymes must recognize their cognate lesions in a vast sea of undamaged DNA. This highly specific, multi-step process can be followed by structural analysis of species along the catalytic reaction pathway of such enzymes. We have undertaken such a study on the DNA glycosylases of the Fpg family. This family, characterized by an N-terminal homology and H2TH motif, commonly with a C-terminal zinc finger, consists of more than 40 bacterial Fpg homologs, 4 bacterial Nei homologs, and several homologs found recently in mammalian cells.

The 3D structure and catalytic mechanisms of action of Fpg and Nei (and probably their mammalian homolog) are essentially identical, yet their substrate specificities are quite different. Physiologic substrates for Fpg include 8-oxoguanine, FapyG and FapyA; Nei selectively excises thymine glycol and other oxidized pyrimidines. The mammalian Nei homolog functions as a hybrid, preferentially removing both thymine glycol and formamidopyrimidines from DNA. When embedded within DNA, these lesions, may be distinguished from unmodified bases by their external H-bonding potential, a mechanism likely to be involved in the initial recognition event. Members of the Fpg family also display a preference for the base positioned opposite the lesion. Such recognition takes place during eversion of the damaged nucleotide. These studies represent an initial effort to solve a challenging problem in DNA repair.
L.1.2. MOLECULAR MECHANISMS of UV-INDUCED MUTATIONS as REVEALED by the STUDY of HUMAN CELLS EXPRESSING DNA POLYMERASE η

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Solar UV induces lesions at bipyrimidine sites in genomic DNA. These photoproducts are normally removed or tolerated by error-free processes. However lesions that escape these error-free mechanisms can give rise to mutations. Thus cells from patients with xeroderma pigmentosum (XP), a rare autosomal, recessive human syndrome characterized by early and numerous skin lesions and cutaneous tumours on sun-exposed body sites, are hypermutable after UV-irradiation due to a defect in Nucleotide Excision Repair (NER). Cells from XP variant (XPV) patients are also UV-hypermutable even though NER is not deficient. XPV cells are deficient in DNA polymerase η which is able to carry out « error free » bypass of cyclobutane pyrimidine dimers. It is likely that in the absence of pol η in XPV cells, other recently discovered polymerases are able to bypass the DNA lesions, but with lower efficiency and lower fidelity. The high rate of misincorporation by these polymerases can explain the cancer-proneness of the XP variant patients. DNA polymerases involved in translesion synthesis in human cells appear to be constitutively expressed, in contrast to what has been found in bacteria. We have used UV-irradiated shuttle vectors to determine the mutagenic characteristics of DNA polymerases implicated in translesion synthesis in normal cells, XPV cells or XPV cells complemented with the wild type pol η gene. In normal cells, the bypass by pol η of UV-induced DNA lesions containing Cs appears to be more error prone than those containing Ts. In contrast, in XPV cells, deficient in pol η, the proportion of mutations at lesions containing Ts is increased, suggesting that pol η bypasses lesions containing Ts in a relatively error-free manner. UV-irradiation of host cells prior to transfection with unirradiated shuttle vectors increases the mutation frequency substantially in normal, XPV and corrected XPV cells to similar extents. This demonstrates that mutagenic polymerases are activated after UV-irradiation and produce mutations in undamaged DNA. The similar increase in normal and XP-V cells suggests that pol η is unlikely to be responsible for this increase.

In a separate study, we have analysed the mutation spectrum in key genes isolated from XP skin cancers, in which a high level of base substitutions was detected in ras oncogenes, p53, INK4α and PTCH tumour suppressor genes. Our analysis clearly indicates that the key mutations were generated from unrepaired UV-induced DNA lesions. These mutations are targeted at bipyrimidine sites, which constitute hotspots of DNA lesions after UV-irradiation and correspond essentially to C to T or CC to TT tandem transversions. These results correlate pretty well with data obtained with shuttle vectors replicating in human fibroblasts from normal individuals or XP patients.
L.I.3. ATM: A SENTRY at the GATE of GENOME STABILITY

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Our concept of the DNA damage response has evolved considerably since the early days of the DNA repair field. We now understand that this response comprises an intricate web of signaling pathways that spans many processes within the cell. As such, the alarm for DNA damage is conveyed to multiple downstream effectors via transducer molecules capable of simultaneous communication with them all. One of the most cytotoxic DNA lesions, the double strand break (DSB), is formed in the course of normal DNA turnover or caused by internal and external DNA damaging agents, e.g., ionizing radiation or oxygen radicals. The chief conveyer of the DSB alarm to systems throughout the cell is the ATM protein. ATM is a versatile protein kinase which is encoded by the gene mutated in the human genetic disorder ataxia-telangiectasia (A-T). A-T is characterized by cerebellar degeneration, immunodeficiency, genomic instability, radiation sensitivity and cancer predisposition. We found that a fraction of nuclear ATM adheres to the sites of damage and that its kinase activity is enhanced in response to DSBs. Activated ATM then phosphorylates a wide array of substrates that cooperatively regulate many interlocking signaling pathways involved in the damage response. Concomitantly, ATM mediates the profound alterations in gene expression patterns typical of the DSB response by controlling the action of key transcription factors. Notwithstanding its major role in the DSB response, evidence is emerging of ATM's possible involvement in other aspects of cellular homeostasis, among them the control of oxidative stress, cellular responses to growth factors and hormones, and the maintenance of the cellular cytoskeleton. ATM thus represents a sophisticated relay conduit for conveying critical cues to multiple physiological processes following the induction of DSBs and possibly other stresses as well. Our attempts to understand the ATM-dependent signaling web are based on the identification of new ATM targets, ATM-associated proteins and ATM-controlled genes, as well as on the establishment of double-knockout mice lacking Atm and other proteins. The results of this analysis will be presented.
L.II.1. YEAST ORIGINS ESTABLISH a STRAND BIAS for REPLICATIONAL MUTAGENESIS

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Genetic stability depends on the fidelity of chromosomal DNA replication. It has long been known that replication error rates vary depending on the local DNA sequence environment. This type of position effect normally operates over a distance of a few base pairs and reflects sequence-dependent differences in DNA structure and protein-DNA contacts at and near the active sites of DNA polymerases (1). Here we investigate a different type of position effect, namely whether mutagenesis resulting from replication of the yeast genome is influenced by the positions of active origins. To study this (2), a reporter gene was placed in two orientations at multiple locations within a 39,000-base-pair region of chromosome III encompassing two strong replication origins. The frequency of mutations resulting from misincorporation of dAMP opposite template 8-hydroxyguanine differed by 3- to 10-fold, depending on the gene orientation and its distance from the flanking replication origins. A reciprocal pattern of strand biases was observed for incorporation of 6-hydroxylaminopurine opposite template cytosine present in the other strand. The pattern of strand biases changes in a predictable manner upon inactivation of one or both flanking origins. The results indicate that active origins establish a mutational strand bias that is maintained over at least 70,000 base pairs. The bias results from higher mutagenesis associated with replication of the leading strand. This may reflect lower nucleotide selectivity, less efficient exonucleolytic proofreading or less efficient mismatch repair of errors made by the leading strand machinery. Experiments are underway to examine these possibilities.

Recent studies have defined in vivo roles of DNA polymerase β (β-pol) in mammalian base excision repair (BER), i.e., gap filling DNA synthesis and dRP lyase activity. By making use of mouse embryonic fibroblasts (MEFs) carrying a β-pol gene deletion, it has been proposed that un-repaired BER intermediates give rise to increased DNA damaging agent-induced cytotoxicity and genomic instability, such as chromosomal aberrations; these un-repaired BER intermediates are processed by β-pol in wild type cells and are β-pol substrates in vitro. In particular, the un-repaired gapped DNA intermediate with a 5' dRP group was proposed to cause the elevated cytotoxicity of methylmethane sulfonate (MMS) in β-pol null cells. And, experiments with cellular extracts and purified BER proteins have indicated that removal of the 5' dRP group is rate-limiting for the overall BER pathway, in the presence of excess DNA ligase. These results are consistent with a model where methylating agent cytotoxicity in MEFs is caused by the 5' dRP containing BER intermediate, rather than by the methylated DNA lesions themselves. Thus, we predicted that in order for methylated bases to become cytotoxic in MMS treated β-pol null cells, the activity of the methylated base – specific DNA glycosylase would be required. Further, this prediction implies that little spontaneous damaged-base loss occurs and that production of the BER intermediate is due mainly to DNA glycosylase mediated base removal. In the studies to be described, we created cellular systems to test this prediction. Our results indicate that DNA polymerase β protects MEFs from cytotoxicity and genotoxicity induced by products of DNA glycosylase activity in vivo.
Human mitochondrial DNA is replicated by the two-subunit DNA polymerase gamma. We investigated the fidelity of DNA replication by pol gamma, with and without exonucleolytic proofreading and the p55 accessory subunit. Pol gamma has high base substitution fidelity due to efficient base selection and exonucleolytic proofreading, but low frameshift fidelity when copying homopolymeric sequences longer than four nucleotides. We have also determined that the majority of errors produced in vivo are produced through DNA replication.

Progressive external ophthalmoplegia (PEO) is a rare disease characterized by the accumulation of large deletions in mitochondrial DNA. Recently, several mutations in the polymerase and exonuclease domains of the human pol gamma have been shown to be associated with this disease. We are analyzing the effect of these mutations on the human pol gamma enzyme. In particular, three autosomal dominant mutations occur in amino acids located in motif B of pol gamma. These residues are highly conserved among Family A DNA polymerases, which include T7 DNA polymerase and E. coli pol I. These PEO mutations have been generated in pol gamma and analyzed for polymerase function and the fidelity of DNA synthesis. One mutation in particular, Y955C, has been found in several families throughout Europe, including one Belgium family and five unrelated Italian families. The Y955C mutant pol gamma retains a wild-type catalytic rate but suffers a 45-fold decrease in apparent binding affinity for the incoming dNTP. The Y955C derivative is also much less accurate than is wild-type pol gamma, with error rates for certain mismatches elevated by 10- to 100-fold. The error prone DNA synthesis observed for the Y955C pol gamma is consistent with the accumulation of mtDNA mutations in patients with PEO. The effect of other pol gamma mutations associated with PEO will be discussed.
L.II.4. MUTATION RATES among BASE PAIRS, GENOMES, PHYLA and KINGDOMS

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Genomic mutation rates are moderately to severely invariant within certain broad groups of organisms. They are highest in riboviruses, a little lower in retroelements, and lowest in DNA-based organisms. The previous estimate for riboviruses has been refined with a new theory and by the acquisition of the first mutational spectrum for a ribovirus, a spectrum with a striking excess of double and triple mutations. The least variable mutation rates are found among DNA-based microbes, an observation reinforced by an analysis of spontaneous mutation in a hyperthermophilic archaeon. Multiple mutations arise more often than randomly in a variety organisms, sometimes in mutator mutants and sometimes not. Transient phenotypic mutator states may contribute to complex evolutionary advances and to carcinogenesis not associated with mutator mutations.
L.II.5. ROLE of DIFFERENT DNA POLYMERASES in MODULATION of DNA REPLICATION FIDELITY in E. coli

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One important question with regard to the mechanism by which mutations occur concerns the relative contribution of different DNA polymerases to the fidelity of leading and lagging strand replication. E. coli, an excellent model organism to study mechanism of mutagenesis, possesses five DNA polymerases. In contrast to Pol I, Pol III and Pol V (UmuD2'C), which physiological functions in DNA replication are well recognized, the role of Pol II and Pol IV (DinB) is poorly understood. It has been shown that even without induction of SOS regulon, E. coli cells possess ~ 50 molecules of Pol II and ~ 250 molecules of Pol IV. Whether such high intracellular concentration of these two DNA polymerases influence overall DNA replication fidelity is still an open question.

In the present study, we used chromosomal system which allows to determine the differences in fidelity between two replicating DNA strands (leading and lagging) to test the contribution of Pol II and Pol IV polymerases to chromosomal DNA replication. We used in these studies E. coli strains carrying either wild type Pol III HE or strains with impaired Pol III HE. Based on our results we propose a model describing the role of different DNA polymerases in chromosomal replication.
L.II.6. DNA REPLICATION FIDELITY in *E. coli*

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The fidelity of DNA replication is a subject of significant interest. Studies on purified DNA polymerases have revealed that they promote fidelity by at least two mechanisms: base selection and exonucleolytic proofreading. However, little is known about the fidelity of the higher-order multimeric complexes (replisomes) responsible for the simultaneous synthesis of leading and lagging strands at *in vivo* replication forks. To address this question, we have undertaken a genetic analysis of the role in fidelity, if any, of the accessory subunits of DNA polymerase III holoenzyme (HE), responsible for the replication of the *E. coli* chromosome. HE is a dimeric polymerase containing 18 subunits of which 10 are distinct. Within HE, the tau subunit (the product of the *dnaX* gene) plays a central role. Tau dimerizes the two core polymerases, it is the scaffold for the gamma clamploader complex, and it coordinates the HE with the DNA helicase that propels the fork. Our studies have revealed increased mutation rates (a mutator effect) in a particular series of *dnaX* mutants. This mutator effect is characterized by an unusual specificity: they preferentially enhance transversion base substitutions and (-1) frameshift mutations. The combined mutational data suggest that tau subunit plays an important role in controlling the fidelity of the replication complex. We have also performed *in vitro* fidelity studies with pol III HE, which have shown an unusual error specificity for HE in vitro (-1 frameshifts primarily). The combined *in vitro/in vivo* data have been used to develop a model in which the tau subunit operates within the replication fork *in vivo* as (i) a sensor for polymerase misinsertion errors and (ii) a coordinator for the controlled removal of the offending mismatch.
L.II.7. BIOCHEMICAL PROPERTIES of DNA POLYMERASE λ: IMPLICATIONS for REPAIR and MUTAGENESIS

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DNA polymerase λ is a recently identified eukaryotic DNA polymerase in the Pol X family (1). Pol λ shares 33 % sequence identity with Pol β and is predicted by modeling to have a similar fold and similar fingers, palm, thumb and 8 kDa domains (1). In contrast to Pol β, Pol λ has an additional N-terminal BRCT domain. Pol λ mRNA is highly expressed in testis, and is expressed at lower levels in all tissues tested. In addition to its template-dependent DNA polymerase activity, Pol λ has a dRP lyase activity consistent with a β-elimination mechanism (2). In reactions reconstituted with uracil-DNA glycosylase, AP endonuclease and DNA ligase, Pol λ can substitute for Pol β to repair uracil in DNA. Pol λ synthesis during primer extension on an open template is distributive, but it is processive during synthesis to fill short gaps containing a 5′-phosphate group (3). Together these properties suggest that Pol λ participates in base excision repair in vivo. Pol λ has high affinity for dNTPs, consistent with its possible participation in DNA transactions occurring at low dNTP concentrations, e.g., in quiescent cells. We have examined the fidelity of Pol λ during gap-filling synthesis in vitro using DNA substrates that allow detection of a wide range of errors. These measurements demonstrate that the error rate and error specificity of Pol λ differs significantly from that of Pol β. Thus, despite several common properties, significant sequence identity and possibly related structures, these two polymerases differ in specific interactions with the template-primer and/or the incoming dNTP that control fidelity.

L.II.8. TRANSLESION SYNTHESIS in PROKARYOTES and EUKARYOTES: a PROCESS INVOLVING MULTIPLE DNA POLYMERASES

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The biochemistry and genetics of translesion synthesis and, as a consequence, of mutagenesis has recently received much attention in view of the discovery of new DNA polymerases, most of which belong to the Y family. These distributive and low fidelity enzymes assist the progression of the high fidelity replication complex in the bypass of DNA lesions that normally hinder its progression. In *E. coli*, in addition to Pol III replicase, all three SOS-inducible DNA polymerases (Pol II, IV and V) are involved in translesion synthesis and mutagenesis. The genetic control of induced mutation pathways appears to be highly variable depending upon the nature of the lesion and its sequence context. We will show various examples of mutation pathways i) with absolute requirements for a specific combination of DNA polymerases and, ii) other examples where two DNA polymerases exhibit functional redundancy within the same pathway. The involvement of multiple DNA polymerases during lesion bypass will also be shown in *S. cerevisiae*. We suggest that cells respond to the challenge of replicating DNA templates potentially containing a large diversity of DNA lesions by using a pool of accessory DNA polymerases in various combinations to assist the high fidelity replicase.

3. Lenne-Samuel et al., EMBO Reports 3 (2002) 45;
L.II.9. STRUCTURAL INSIGHTS into the FUNCTIONS of Y-FAMILY DNA POLYMERASES

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Y-family DNA polymerases are found in all kingdoms of life. They are best characterized by their low-fidelity DNA synthesis on undamaged templates and their ability to traverse lesions in DNA that normally block higher fidelity enzymes. Interestingly, in vitro studies reveal that the Y-family polymerases often vary in their ability to bypass any given lesion. Pol eta, for example, bypasses a cis-syn thymine-thymine dimer accurately and efficiently. In contrast, pol iota, a paralog of pol eta, bypasses the same lesion inefficiently and inaccurately. However, we have recently discovered that the efficiency of pol iota-dependent lesion bypass depends upon the local template sequence context and is greatly enhanced when the 5’ template base is Adenine. Our in vitro results raise the possibility that pol iota may play a larger role in the bypass of cyclobutatane pyrimidine dimers in vivo, than previously thought.

In an attempt to understand the molecular basis for the reported differences in lesion bypass facilitated by the Y-family polymerases, we have cloned, overproduced and crystallized a thermostable Y-family DNA polymerase, Dpo4, from the archaeon Sulfolobus solfataricus. Although phylogenetically related to the DinB-like polymerases, Dpo4 shares lesion-bypass properties akin to UmuC-like and pol eta-like polymerases in its ability to bypass a variety of DNA lesions. We have recently been able to crystallize Dpo4 in a ternary complex with lesion-containing DNA and incoming nucleotide. Our progress in solving these ternary structures will be described.
L.III.1. OXIDATIVE DAMAGE to DNA: FORMATION, MEASUREMENT and DNA REPAIR SPECIFICITY

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Major progress was recently made on the delineation of mechanistic aspects of the formation of oxidative damage within the guanine moiety of DNA and model compounds as the result of one-electron abstraction, $^1$O$_2$ oxidation and OH radical reactions. It was found that 8-oxo-7,8-dihydroguanine (8-oxoGua) that is generated by either hydration of the guanine radical cation or OH addition at C8 of the imidazole ring is a preferential target for further reactions with $^1$O$_2$ and one-electron oxidants including the highly oxidizing oxyl type guanine radical. Interestingly, tandem base lesions that involve 8-oxoGua and a vicinal formylamine residue were found to be generated within DNA as the result of a single OH radical hit. The likely mechanism of formation of the latter lesions involves the transient generation of 5-(6)-hydroperoxy-6-(5)-hydroxy-5,6-dihydropyrimidyl radicals that may add to the C8 of a vicinal guanine base prior to undergo rearrangement. Another major topic which will be addressed deals with recent developments in the measurement of oxidative base damage to cellular DNA. This was mostly achieved using the accurate and highly specific HPLC method coupled with the tandem mass spectrometry detection technique. Interestingly, optimized conditions of DNA extraction and subsequent work-up allow the accurate measurement of 11 modified nucleosides and bases within cellular DNA upon exposure to oxidizing agents including UVA and ionizing radiations. Improvement in the modified comet assay that involves the use of DNA repair enzymes to reveal oxidized purine and pyrimidine bases was recently achieved using YOYO-1 as the fluorescent dye. Interestingly, formamidopyrimidine DNA N-glycosylase- and endonuclease III-sensitive sites were detected in the DNA of isolated cells upon exposure to 0.2 Gy of gamma rays. Finally, recently available data on the substrate specificity of DNA repair enzymes belonging to the base excision pathways will be briefly reviewed. For this purpose modified oligonucleotides in which cyclopurine, cyclopyrimidine and hydantoin nucleosides were site-specifically inserted were synthesized.
L.III.2. REPAIR of OXIDATIVELY DAMAGED DNA BASES in *Saccharomyces cerevisiae*: CROSSTALK between REPAIR MECHANISMS

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In *Saccharomyces cerevisiae*, three DNA N-glycosylases / AP lyases, Ntg1, Ntg2 and Ogg1, are involved in the repair of oxidatively damaged DNA bases. Ntg1 and Ntg2 are closely related to each other and to *E. coli* Nth. Ntg1 and Ntg2 display a broad substrate specificity, releasing a great number of oxidized pyrimidines such as 5-hydroxycytosine and thymine glycol in DNA. They also excise purine-derived lesions such as FapyGua and FapyAde. Ntg1 also excises 7,8-dihydro-8-oxoguanine (8-oxoG) paired with a guanine whereas Ntg2 does not. On the other hand, Ogg1 exhibits a narrower substrate specificity, catalyzing the removal of 8-oxoG and FapyGua in DNA. Ogg1 also excises 7,8-dihydro-8-oxoadenine (8-oxoA) paired with a cytosine. Finally, Ntg1, Ntg2 and Ogg1 incise DNA at AP sites via a β-elimination reaction. The biological functions of Ntg1, Ntg2 and Ogg1 have been investigated by analyzing the phenotypes of mutant strains. Our results show that a *ntg1 ntg2* double mutant is not unusually sensitive to H2O2, nor exhibits a mutator phenotype. However, a *ntg1 ntg2 rad14* triple mutant exhibits a mutator phenotype and enhanced sensitivity to the lethal effect of chemical oxidants. Therefore, oxidatively damaged pyrimidines are not only removed by BER enzymes such as Ntg1 and Ntg2 but also by nucleotide excision repair (NER). On the other hand, inactivation of Ogg1 results in a GC to TA mutator phenotype, which is probably due to 8-oxoG in DNA. Recent studies suggest that the mutagenic action of endogenous oxidative DNA damage is also counteracted by the mismatch repair pathway (MMR). The prevention of GC to TA transversion events required functional Msh2, Msh6 and Mlh1. These results suggest that MMR eliminates adenine residues incorporated opposite 8-oxoG. MMR could also remove 8-oxoG incorporated by a DNA polymerase using 8-oxo-dGTP as a precursor in the course of DNA replication. Therefore, MMR could be the functional homologue of the bacterial MutY and MutT in *S. cerevisiae*. In addition, we demonstrated that Ntg2 interacts with the mismatch repair protein Mlh1. Taken together, the results show that in *S. cerevisiae*, BER, NER and MMR are involved in the repair of oxidatively damaged DNA bases, thus, preventing their deleterious action.
L.III.3. ENDOGENOUS OXIDATIVE DNA DAMAGE and its CONSEQUENCES


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Basal steady-state levels of oxidative DNA base modifications such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG) are observed in all types of cells, and it has long been suspected that they might play an important role in the initiation of carcinogenesis. Recently, the generation of mice deficient in Ogg1 protein, the repair glycosylase that initiates the base excision of 8-oxoG, has provided a new opportunity to test this hypothesis, since the consequences of elevated steady-state levels caused by the repair defect can be studied without variation of the cellular redox state and its associated epigenetic effects. However, the Ogg1 defect alone had only mild phenotypic effects due to the presence of a back-up repair mechanism for 8-oxoG, the nature of which has not yet been established. We now have demonstrated that the Ogg1-independent repair involves the Cockayne syndrome protein Csb. Thus, the steady-state levels of 8-oxoG in the hepatocytes of $csb^{-/-}oggl^{-/-}$ double knockout mice – determined by means of an alkaline elution assay in combination with the bacterial repair glycosylase Fpg protein as a probe – were found to be severalfold higher than in $oggl^{-/-}$ mice and to increase with age. In contrast, the levels in cells from $csb^{-/-}$ mice were not different from those in wild-type mice. Accordingly, the repair of additional oxidative DNA base modifications induced by photosensitization in immortalized embryonic fibroblasts (mostly 8-oxoG) was only slightly retarded in $csb^{-/-}$ cells, more compromised in $oggl^{-/-}$ cells, but virtually absent in $csb^{-/-}oggl^{-/-}$ cells. The data indicate a role for Csb in the removal of 8-oxoG from the global genome that is independent of base excision repair by Ogg1 and cannot be explained by a mechanism limited to short transcribed sequences. The influence of the elevated oxidative DNA base damage in $csb^{-/-}oggl^{-/-}$ mice on the incidence of cancer and other diseases remains to be established.
L.III.4. OXIDATIVE DNA DAMAGE and REPAIR in NON SMALL CELL LUNG CANCER (NSCLC) PATIENTS

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Cigarette smoke, implicated in the development of 90% of lung tumors induces oxidative damage in humans. Therefore, we carried out comprehensive study concerning oxidative DNA damage in NSCLC. Following parameters were investigated; (i) level of 8-OHdG in DNA isolated from cancerous tissue and from surrounding area free of cancer as well as from leukocytes DNA of cancer patients and control group (HPLC/EC method), (ii) excretion of 8-OHdG and 8-OHG into urine of cancer patients and control group (HPLC- GC/MS – isotope dilution technique), (iii) activity of 8-OHG DNA-glycosylase (hOGG1) in cancerous tissue and in surrounding area free of cancer (control tissue).

The study was conducted in three groups. Two control groups consisted of 38 healthy non-smokers and 26 smokers and the lung cancer group comprised 46 patients. There were no significant differences in diet, antioxidant use, age, sex and weight between the control groups and the group of cancer patients.

The mean level of 8-OHdG in DNA isolated from cancerous tissues reached the value of 7.3 per 10⁶dG. It was significantly lower than in DNA of the control tissue where the mean level was 12.4 per 10⁶dG. The mean level of 8-OH dG in leukocytes DNA of cancer patients was significantly higher than this in control groups.

The mean level of 8-OHG in urine samples of cancer patients was 177 nmol/24h and was very similar to that characteristic for healthy smokers. However, 8-OHG level in non smoking subjects was 138nm/24h. This value was significantly lower than in two other groups. The mean levels of 8-OHdGuo in the control groups and in cancer patients did not significantly differ.

The level of 8-OHdG in cellular DNA significantly correlated with the activity of 8-OHG-DNA glycosylase. Our results suggest that reduced efficiency of BER (most likely reduced human OGG1 involvement) may be the factor that predispose cigarette smokers to NSCLC development.
L.III.5. ESTROGEN METABOLISM, DNA DAMAGE and CANCER RISK

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Estrogens are metabolised by a number of cytochrome P450'ies, including CYP1B1, CYP3A4 and CYP1A2. The metabolic pathways may result in products with sustained estrogenic activity and/or propensities to generate reactive oxygen species through redox cycling.

In general CYP1A2 generates metabolites, including 2-hydroxyestrone, with low risk in this aspect. CYP1A2 activity is inducible by environmental factors, including tobacco smoking, organochlorine exposure, strenuous exercise and diets rich in fried or charcoal broiled material or cruciferous vegetables. Accordingly, such factors may modify cancer risk in complicated manners. The CYP1A2 inducibility has been suggested to be polymorphic and depend on other detoxification enzymes, such as GSTM1. In a cross-sectional study the GSTM1 null genotype conferred a high CYP1A2 activity among 61 smokers. However, in a smoking cessation study with 169 subjects the decrease in CYP1A2 activity was unimodal and not dependent on the GSTM1 genotype.

Women with low CYP1A2 activity and/or a metabolic profile of estrogens favouring receptor effects and redox cycling may have an increased risk of breast cancer. However, the epidemiological studies published so far have been equivocal. We are currently conducting a case-control study involving 400 breast cancer cases appearing in a large prospective cohort study focusing on estrogen metabolites and CYP1A2 activity measured in stored urine samples as well as metabolic polymorphisms and interactions with diet.

In a case-control study with 378 subjects we have shown that low CYP1A2 activity is an independent risk factor for testicular cancer, which has also been suggested to be related to estrogen exposure at some stage during development.

In experimental studies we have shown that ethinylestradiol can induce oxidative damage to DNA in testicular cells. The effect appeared to be estrogen receptor mediated occurring at low concentrations. Estrogen receptors and CYP3A4/5 are abundant in testicular cells.

Metabolism of endogenous and exogenous estrogens may be important for the risk of a number of cancers.
L.IV.1. DNA DAMAGE and ALTERATIONS of GENE EXPRESSION in CHRONIC-DEGENERATIVE DISEASES

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Pathophysiological changes, such as ageing, and chronic degenerative diseases, such as cancer, atherosclerosis and glaucoma, recognise a variety of interacting risk factors, both of exogenous and endogenous source.

Ageing. A progressive accumulation of oxidative molecular alterations occurs lifetime in organs constituted by perennial cells. We examined 8-OH-dG, DNA adducts and multigene expression by cDNA array technology, and showed that this accumulation already begins during the early life steps, as indicated by the occurrence of an oxidative burst during the first hours after delivery. The transplacentar exposure to environmental cigarette smoke (ECS) consistently increased the formation of DNA adducts and 8-OH-dG in foetus liver before delivery. As a consequence, the gene expression profile was modified towards a decrease of cell replication rate and an increase of DNA repair. These findings demonstrate that the transplacentar exposure to ECS represents a significant risk factor for growth retardation of newborns.

Cancer. Light is a cancer risk factor having a great variety of biological effects. Exposure of hairless mice to light significantly modified the following end-points: (a) skin: DNA adducts, 8-OH-dG, thymine dimers, proliferation cell nuclear antigen (PCNA), apoptosis (TUNEL), multigene expression; (b) lung: DNA adducts; (c) bone marrow: DNA adducts, micronuclei in polychromatic erythrocytes (PCE); (d) blood: micronuclei in normochromatic erythrocytes. These results indicate that light induces a genotoxic damage not only in skin but also in distant organs.

Cigarette smoke. To evaluate the influence of the P53 status towards ECS effects, wild type (P53+/+) or transgenic mutant (P53+/-) A/J mice were exposed to ECS for 4 weeks. A slightly more consistent increase of DNA adducts (lung and heart) and PCE micronuclei was detected in P53+/- vs. P53+/+ mice. Experiments are in progress to ascertain the patterns of 8-OH-dG, DNA adduct removal, multigene expression, cell proliferation, apoptosis, and cancer development.

Glaucoma. DNA damage represents a risk factor for proliferative diseases, such as cancer and atherosclerosis, when occurring in dividing cells. It may represent a risk factor for degenerative diseases when occurring in non-dividing cell populations. Eye trabecular meshwork (TM) is a non-cycling tissue which regulates the aqueous humor outflow from the eye anterior chamber. Its alteration determines deregulation of intracocular pressure and the development of glaucoma, the main cause of irreversible blindness worldwide. We collected 45 TM specimens form glaucoma patients and 46 TM specimens from unaffected matched controls. Oxidative damage (8-OH-dG) in TM was increased in glaucoma and in subjects carrying a homozygous GSTM1 gene deletion, which was significantly more frequent in glaucoma patients than in controls. These results suggest that GSTM1 deletion represents a possible risk factor for glaucoma development and oxidative DNA damage in TM cells.
L.IV.2. OXIDATIVE DNA DAMAGE and CELL DEATH INDUCED by SELECTED ENDOGENOUSLY PRODUCED TOXICANTS

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A group of naturally occurring tetrahydroisoquinoline alkaloids have been detected in certain regions of mammalian brain. Examples are salsolinol (SAL) and tetrahydropapaveroline (THP). These endogenous isoquinoline derivatives have been implicated in the pathophysiology of Parkinsonism and chronic alcoholism. Recent studies from this laboratory have revealed that SAL in combination with cupric ion induces strand scission in pBR322 supercoiled DNA and causes apoptotic or necrotic death in cultured PC12 cells (H.-J. Kim et al., Mol. Pharmacol., 60: 440-449, 2001; Y.-J. Jung and Y.-J. Surh, Free Radical Biol. Med., 30: 1407-1417, 2001). PC12 cells treated with SAL and cupric ion exhibited higher levels of malondialdehyde and 8-oxoguanine than did control cells treated with SAL alone. SAL also induced DNA damage and chromosomal aberrations in Chinese hamster lung fibroblasts. Likewise, THP has the ability to cause DNA strand scission and apoptosis. Addition of reduced glutathione or N-acetyl-L-cysteine or overexpression of antiapoptotic Bcl-2 led to substantial protection against THP-induced PC12 cell death. THP treatment caused transient activation of ERK1/2 and p38 mitogen-activated protein (MAP) kinases and NF-kappa B, which eventually led to induction of inducible nitric oxide synthase.

Beta-Amyloid peptide has been considered to be responsible for the formation of senile plaques that accumulate in the brains of Alzheimer’s disease. Treatment of PC12 cells with beta-amyloid resulted in increased intracellular accumulation of reactive oxygen species (ROS) and caused apoptotic death and oxidative/nitrative DNA damage. Beta-Amyloid treatment also induced activation of NF-kappa B which was preceded by differential activation via phosphorylation of ERK1/2, p38, and JNK.

Catechol estrogens formed by cytochrome P450-catalyzed oxidative metabolism of 17-beta-estradiol have been proposed to undergo redox cycling to produce ROS that could cause oxidative DNA damage associated with hormonal carcinogenesis. In the present work, immortalized human breast epithelial cells treated with 2-hydroxy- or 4-hydroxyestradiol underwent apoptosis as determined by morphological features, positive in situ terminal nick end-labeling (TUNEL) and poly(ADP-ribose)polymerase cleavage.
L.IV.3.  ANTIMUTAGENESIS STUDIES USING DIFFERENT SYSTEMS

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There are several strategies against cancer considering multistep carcinogenesis processes. Strategies addressed, not only to minimize exposure to environmental carcinogens, but also in secondary and tertiary prevention. Primary prevention constitutes one of the most important aspect in the fight against tumour development. This procedure encloses a variety of end points and systems from human to prokaryotes. Information obtained are determinant in the evaluation of risk assessment and, consequently, the measure to be adopted to reduce or, defeat, cancer. Antimutagenesis studies constitute an important tool to evaluate the mechanisms and potentiality of different compounds, such as natural and synthetic products. This investigation results obtained using different systems such as mammalian cell line and strains of *Saccharomyces cerevisiae* in the study considering several microelements. In particular selenium, magnesium, vitamins A, C, E and lipoic acid were investigated. Selenium is a compound with double face in consideration of fact that used at low concentration exerts benefit effects, while, at high doses employed is toxic and mutagenic. It was indicated the dose for human use that is 50 µg/day. The same results were obtained using lipoic acid, an important compound that enters in new life style, particularly in sport activity. The study was performed in combination with other microelements in particular vitamins in order to evaluate eventual synergistic effects. Investigations were performed using strong oxidants such as hydrogen peroxide. The mechanism through which the compounds studied act, mainly is an antioxidative process.

L.IV.4. DNA REPAIR CAPACITY among SISTERS DISCORDANT for BREAST CANCER

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The mutagen sensitivity assay is one of the approaches used to investigate individual DNA repair capacity. This method is based on the premise that after in vitro treatment with a test mutagen, DNA from subjects with defective repair will be more damaged than DNA from those with an efficient repair system. However, very little is known about unmeasured processes that occur between cell treatment and final assessment of DNA damage.

To develop a more precise assay, we modified the traditional mutagen sensitivity assay to also include measurement of DNA damage after culturing cells in the absence of mutagen. First we treated apparently normal and Xeroderma Pigmentosum lymphoblastoid cell lines with various doses of benzo(apyrene diol epoxide (BPDE), and harvested cells at different time points. A polyclonal antiserum against BPDE-DNA was used to quantitate levels of adducts by immunoslot-blot and immunohistochemistry. Selected conditions included treatment with 10 μM BPDE, a 4h culture in mutagen-free medium, and immunohistochemical measurement of BPDE-DNA adducts.

The method was then applied in a pilot study to 50 lymphoblastoid lines from sisters discordant for breast cancer. There was no significant difference between cases and controls in the level of BPDE-DNA adducts in lymphoblasts harvested immediately after BPDE treatment. However, after a 4h culture in mutagen-free medium, the level of adducts was significantly higher (p=0.006) among cases than in controls. There was a two-fold increase in mean adduct removal in lines from non-affected as compared to affected sisters (44% and 22% decrease, respectively). DNA repair capacity was predictive of case status (p=0.04) in logistic regression analysis. This method, which can be easily applied to large numbers of samples should be useful in studies to investigate the role of DNA repair in cancer risk.
L.IV.5. The CONTRIBUTION of DNA ADDUCTS and RADICAL-INDUCED DNA DAMAGE to LATER BIOLOGICAL EFFECTS

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DNA damage is caused both by exposure to exogenous carcinogens and to endogenous agents. Although the extent of the latter damage, such as DNA base modification by hydroxyl radicals, is often greater than that of exogenously produced damage, the relative biological importance of the two types of damage is not understood. We are exploring this in vivo, using populations exposed to urban pollution, and in vitro, using forward mutation assays with the supF gene.

The in vivo study is an EU funded project (synonym EXPAH, partners: R Šrám, I Kalina, T Popov, S Garte, A Gabelova, A Cebulska-Wasilewska) and involves evaluation of human exposure to air pollution, with particular emphasis on PAHs, in 3 cities in Czech and Slovak Republics and in Bulgaria. Personal exposure monitors were used and biomarkers of exposure (e.g. DNA adducts), effects (e.g. chromosome aberrations by FISH) and susceptibility (e.g. genetic polymorphisms of metabolising enzymes) are being determined. Radical-induced DNA damage (8-hydroxyguanine and malondialdehyde adducts) is being measured and its relationships with exogenous damage and biomarkers of exposure, effect and susceptibility investigated. A new approach to measure 8-hydroxyguanine using immunoaffinity chromatography and LC-MS-MS has been developed.

The in vitro studies involve mutation induction in the supF gene. A plasmid containing supF was adducted with the genotoxic carcinogen benzo(a)pyrene diol epoxide (BPDE), or exposed to UVC radiation, or both, and allowed to replicate in human cells to allow for mutagenesis. After recovery, plasmid was screened for the presence of mutant supF in MBM7070 E.coli. Both UVC and BPDE increased the mutation frequency, and the combination gave a synergistic effect. Treatment with BPDE before UVC gave a 10-fold higher enhancement of mutation frequency compared to treatment with UVC before BPDE, suggesting possible photoactivation of BPDE adducts by UVC.

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Radicals generated during oxidative stress are involved in aging as well as in the pathogenesis of different degenerative diseases. Oxidative stress enhances lipid peroxidation (LPO), implicated in initiation, promotion and progression stages of carcinogenesis, in particular in chronic inflammation and infection. Oxidative stress and LPO are also entailed in the development of atherosclerosis and coronary heart disease. In the last decade it has become evident that reactive aldehydes such as trans-hydroxy-4-nonenal and malondialdehyde generated during LPO can react with nucleic acid bases to form exocyclic DNA adducts. We have used an ultrasensitive immunoaaffinity-32P-postlabelling method, to investigate formation and persistence of exocyclic etheno-DNA adducts such as 1,N⁶-ethenodeoxyadenosine (εdA) and 3,N⁴-etheno-deoxycytidine (εdC). Several human diseases with enhanced oxidative stress that show a high risk in developing into malignancy were investigated i.e., metal storage diseases (Wilson’s disease and primary hemochromatosis), ulcerative colitis, Crohn’s disease and chronic pancreatitis. In all these diseases the etheno-DNA adduct levels were found to be significantly increased in tissues with pathological manifestation of the disease when compared to the normals. Further, detection methods have been developed to measure εdA, in urine using an immuno-enriched-HPLC-fluorescence technique and in tissues using immuno-histochemistry. Using these two techniques, the role of hepatitis (viral or alcohol induced) in the formation of etheno-DNA adducts in humans were investigated. Urine samples from Thai subjects who were diagnosed with chronic hepatitis (CH), cirrhosis (CIR) or hepatocellular carcinoma (HCC) and from asymptomatic HBV carriers (NC) . When compared to NC subjects, urinary εdA levels in these patients were ~ 20-, ~90 and ~ 35- times higher for CH, CIR and HCC, respectively. Immunohistochemistry of human liver biopsies revealed increased εdA levels in fatty and cirrhotic/fibrotic liver, besides in the liver of Wilson’s disease and primary hemochromatosis patients. Taking all the data together, we have clearly demonstrated that oxidative stress/LPO induces etheno-DNA adducts in target organs and in patients suffering from diseases with a high risk to turn in malignancy. Formation of miscoding etheno-DNA adducts is one of the earliest detectable damage to the genome in the cells; if not repaired, adducts can cause mutations, disrupt genomic integrity and lead to the development cancer (Supported in part by EU grant ENV4-CT97-0505).
The etheno ring system is formed by the attack of reactive bifunctional epoxides or aldehydes at a nitrogen of the DNA base, followed by dehydration and ring closure. The ε-derivatives of purine and pyrimidine bases (e.g. ε-A, N^2ε-G, 1,N^2ε-G, and ε-C) are generated in cellular DNA by reaction with epoxides that result from the metabolism of various industrial pollutants. The highly mutagenic and genotoxic properties of ε-adducts have been established in vitro by analyzing steady-state kinetics of primer extension assays and in vivo by site-specific mutagenesis in mammalian cells.

The increasing interest in exocyclic DNA adducts has been triggered by the discovery that they can be formed by endogenous processes through the interaction of lipid peroxidation-derived aldehydes and hydroxyalkenals with DNA. ε-adducts are ubiquitous and have been found in DNA isolated from tissues of untreated rodents and human controls. However, ε-A and ε-C levels were significantly increased by cancer risk factors contributing to lipid peroxidation and oxidative stress, such as dietary ω-6 fatty acid intake, chronic infections, and inflammatory conditions. Therefore the repair processes eliminating exocyclic adducts from DNA should play a crucial role in maintaining the stability of the genetic information.

The DNA glycosylases implicated in the repair of ethenoadducts have been identified. Human and the E.coli 3-methyladenine (3-meA)-DNA-glycosylases (ANPG and AlkA proteins, respectively) excise εA residues. We have identified two homologous proteins present in human cells and E.coli that remove εC residues by a DNA glycosylase activity. The human enzyme is an activity of the mismatch-specific thymine-DNA glycosylase (hTDG) while the bacterial enzyme is an activity of the mismatch-specific uracil-DNA glycosylase (MUG), i.e., the homologue of hTDG. Recently, we have found that two structurally unrelated proteins, bacterial MUG and human ANPG, can both release 1,N^2ε-G from defined oligonucleotides containing a single modified base. A comparison of the kinetic constants of the reaction indicates that the MUG protein removes the 1,N^2ε-G lesion more efficiently (k_{cat}/K_{m} = 0.95 \text{ min}^{-1} \text{ nM}^{-1}) than the ANPG protein (k_{cat}/K_{m} = 0.1 \text{ min}^{-1} \text{ nM}^{-1}). Additionally, while the non-conserved N-terminal 80 amino acids of the ANPG protein are not required for activity on 1,N^2ε-G, hypoxanthine, or N-methylpurines, we show that they are essential for 1,N^2ε-G-glycosylase activity. Using cell-free extracts from genetically modified E. coli and murine embryonic fibroblasts lacking MUG and mANPG activity, respectively, we show that the incision of the 1,N^2εG-containing duplex oligonucleotide has an absolute requirement for MUG or ANPG. Taken together, these observations suggest a possible role for these proteins in counteracting the genotoxic effects of 1,N^2εG residues in vivo.
L.V3. ETHENO DNA ADDUCTS: PERSISTENCE and CARCINOGENESIS in DNA REPAIR DEFICIENT MICE


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Etheno DNA adducts are promutagenic lesions thought to be involved in the initiation of carcinogenesis by chemicals such as vinyl halides and urethane. Recently, the development of sensitive techniques for the measurement of these exocyclic DNA adducts has also revealed the presence of background levels of etheno adducts in genomic DNA from tissues of unexposed humans and rodents. However, the factors that modulate induced or endogenous levels of etheno adducts are still largely unknown. The availability of several mutant mouse strains deficient in specific DNA repair activities provides the means to investigate the effects of different DNA repair pathways on the formation and persistence of etheno adducts and to obtain further insight into their role in carcinogenesis. Thus, wildtype and DNA repair-deficient mice were treated with vinyl carbamate (Vcar), the proximate metabolite of urethane and the formation and persistence of 1,N6-ethenoadenine (εA) and 3,N4-ethenocytosine (εC) in DNA was measured by immunoaffinity/32P-postlabelling. A first series of experiments was carried out on APNG-/− mice, deficient in alkylpurine DNA N-glycosylase, an enzyme known to excise εA but not εC in vitro. Following Vcar treatment, εA was formed at higher levels and persisted for longer in DNA from APNG-/− mice compared to wildtype mice. No such strain difference was observed for εC. Base excision repair is thus involved in the repair of εA in vivo. As εA levels decreased after Vcar exposure even in APNG-/− mice, it is likely that there is an alternative mechanism for its removal that remains to be identified. Studies are underway to determine the contribution of nucleotide excision repair (see Abstract by Wang et al, these Proceedings). The carcinogenicity of Vcar was also compared in APNG-/− and wildtype mice. Following a single i.p. injection, the incidence of hepatocellular carcinomas was not significantly different after one year in wildtype and APNG-/− mice, suggesting that, in addition to the formation/repair of etheno DNA adducts, other factors determine tumour outcome.

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L.V.4. EXOCYCLIC DNA ADDUCTS: FORMATION, REPAIR and BIOLOGICAL EFFECTS in Drosophila

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A mandatory requirement for understanding the action of genotoxic carcinogen is information on its DNA-adduct spectra and the resulting genetic activity profile (GAP), i.e., the totality of adverse genotoxic effects the carcinogen can produce. In spite of the ubiquity of DNA etheno-bases in tissues of different species, data on genotoxic effects of ε-adduct forming chemicals in somatic cells of in vivo mammalian assays are barely available, and attempts to induce by vinyl chloride (VC1) and ethyl carbamate (EC) genetic damage in germ cells of mice were unsuccessful. Thus pertinent questions are (i) which of the numerous metabolites of vinyl halides and mechanistic analogues have genotoxic properties in vivo, and (ii) are ε-adducts involved in the induction of genotoxic damage and if so which ε-adduct(s) in specific.

The GAPs of ε-adduct forming chemicals were investigated by estimating forward mutation spectra, structural chromosome aberrations and mitotic recombination, utilizing germ cells and somatic cells of Drosophila melanogaster as in vivo targets. These genetic endpoints were correlated with adduct levels of εdA and εdC in DNA of larvae tissue measured by the immunoaffinity-32P-postlabelling method. Etheno DNA-adduct forming compounds are bifunctional by definition, and therefore formation of DNA cross-links and contribution of these adducts to mutation induction can not be excluded. However, GAP data of bifunctional agents, known to form cross-links within the DNA, revealed that etheno-adducts and not DNA cross-links are involved in mutation induction by ε-adduct forming chemicals.

L.V.5. POLYUNSATURATED FATTY ACIDS as SOURCES for ENDOGENOUS EXOCYCLIC DNA ADDUCTS

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Our studies have shown that the exocyclic 1,N\(^2\)-propanodeoxyguanosine adducts of acrolein (Acr), crotonaldehyde (Cro), and \(\alpha\)-hydroxy-2-nonenal (HNE) are endogenous DNA lesions caused by lipid peroxidation. In this study, we investigated the role of different types of fatty acids for their formation. The reactions were carried out at pH 7 and 37°C with deoxyguanosine 5'-monophosphate and \(\omega\)-3 polyunsaturated fatty acids (PUFAs); including docosahexaenoic acid (DHA), linolenic acid (LNA), and eicosapentaenoic acid (EPA); or \(\omega\)-6 PUFAs, including linoleic acid (LA) and arachidonic acid (AA), each in the presence of ferrous sulfate. The formation of Acr, Cro, and HNE-derived 1,N\(^2\)-propanodeoxyguanosine adducts (Acr-, Cro-, and HNE-dG) in the incubation mixture was determined by reversed-phase HPLC analysis. The results showed that Acr and Cro adducts are primarily derived from \(\omega\)-3 PUFAs, although Acr adducts are also formed to a lesser extent from oxidized AA and LA. HNE-dG adducts were formed exclusively from AA. The rate of Acr adduct formation was about 5- to 10-fold that of Cro adducts, depending on the type of PUFAs, and the rate of formation of HNE adducts from AA was also considerably slower than that of Acr adducts. Unlike other cyclic adducts, the formation of Acr adducts was independent of types of PUFAs, but its yield was proportional to the number of double bonds in the fatty acid. Two previously unknown cyclic adducts, one derived from pentenal and the other from heptenal, were also detected as products from \(\omega\)-3 and \(\omega\)-6 fatty acids, respectively. However, whether these adducts are formed \textit{in vivo} is not yet known. This study demonstrated the specificity for the formation of the cyclic adducts of Acr, Cro, and HNE and other related enals by oxidation of \(\omega\)-3 and \(\omega\)-6 PUFAs. Through our collaboration with M.-S. Tang at New York University and R. Roy in our institute, questions regarding the biological significance of HNE-dG adducts from \(\omega\)-6 PUFAs were addressed with respect to HNE binding site(s) in the p53 gene and the repair of HNE-dG adducts. Some of the results from these studies will be discussed.
Oxidative stress enhances lipid peroxidation (LPO), implicated in the promotion and progression stages of carcinogenesis. One of the most abundant products of LPO, trans-4-hydroxy-2-nonenal (HNE), was shown to be mutagenic and carcinogenic, however, its oxidised derivative, 2,3-epoxy-4-hydroxynonalal (EH), is much more potent mutagen and carcinogen. Our studies on M13 phage have revealed that all four DNA bases, A, C, G and T, are targets for HNE. DNA synthesis by T7 DNA polymerase is stopped at A, C, G and T sites in M13 DNA pretreated with HNE, albeit the reactivity of bases is different and follows the order G ≥ C > A ≥ T. Mutagenicity studies in M13 phage system showed increased mutation rates, among which mainly recombination events were observed, followed by base substitutions and frameshifts, the latter occurring in runs of A, C or G. Over 50% of base substitutions were C → T transitions, followed by G → C and A → C transversions. In E. coli strain recA deficient the frequency of recombination events decreased significantly, suggesting that recombination repair system is involved in removing HNE/EH adducts to DNA bases. Since at present, only reaction of HNE with dG was described in literature, we have undertaken studies on all four deoxynucleosides. The HPLC analysis of reaction mixtures showed formation of several products in each case, and reactivity follows the order dG > dC > dT ≈ dA. MS of the reaction mixtures showed peaks corresponding to HNE-dN 1:1 adducts, and in lower abundance, to 2:1 and 3:1 adducts. In dA, dC and dG reactions peaks corresponding to heptyl-substituted ε-adducts were detected, what indicates that during reaction HNE is oxidized to EH, probably by oxygen from air. The four most abundant products of the HNE-dC reaction were temporarily characterised on the basis of MS, UV and pKₐ evaluation: A is N3-substituted HNE-dC (cyclic or linear), B and C are N2-substituted HNE-dC, whereas D is dehydrated heptyl-substituted etheno-dC adduct. Thus, these long chain adducts to DNA bases arrest DNA synthesis, initiate recombination, base substitutions and frameshift mutations.

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L.VI.1. REPAIR of URACIL in DNA REQUIRES SEVERAL DIFFERENT URACIL-DNA GLYCOSYLASES


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BER is probably the most versatile and handles the largest number of DNA damage events among the different pathways, yet its functional importance remains unclear. BER is a major pathway for repair of other types of endogenous damage to DNA bases arising from oxidative stress and alkylating agents. The AP-site generated by a DNA glycosylase is itself mutagenic and toxic and is shielded by the DNA glycosylase until the next enzyme, AP-endonuclease, arrives. APE1 (HAP1). BER of uracil is completed by a short patch pathway, or a long patch pathway, requiring largely different proteins. Deficiencies in BER, recombination repair, as well as translesion synthesis (TLS) reduce cell survival after induction of AP-sites by engineered DNA glycosylases that remove Cs or Ts. This indicates that, at least in E. coli, repair of the AP-site may involve both BER, recombination and TLS (Otterlei et al., 2000).

Uracil in DNA results from deamination of cytosine to uracil, creating a premutagenic U:G mispair, or from misincorporation of dUMP instead of dTMP during replication, creating a U:A pair (Krokan et al., 2000). The inappropriate base is removed by a uracil-DNA glycosylase activity (UDG) in the first step of the base excision repair (BER) pathway to restore the correct sequence. Uracil-DNA glycosylase was discovered in Escherichia coli in 1974 in a search for activities that would repair uracil in DNA. This also represented the discovery of the BER pathway. The activity released uracil as a free base, leaving an intact abasic site in DNA. The gene for this activity, ung, is a prototype of the highly conserved family of the major UDGs present in most living organisms examined. However, mammalian cells contain at least three additional enzymes (TDG, SMUG1 and MBD4) that have the capacity to remove uracil from DNA. Nuclear UNG2 and SMUG1 prefer single-stranded DNA as substrate, but also remove U from double-stranded DNA. In contrast, TDG and MBD4 are strictly specific for double-stranded DNA, and have very low turnover numbers. Whereas UNG2 is very selective for uracil and remove related bases very slowly or not at all, SMUG1 also efficiently removes 5-hydroxymethyluracil. In addition, and like TDG, SMUG1 removes 3,N\textsuperscript{4}-ethenocytosine, although less efficiently than uracil and 5-hydroxymethyluracil. The functional roles of the different enzymes are only starting to be understood, as inferred from their properties e.g. substrate specificities and subcellular localisation, as well as from mouse models. This is discussed in the last section of the abstract.

In the S-phase PCNA and RPA co-localize with UNG2 in replication foci and interact with N-terminal sequences in UNG2. It is also present in the nucleoplasm, but is relatively excluded from nucleoli. UNG2 is upregulated in the S-phase. In contrast, SMUG1 is not present in replication foci, but found in the nucleoplasm and nucleoli, where it is more abundant, and it is not cell-cycle regulated. The selectivity of UNG-proteins for uracil is explained by the structure of the active site. The common core catalytic domain in UNG1 and UNG2 contains a conserved DNA binding groove and a tight fitting uracil-binding pocket that binds uracil when the uracil-containing nucleotide is flipped out. Flipping of uracil requires a "pinch-push-pull" mechanism, in which Ser-Pro residues compress the DNA backbone and promote flipping by destabilising the helix. The side chain of a leucine residue pushes (or at least fills the evacuated space) and several active site residues pull uracil into the catalytic pocket through different interactions. The specificity for uracil can be relaxed by single amino acid substitutions thus creating novel enzymes that release uracil and either cytosine, or thymine, depending on which residues are mutated.

It is likely that uracils in different positions relative to the replication fork are repaired by different mechanisms. Thus, deamination of cytosine leading to U:G mispairs probably occurs mainly outside of the S-phase, but must be repaired prior to the replication in order to avoid GC to AT transition mutations. Uracils that are not repaired prior to replication, or uracils resulting from deamination at the short single-stranded area at the fork represent a special problem, since a complementary and informative strand is not available in the form of a duplex. Uracils resulting from misincorporation of dUMP-residues are obviously located post-replicatively in
the newly formed strands. Which of the uracil-removing enzyme carries out the different functions? The answers are starting to become somewhat clearer. TDG is possibly a major enzyme for removal of εC, while both TDG and MBD4 may have main roles in repair of U in special sequence contexts of doublestranded DNA. UNG2 has a distinct role in post-replicative removal of misincorporated Us, as demonstrated by experiment using isolated nuclei and specific antibodies (Otterlei et al., 1999), as well as isolated nuclei from cells derived from Ung-/- knock-out mice (Nilsen et al., 2000). SMUG1 is likely to have a role in pre-replicative removal of uracil from U:G mismatches, but there is no reason from available experimental data to exclude a role for UNG2 in pre-replicative removal of U from U:G mismatches. On the contrary, U in U:G mismatches is the preferred substrate for UNG2 and is present both in replication foci and the nucleoplasm. Most likely UNG2 and SMUG1 complement each other in this important function. U present at single-stranded regions is a very good substrate for UNG2, and also for SMUG1, but only UNG2 accumulates in replication foci. Probably UNG2 removes uracils at the single-stranded part of the fork, leaving a cytotoxic AP-site that stalls replication of this strand and attracts repair and recombination factors. Since APE1 is highly double-strand-specific, the risk of creating a strand break is very low. AP-sites at the replication fork are most likely repaired either by BER subsequent to fork regression, or by recombination using information from the sister chromatid or by TLS. The significance of the UNG-gene is also underlined by the approximately 15-fold increase in spontaneously occurring lymphomas in Ung-/- knock-out mice (unpublished results). To our knowledge this is the first experimental evidence for the functional significance of DNA glycosylases in spontaneously occurring cancer.

Free radicals such as hydroxyl radical react with DNA to give multiple final products from each of the DNA bases and the sugar moiety by a variety of mechanisms. Numerous DNA lesions were identified in living cells exposed to free radical-generating systems. There are repair mechanisms in living cells to repair DNA lesions. Modified DNA bases are mainly repaired by base-excision repair pathway. DNA glycosylases are involved in the first step of this type of repair and remove modified bases from DNA. With the use of the technique of gas chromatography/mass spectrometry, substrate specificities and excision kinetics of numerous DNA glycosylases have been determined. This technique permits the concurrent measurement of the products of all four DNA bases in a given DNA sample. Thus, it enables the determination of substrate specificities of DNA glycosylases by identifying which modified bases are excised or not excised from damaged DNA containing multiple lesions. The use of damaged DNA instead of an oligonucleotide with a single modified base permits a comparison of the substrate specificity and excision kinetics of a DNA glycosylase for a multitude of modified bases under identical conditions. This concept also facilitates a quantitative comparison of different DNA glycosylases. The studies showed that some DNA glycosylases are specific for pyrimidine-derived lesions, whereas others excise purine-derived lesions. There are also repair enzymes with cross reactivity excising both pyrimidine- and purine-derived lesions. Excision kinetics depends on the enzyme and the nature of DNA substrates. Single mutations in genes that encode DNA glycosylases significantly alter the substrate specificities. Prokaryotic enzymes appear to possess broader substrate specificities than eukaryotic enzymes, indicating that additional repair enzymes might exist in eukaryotes yet to be discovered.
L.VI.3. PATHWAYS for THE REPAIR of OXIDATION and ALKYLATION DAMAGE to DNA

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Whereas general mechanisms exist for the repair of DNA damage at large, specialised systems have evolved for the removal of smaller but frequent lesions induced by oxygen radical species and alkylating agents. In the case of oxidation damage, the dominant repair pathway is base excision repair, which is initiated by DNA glycosylases with different specificity against different types of modified base residues. In *E. coli*, typical enzymes are Nth, Fpg/MutM and Nei, which are responsible for removal of oxidized pyrimidines (e.g. thymine glycol), oxidized purines (e.g. 8-oxoguanine) and oxidized pyrimidines/purines, respectively. Nei appears to function as backup for Nth with prime affinity for oxidized pyrimidines, however, a small but significant activity against 8-oxoguanine has also been reported. In mammalian cells, functional counterparts to Nth and Fpg are hNTH1 and hOGG1, which both belong to the helix-hairpin helix superfamily of DNA glycosylases. Fpg and Nei are structurally different and this class of enzymes was until recently thought to be confined to bacterial and plant species. However, recent entries in the EST and genomic databases have revealed the presence of three human homologues; termed hFPG1/2/3 (by us) or hNEH1/2/3 (by Sankar Mitra/Susan Wallace) due to its functional similarity to Nei. We have been investigating hFPG1/2 and shown that both are DNA glycosylases that will remove formamidopyrimidines from DNA implying that there are at least 4 enzymes for repair of this lesion in mammalian cells (Morland et al.). Human FPG1/NEH1 also removes 5-ohC with high efficiency and 8-oxoG with lower efficiency. The 8-oxoG activity is abolished by the presence of A in the opposite strand and hFPG1 thus have properties as required for a backup involved in 8-oxoG repair, although the catalytic efficiency compared to hOGG1 is 10-fold lower. The intracellular localisation of hFPG1 is similar to that observed for hOGG1 with accumulation in the nucleoli during interphase and association to condensed chromatin during mitosis (Luisa Luna, Francoise Dantzer et al.). In contrast, hFPG2 co-localises with RPA and to the replication foci during S-phase. The presence of the Fpg/Nei homologues in mammalian cells probably explains the marginal phenotype associated with knock-out mice defective in mOGG1 or mNTH1.

Base excision repair is also an important mechanism in the repair of methylation damage to DNA and is essential for removal of 3-methyladenine. However, other pathways are also involved such as the methyltransferase catalysed direct removal of methyl groups from O-methylated guanine and thymine. Yet another gene involved in alkylation repair is AlkB, which has several counterparts in mammalian cells. We have now elucidated the function of AlkB in *E. coli* (Pål Falnes et al.) and homologous gene products in mammalian cells (in collaboration with the group of Hans Krokan) and shown that these proteins are involved in a novel repair pathway different from other repair mechanisms previously described.
L.VI.4. STRUCTURAL DETERMINANTS in NUCLEASE SPECIFICITY of HUMAN EXOIII-LIKE PROTEINS: APE1 and APE2


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Abasic sites and non-conventional 3’-ends, e.g. 3’-oxidized fragments (including 3’-phosphate groups) and 3’-mismatched nucleotides, arise at a significant frequency in the genome, due to spontaneous decay, oxidation or replication errors. To avert the potentially mutagenic or cytotoxic effects of such chromosome modifications/intermediates, organisms are equipped with apurinic/apyrimidinic (AP) endonucleases and 3’-nucleases which initiate repair. Ape1, which shares homology with Escherichia coli exonuclease III (ExoIII), is the major abasic endonuclease in mammals and an important, and selective contributor to 3’-end processing. Mammals also possess a second protein with sequence homology to ExoIII, Ape2, but this protein exhibits comparatively weak AP site-specific and 3’-nuclease activities [1].

Prompted by homology modeling studies, we found that substitutions in the hydrophobic pocket of Ape1 (comprised of F266, W280 and L282) reduce abasic incision potency, while introduction of an ExoIII-like pocket into Ape2 enhances its AP endonuclease function [2]. We have also demonstrated that mutations at F266 and W280 of Ape1 increase 3’ to 5’ DNA exonuclease activity. These results, coupled with comparative sequence analysis, permitted ascertaining the active site protein elements that influence the substrate specificity of a diverse set of sequence-related proteins possessing the conserved four-layered α/β-sandwich fold [3]. Absolute conservation of the catalytic residues among these proteins suggests a common, conserved mechanism for cleaving phosphoester bonds, embracing vastly different substrates, including phospholipids, nucleic acids and polypeptides. Finally, we found that wild-type Ape1 excises 3’-mismatched nucleotides at a much higher rate than correctly base-paired nucleotides, depending largely on the structure and sequence of the DNA substrate, suggesting its novel, selective role for the human.

Poly(ADP-ribose) polymerase (PARP-1) is an abundant nuclear protein with a high affinity for single- and double-strand DNA breaks. PARP-knockout cells are extremely sensitive to alkylating agents, suggesting a role for PARP-1 in base excision repair (BER). However, since there is no known enzymatic activity for this protein directly involved in DNA damage processing, the role of this protein in DNA repair remains controversial. We have recently developed an in vitro DNA repair assay that allows monitoring BER at nucleotide resolution. We use this assay to address the involvement of PARP-1 in individual BER pathways and the effect of PARP-deficiency on the BER process. We find that after cleavage of an AP site by AP endonuclease, the formation of a PARP-nicked DNA complex is an integral part of the BER process. In the presence of NAD⁺ this complex readily dissociates and is replaced by repair enzymes, which complete repair. Both short- and long-patch BER are NAD⁺ dependent. “Freezing” of the PARP-DNA complex by removal of NAD⁺ resulted in a 5-10-fold decrease of repair efficiency. We also find that in the PARP-deficient cell extracts both short- and long-patch BER pathways efficiently operate in an NAD⁺ independent manner, suggesting that PARP-1 is not required for DNA synthesis, flap removal or ligation steps of BER. Our observation that PARP-1 is not essential for BER but is always involved in the repair process supports the suggestion that PARP-1 may be a part of a DNA damage signal transduction pathway reporting on the initiation of BER.
L.VI.6. BASE EXCISION REPAIR of OXIDATIVE DNA DAMAGE in MAMMALIAN CELLS

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In all organisms the mutagenic effects of 7, 8-dihydro-8-oxoguanine (8oxoG) lesions, caused by DNA oxidation, are prevented by base excision repair (BER). This repair mechanism is active on 8oxoG/C pairs by a mechanism initiated by the bifunctional DNA glycosylase hOGG1. Replication of unrepaired 8oxoG generate 8oxoG/A mismatches which are also a substrate for BER via a process initiated by the monofunctional DNA glycosylase hMYH. The mechanisms of repair of 8oxoG/C and 8oxoG/A pairs constructed in plasmid genomes were characterized by using human purified enzymes and human cells extracts respectively. We show that efficient and complete repair of 8oxoG/C pairs requires hOGG1, the AP-endonuclease HAP1, DNA polymerase (Pol) β and DNA ligase I. After glycosylase base removal, repair occurred through the AP lyase step of hOGG1 followed by 3’dRP removal by the 3’diesterase activity of HAP1 and precise replacement of a single nucleotide by Pol β. When the proteins involved in long-patch BER like PCNA and FEN1 were added to the repair reaction, the patch of resynthesis was strongly dependent on the presence of DNA ligase I. The assembly of all the core proteins for 8oxoG repair is required to catalyse one major pathway that involves single nucleotide repair patches.

The analysis of repair of 8oxoG/A mismatches by human cell extracts revealed that BER events occur on both DNA strands of the mismatch. At early repair times the specificity of nucleotide incorporation indicated a preferential insertion of C opposite 8oxoG leading to the formation of 8oxoG/C pairs. This was followed by repair synthesis on the opposite DNA strand that is consistent with hOGG1-mediated correction of 8oxoG/C to G/C. Repair synthesis on either strand was completely inhibited by aphidicolin suggesting that a replicative DNA polymerase is involved in the gap filling. This is the first demonstration that repair of 8oxoG/A base pairs is by two BER events likely mediated by Pol δ/ε. We suggest that the Pol δ/ε-mediated BER is the general mode of repair when BER lesions are formed at replication forks.
Base excision repair is thought to be the principal route of repair for oxidant-derived DNA damage in mammalian cells. Together, the mammalian DNA N-glycosylase / lyases Ogg1 and Nth1 remove a wide range of oxidatively damaged purines and pyrimidines from cellular DNA. However, despite the known toxic and mutagenic properties of many of these adducts, mice lacking both Ogg1 and Nth1 are viable and show no clear deleterious effects of the gene disruption. To investigate the biochemical basis behind this apparent lack of phenotype, we have tested cell-free extracts from these mice for their ability to incise a number of monosubstituted oligonucleotides containing different oxidised bases. Interestingly, initial results suggest significant differences in the ability of extracts from different tissues to excise the various modified bases. Thus, while all [Nth1,Ogg1]-/- extracts lack any measurable activity against thymine glycols, significant incision of a 5-hydroxycytosine containing oligonucleotide was observed for some of the extracts. Results obtained from comet assays performed on H2O2-treated murine embryonic fibroblasts (mefS) prepared from Nth1-/- mice are also highly suggestive of an alternative pathway for the repair of oxidative base damage. Consequently, under alkaline conditions more DNA was found in the comet tail at longer times in Nth1-/- cells than in Nth1+/+ cells, indicating either the persistence of enzyme induced abasic sites or DNA strand breaks in the null cells. Studies in vivo have also proved illuminating. Initial experiments using a split low dose (2 x 0.1 Gy) of ionising radiation indicate that the null animals are more resistant to apoptosis in the gut crypt than normal littermates. This indicates that under conditions of chronic oxidative stress, actively dividing cells are normally prone to apoptosis. However, should the DNA repair pathways become compromised, (e.g. during chronic inflammation) cells may escape apoptosis and deleterious genetic changes become fixed, leading to a disease phenotype. The nature of the compensatory pathways and the long-term biological consequences of a reduction in apoptosis remain to be elucidated and are the focus of our work.
L.VL8. RELATIONSHIP between EXOCYCLIC ADDUCT STRUCTURE and REPAIR RECOGNITION/EFFICIENCY

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Exocyclic adducts are formed by metabolites of a group of carcinogens including vinyl halides, bis-chloroethyl nitrosourea, glycidol ethers and mucochloric acid. The products of reaction are cyclic adducts of dA, dC and dG which contain one or two extra rings. Adducts with an extra 5-membered ring (etheno, ethano, hydroxymethyl-etheno) are excised through DNA glycosylase action while p-benzoquinone (pBQ) adducts with two extra rings are repaired by DNA endonucleases. Purine and pyrimidine 5-membered exocyclic adducts are repaired by different glycosylases while all three pBQ adducts are repaired by the AP endonucleases. Etheno C and etheno A which have the same modification are repaired by different repair enzymes, while diverse structures (pBQ adducts vs. an AP site) are repaired by the same enzyme.
L.VII.1. DNA REPAIR and its INTERPLAY with TRANSCRIPTION

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Nucleotide excision repair (NER) is a versatile and highly conserved repair system capable of removing a wide range of DNA helix distorting lesions. Detailed analysis of human cells defective in NER have uncovered three unexpected links with transcription: (i) active genes are repaired much faster than non-transcribed parts of the genome, (ii) the basal transcription factor TFIIH is required for transcription initiation by RNA polymerase II and involved in NER and (iii) upon induction of DNA damage transcription is transiently repressed, suggesting tight coordination between repair and transcription regulation. The repression of transcription by DNA damage is likely to be a consequence of blockage of transcription elongation and/or initiation. The mechanism of UV induced transcription repression and repair was further examined by in vitro transcription, by chromatin immuno-precipitation and by a technique that allows to irradiate only part of a cell nucleus. Taken together the results demonstrate that (i) repair and transcription factors are recruited to sites of damage in active and inactive sequences,(ii) that inhibition of transcription after UV-irradiation is at least partially due to repression of transcription initiation via a reduction of hypophosphorylated RNA Polymerase and (iii) that inhibition of transcription by trans effects is limited to short distance. Moreover, our findings suggest that NER is mediated by sequential assemblage of repair protein complexes at the site of the DNA lesions rather than by the action of a preassembled repairosome.
L.VII.2. A ROLE OF THE HAMSTER ERCC3/XPB PROTEIN IN THE NUCLEOTIDE EXCISION REPAIR AND TRANSCRIPTION

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Mutation of the XPB gene in humans gives rise to the distinct, autosomal recessive disorder, with a striking clinical heterogeneity: xeroderma pigmentosum associated with Cockayne's syndrome (XP/CS) and trichothiodystrophy (TTD). XPB is a subunit of a multifunctional RNA polymerase II general initiation factor TFIH and codes for 3'→5' DNA helicase essential for both nucleotide excision repair (NER) and transcription. In NER, it unwinds DNA around the damage by about 25-30 base pairs forming open complex. In transcription, XPB functions at multiple steps to promote efficient initiation and promoter escape by RNA polymerase II. There have been only five XP-B patients with three naturally occurring mutations identified so far. The cells from these patients exhibit different levels of DNA repair and transcription. Since XPB defective human disease is extremely rare, Chinese hamster (CHO) mutant cell lines belonging to the 3rd rodent complementation group (CG3; the hamster ERCC3 gene is the homologue of the human XPB gene) are a unique resource for analyzing structure-function relationships in the ERCC3/XPB protein. There are nine CHO mutant cell lines assigned to CG3 that have never been characterized.

We have amplified (by 5' and 3' RACE PCR), cloned (into T/A-type cloning vector pCRII) and sequenced the ERCC3 genes from wild type and 2 CHO mutant cell lines and identified the sites of the respective mutations: i) A1075G transition (K359E), located between the helicase domains I and Ia, causes deficiency in open complex formation and in 3', 5' and dual incisions during NER; ii) C2215T transition (Q739STOP) causes the truncation of the C-terminus of the protein, responsible for the 5' incision, by 44 amino acids. The effects of the above mentioned mutations on cell survival, RNA synthesis recovery and DNA repair capacity of the hamster ERCC3 mutant cell lines after UV irradiation will be discussed.

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L.VII.3. DUAL ROLE of the Ku70/80 AUTOANTIGEN in HUMAN CELLS

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Ku70/80 heterodimer that belongs to the DNA-dependent protein kinase (DNA-PK) is expressed mostly in the cell nucleus. The Ku complex binds to DNA double-stranded breaks and recruits the catalytic sub-unit DNA-PKcs (p460 protein). The minimal set of proteins acting in repair of DNA double strand breaks by the non homologous end-joining pathway (NHEJ) requires in addition to DNA-PK, DNA-ligase IV and XRCC4 proteins. By monitoring protein assembly from human nuclear cell extracts on DNA ends in vitro, we show that recruitment to DNA ends of the XRCC4-ligase IV complex responsible for the key ligation step is strictly dependent on the assembly of both the Ku and p460 components of DNA-PK to these ends. Based on coimmunoprecipitation experiments, we conclude that interactions of Ku and p460 with components of the XRCC4-ligase IV complex are mainly DNA-dependent. In addition, under p460 kinase permissive conditions, XRCC4 is detected at DNA ends in a phosphorylated form. This phosphorylation is DNA-PK dependent. However, phosphorylation is dispensable for XRCC4-ligase IV loading to DNA ends since stable DNA-PK/XRCC4-ligase IV/ DNA complexes are recovered in the presence of the kinase inhibitor wortmannin. These results substantiate the hypothesis of a scaffolding role of DNA-PK towards other components of the NHEJ DNA repair process.

Ku70/80 heterodimer is also expressed in the cytoplasm and at the plasmique membrane. By looking for the Ku partners we found using the double-hybrid methodology an interaction with a metalloprotease that degrades the extracellular matrix. Ku may play a role of anchorage protein in order to increase the metalloprotease efficacy.

In conclusion Ku70/80 heterodimer can play a dual role, one as a major protein in the mechanism of double-strand breaks repair induced by ionising radiations, the other as a partner of a collagenase that plays a role in the progression of surviving tumoral cells to the ionising radiation exposure.
DNA non-homologous end-joining (NHEJ) is the major mechanism for the repair of DNA double strand breaks in mammalian cells. Five proteins have been identified that function directly in NHEJ; namely the two subunits of Ku (Ku80 and Ku70), the DNA-dependent protein kinase catalytic subunit, Xrcc4, and DNA ligase IV. Ku has double stranded (ds) DNA end-binding activity and, via an ability to translocate along the DNA molecule, multiple Ku molecules can bind to a single DNA substrate. DNA ligase IV and Xrcc4 form a complex termed LX. LX has previously been reported to be a poor ds ligase. We show that, in fact, LX is a good ds ligase but its activity is critically dependent upon the substrate length with very short substrates being inefficient for ds ligation. Ku can stimulate LX ds ligation activity provided only few (1-2) Ku molecules are bound to the DNA substrate. There is a strong inhibition of ds ligation when multiple Ku molecules are bound to the DNA substrate. Evidence will be presented suggesting that Ku binds to DNA ends, helps to recruit the LX complex and translocates inwards to provide free DNA ends for LX ligation. LX and Ku act additively to protect DNA ends from nucleolytic degradation.

Two approaches have been taken to investigate the contribution of defects in NHEJ to human disease. A homozygous mutation in DNA ligase IV was identified in a leukaemia patient who dramatically over-responded to radiotherapy. This patient was clinically normal until the onset of leukaemia at age 14. Several additional patients with hypomorphic mutations in DNA ligase IV have also recently been identified. These patients display immunodeficiency, developmental and growth delay, features which resemble those present in Nijmegen Breakage Syndrome (NBS). The disorder has been called LIG4 syndrome. The genotype/phenotype relationship of these patients and the cell lines established from them will be discussed and compared to NBS.
L.VII.5. ROLE of HOMOLOGOUS RECOMBINATION in PROVIDING CHROMOSOMAL STABILITY and PROTECTION against DNA CROSS-LINKS in MAMMALIAN CELLS

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Homologous recombination (HR) plays an essential role in the maintenance of genome integrity and in the cellular response to DNA damage. Recently, compelling evidence suggest that HR also plays a role in processing DNA interstrand cross-links (ICLs) that are introduced by commonly used anti-cancer drugs such as cisplatin or mitomycin C. The existence of multiple complementation groups in MMC-sensitive human disorders and rodent cell mutants indicates complexity of the mechanisms involved. Rad51 plays a key role in HR and displays a highly dynamic distribution in nuclear foci following DNA damage. Rad51 interacts with many proteins, including the proteins encoded by the breast cancer susceptibility genes, \textit{BRCA1} and \textit{BRCA2}. Following DNA damage, Rad51 forms nuclear foci in which Xrcc2, Xrcc3, Rad51B, Rad51C, Rad51D, Rad52 and Rad54 are also involved. Recently, we identified two MMC-sensitive hamster cell mutants that are impaired in Rad51 foci formation in response to DNA damage. We found that one is defective in Brca2 and the other in Rad51C. The phenotype of these mutants indicates that both proteins function in DNA-cross-link resistance and genomic stability, however Rad51C is also involved in sister chromatid cohesion. Like the MMC-sensitive hamster cell mutants, cells derived from Fanconi anemia (FA) patients are characterized by chromosomal instability and a specific hypersensitivity to ICLs. Therefore, we also examined Rad51 foci formation in response to DNA damage in all complementation groups of FA. Clearly, only fibroblasts derived from FA complementation group D1 are impaired in this process. FA is an autosomal recessive disorder characterized by progressive bone marrow failure, various congenital malformations and marked predisposition to malignancies. Despite cloning of six FA genes, this pathway remains mysterious. Thus, our observations imply, that unlike all other FA proteins known to date, the product of the \textit{FANCD1} gene is involved in the assembly and/or stabilization of the Rad51 protein complex, suggesting that FANCD1 is likely to be involved in HR-dependent ICL repair. Our results underline the role of HR in the protection of mammalian cells against ICLs and in maintaining chromosome integrity by controlling several different processes, such as mutagenesis, cell cycle progression, centrosomes and sister chromatid cohesion.
L.VII.6. **MOLECULAR DEFECTS in AGING: STUDIES on the MOLECULAR BIOCHEMISTRY of PREMATURE AGING PROTEINS**

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Patients suffering from the premature aging syndromes appear much older than their chronological age. Further understanding of the molecular defects in those conditions leads to important insight into the aging process. The diseases of premature aging include Werners syndrome and Cockayne syndrome. The genes that are mutated in these disorders have been identified and studies of the gene products are done at the cellular and molecular level. We are studying the cellular defects and molecular biochemistry of the Werner and Cockaynes proteins with a special focus on the protein interactions involved. Insight into the protein interactions lead to insight into the molecular pathways that are involved and that are then defective in aging.
MODELLING the CONSEQUENCES of mtDNA DEFECTS for the AGING PROCESS

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Mitochondria are not only the main source of metabolic energy for most eukaryotic cells, but also the main source of free radicals. These reactive molecules can damage all components of a cell such as membranes, proteins and DNA. Therefore they have long been suspected to be involved in the biological aging process. The fact that mitochondria possess their own genetic material (mtDNA) and that they only have a reduced set of DNA repair processes makes them one of the prime targets for reactive oxygen species. The idea that genetically damaged mitochondria accumulate with time and are causally responsible for the aging phenotype via a disturbed energy budget is at the core of the so called mitochondrial theory of aging. In recent years this idea has gained impetus from the discovery of mitochondrial diseases and mtDNA deletions in old organisms. However, there are still many open questions regarding the mechanism of the accumulation of these deletions and their physiological relevance. In my presentation I will discuss the results of computer simulations, which show that the cell division rate and the speed of the mitochondrial turnover are the most important factors controlling the fate of a mitochondrial population. Furthermore, the relevance of these findings with respect to recent experimental observations will be examined.
L.VII.8. IMPAIRED DNA REPAIR CAPACITY and its EFFECT on CANCER SUSCEPTIBILITY in HUMANS

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Individual repair rates of DNA damage can be a crucial determinant of cancer susceptibility. In order to measure individual cellular DNA repair capacity in human studies, we used the alkaline comet assay as phenotypical repair test and bleomycin as a mutagen. We have screened peripheral blood lymphocytes in different cancer case-control studies of which one included 160 non-small cell lung cancer patients and 180 controls. After adjustment for age, gender and smoking status, we observed a statistically significant >2fold increase in cancer risk for individuals with a reduced DNA repair capacity (Int. J. Cancer 95:86, 2001). In order to identify the impaired DNA repair enzymes, the phenotypic repair capacity is compared with data generated either by the use of cDNA arrays to analyse expression profiles of DNA repair genes (DNA Repair 1:237, 2002), or by the analysis of DNA polymorphisms in DNA repair genes for which an association with an increased lung cancer risk has been reported. So far, 284 lung cancer patients and controls have been analysed for variants in XRCC1, XRCC3 and XPD. Our results indicate that a combination of sequence variations in these genes may effect both cellular DNA repair capacity and lung cancer risk. A further approach to identify repair genes involved in increased cancer susceptibility is to analyse the activity of single repair enzymes. As the individual repair capacity in our studies is determined by the alkaline comet assay, and this assay measures primarily DNA strand breaks and alkaline labile sites, we studied the activity of poly(ADP-ribose)polymerase, an enzyme involved in the repair of these lesions in a population-based case-control study (Int. J. Cancer 98:780, 2002): Peripheral blood lymphocytes from laryngeal cancer patients (n=69) showed significantly (p=0.01) less bleomycin-induced polymer formation than cells from healthy controls (n=125). In conclusion, our data provide further evidence that individuals with an impaired DNA repair capacity are at increased cancer risk, and that assays are becoming available which could identify those subjects.

In collaboration with Thoraxklinik Heidelberg-Rohrbach, Dept of Otolaryngology/University of Heidelberg, and Axaron Bioscience AG, Heidelberg
L.VII.9. \( \textit{O}^6 \)-ALKYLGUANINE-DNA ALKYLTRANSFERASE INACTIVATION: LAB to CLINIC

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Antitumour agents such as the DNA methylating drug Temozolomide (Tz) mediate their cytotoxicity mainly through reaction at the \( \textit{O}^6 \)-position of guanine in DNA. The DNA repair protein, \( \textit{O}^6 \)-alkylguanine-DNA alkyltransferase (ATase/MGMT) is an important mechanism of tumour resistance to such damage. Strategies to improve tumour response include attenuating ATase activity using pseudosubstrates. One such agent, \( \textit{O}^6 \)-(4-bromothenyl)guanine (PaTrin-2), is a highly effective ATase inactivator in vitro. In human tumour xenograft models, PaTrin-2 can increase the sensitivity of Tz-responsive tumours and reverse the resistance of Tz-resistant tumours. A Phase I clinical trial of PaTrin-2 has been undertaken to assess safety, bioavailability and pharmacokinetics of a five day schedule of intravenous and/or oral PaTrin-2 in combination with Tz. ATase activity was in most cases completely ablated in peripheral blood mononuclear cells and in tumour biopsies following PaTrin-2 administration by both routes. The myelosuppressive effect of Tz was increased by PaTrin-2, requiring a dose reduction of Tz. Additional strategies to protect bone marrow stem cells could allow dose intensification of Tz. These studies have established a regimen of oral PaTrin-2 and Tz suitable for Phase II evaluation.

Supported by Cancer Research UK.
L.VIII.1. The ROLE of 20s PROTEASOME in DNA REPAIR of *S. cerevisiae*

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26S proteasome is multi-protein molecular machinery designed for controlled proteolysis and is required for turn over of majority of proteins in eukaryotic cells. The role of proteasomal activity in regulation of fundamental cellular processes like cell cycle progression, differentiation, immunological or heat shock response is well documented, in much more cellular processes the role is postulated (Hershko and Ciechanover, 1998). Proteasomal activity is involved in etiology of some human diseases like Cystic Fibrosis and, on the other hand, this activity seems to be a good target for anticancer drugs, there is developed a whole line of anticancer drugs, of new generation, which are proteasome inhibitors like LPD-341. Therefore, the investigation of consequences of proteasome dysfunction to the genetic stability seems to be important.

With the use of *S. cerevisiae* as a model, we have analyzed the DNA repair phenotypes of cells carrying mutations in genes affecting the activity of 20S proteolytical core of yeast proteasome. We have previously shown that the activity of Ump1, the 20S proteasome maturase, is required for the normal resistance of yeast cells to UV radiation (Mieczkowski et al. 2000). On the basis of epistatic analysis, we are revealing now that, with the respect to UV sensitivity, *UMP1* belongs to the *RAD6* group of genes encoding proteins involved in post-replicative DNA repair. This repair pathway supports both error prone and error free repair by mechanisms allowing filling in the gaps opposite damaged sites in newly synthesized DNA. The pivotal role in this pathway play enzymes engaged in controlled ubiquitination. Although the attachment of the ubiquitin is the main modification targeting proteins to proteasomal degradation, the role of proteasome in modulation of post-replicative repair has been till now neglected. We show that yeast cells deprived of active proteasome maturase are not only hyper sensitive to UV radiation but, similarly to majority of mutants impairing post-replicative repair pathway, exert spontaneous mutator phenotype. The same phenotypes, as found for *ump1* mutants, exert yeast strains carrying mutations in the active centers of chymotrypsin-like and trypsin-like proteasomal peptidases. The results implicate the role of proteolytically active 20S proteasome in modulation of post-replication DNA repair pathway

L.VIII.2. ROLE of the MGS1 GENE in the RESCUE of ARRESTED REPLICATION FORK

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Orthologs of the Saccharomyces cerevisiae MGS1 gene are highly conserved from bacteria to human. Mgs1 protein, which possesses DNA-dependent ATPase and single strand DNA annealing activities, plays a role in maintaining genomic stability (1). mgs1 mutation causes synthetic lethality with rad6 mutation and exhibits a synergistic growth defect with rad18 and rad5 mutations, which are members of the RAD6 epistasis group important for tolerance of DNA damage during DNA replication. The mgs1 rad5 double mutant shows a high genomic instability and increased sensitivity to hydroxyurea. The growth defect of the mgs1 rad18 double mutant is suppressed by a mutation in SRS2, encoding a DNA helicase, or by overexpression of Rad52. These suppressions require the function of RAD51. From these results, we proposed that MGS1 constitutes a third pathway for the rescue of arrested replication fork, which is different from the RAD6-RAD18 pathway and the recombination repair pathway (2).

We studied the interactions of Mgs1 with the components of the replication machinery mgs1 mutation suppresses the temperature sensitivity of mutants in POL3, encoding DNA polymerase delta, and also suppresses the growth defect of a pol3 mutant caused by expression of the E coli Holliday junction resolvase RuvC. We propose that Mgs1 may play a role in the formation of “chickenfoot” at the replication fork by promoting annealing of newly synthesized strands when the fork is arrested in the pol3 mutant. This function of Mgs1 may be related to its role in the rescue of arrested replication fork.

The ortholog of MGS1 in E. coli is mgsA. Like Mgs1, MgsA has DNA-dependent ATPase and single strand DNA annealing activities. mgsA mutation causes a severe synergistic growth defect with recA mutation. Overexpression of MgsA causes growth inhibition and hypermutation similar to DinB overexpression. These results suggest that MgsA, like Mgs1, plays a role in resolving fork block in a recombination-independent manner.

L.VIII.3. DNA REPAIR and CHROMATIN STRUCTURE in YEAST: OVERLAP APPROACHES and NEW INSIGHTS

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A technology for analysing DNA damage at nucleotide resolution has been developed by us (1-5). This has now been adapted for the high resolution mapping of nucleosomes and regulatory proteins that bind to DNA (6). Thus we are in a position to examine how DNA repair proceeds in relation to chromatin structure (7).

How DNA repair enzymes or complexes gain access to chromatin is still not understood. Here we report on the role of the histone acetyltransferase Gcn5 in photoreactivation and nucleotide excision repair (NER) at the level of the yeast genome, the MFA2 and RPB2 genes, and at specific nucleotides within MFA2. The deletion of GCN3 markedly influenced the PR and NER of UV induced cyclobutane pyrimidine dimmers (CPDs) in MFA2 but much less so in RPB2, whereas no detectable defect was seen for the genome overall. These effects cannot be accounted for by changes in transcription of the MFA2 gene, the RPB2 gene, or the DNA repair genes in the gcn5 mutant. We conclude that the histone acetyltransferase Gcn5 directly influences photoreactivation and NER at MFA2 in both its transcribed and nontranscribed DNA, yet it has little effect on these processes for most of the genome, thus providing the first evidence for the role of a histone acetyl transferase in facilitating efficient access of NER to CPDs in a specific part of the yeast genome (8).

5. S. Yu, Y. Teng, N. F. Lowndes and R. Waters Mutation Research 485, 229-236 (2001)
L.VIII.4.  MISMATCH REPAIR, GENOME INSTABILITY and DRUG SENSITIVITY

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The primary role of DNA mismatch repair (MMR) is the maintenance of genomic stability by removing replication errors. The critical proteins involved in this activity (MSH2, MSH6, MLH1 and PMS2) are organised in two heterodimers, hMutSα and hMutLα, which participate in the repair of a variety of DNA substrates (base:mismatches and extrahelical bases). MMR defects are associated with both hereditary and sporadic cancer and inactivation of hMutSα and hMutLα in human tumors confers a spontaneous mutator phenotype and large increases in the rate of frameshift mutation at repetitive DNA sequence. In addition loss of MMR modifies the cytotoxic response to a variety of DNA damaging agents. The relationship between inactivation of MMR and drug resistance is particularly obvious for methylating agents. Since drug resistance is not due to increased removal of DNA damage this phenomenon has been named methylation tolerance. A defective MMR also appears to be associated with a lower level of resistance to a number of other therapeutic DNA damaging agents, including cisplatin and doxorubicin. On the other hand loss of MMR can also be associated with hypersensitivity to some chemotherapeutic drugs, possibly because of its role in recombinational repair. Recent evidence indicates that MMR is also involved in the control of oxidative DNA damage. Oxidation produces several DNA alterations against which cells deploy multiple protective strategies. Base excision repair is the main pathway involved in the removal of the oxidised purine 8-oxoguanine (8-oxoG) from DNA. hMTH1 -an 8-oxodGTPase that eliminates 8-oxodGTP from the dNTP pool- provides additional protection by minimising 8-oxodGMP incorporation during replication. Both steady-state and oxidant-induced DNA 8-oxoG levels are higher in MSH2- or MLH1-defective cells than in their repair-proficient counterparts. These data strongly suggest that the MMR pathway contribute significantly to the removal of 8-oxoG from DNA. Increased expression of hMTH1 in MMR-defective cells significantly reduces steady-state and H_2O_2-induced DNA 8-oxoG levels. This reduction dramatically diminishes the spontaneous mutation rate of Msh2- mouse embryo fibroblasts. Molecular analysis of spontaneous mutations at the HPRT gene indicate that several classes of mutation, including frameshifts, are modulated by hMTH1 overexpression. These results suggest that a large part of spontaneous mutations in MMR defective cells is due to DNA damage derived from an oxidized dNTPs pool.
L.VIII.5. TP53 and MUTATIONS in HUMAN CANCER

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TP53 is the most frequently mutated gene in human cancer, with a predominance of missense mutations scattered over 200 codons. In many cancers, specific mutation patterns can be identified, which are shaped by site-specific mutagenesis and by biological selection. In tobacco-related cancers (lung, head and neck), organ-specific patterns are observed, with many mutations compatible with the ones experimentally induced by tobacco carcinogens. In several other cancers, such as squamous cell carcinoma of the oesophagus, mutation patterns show geographic variations between regions of high and low incidence, suggesting a role for region-specific factors of risk. These assessments are useful in generating clues on the mutagenic mechanisms involved in human cancer.

The TP53 gene belongs to a family with complex and overlapping function in growth control, development and differentiation. Two TP53 homologues, TP73 and P63, encode several splicing variants involved in essential growth regulatory functions. Although neither of these genes is a frequent mutation target in cancer, there is evidence that deregulation of P63 is important in the pathogenesis of squamous carcinomas.

The p53 protein is a central tumour suppressor that plays anti-proliferative and repair-promoting roles in response to multiple stress conditions. At least three overlapping pathways of p53 induction have been identified. One is activated in response to many forms of DNA damage (genotoxic stress). Another is triggered by constitutive activation of growth stimulatory pathways, such as Wnt/βcatenin or H-Ras dependent pathways (oncogenic stress). The third pathway is activated in response to non-DNA damaging stress signals and is essentially mediated through the c-Jun N-terminal Kinase (JNK). The latter pathway may be particularly interesting for the pharmacological modulation of p53 activity.

There is evidence that the contribution of TP53 mutation to cancer is not limited to inactivation of wild-type p53 protein functions. Many mutant proteins accumulate in cancer cells and are thought to exert growth-promoting effects. Recent data suggest that some of these effects may result from specific interactions between mutant p53 and homologue proteins p63 and p73. These findings further emphasise the essential role of p53 in molecular carcinogenesis.
L.VIII.6. ENDOGENOUS MUTAGENS and GENOTOXICANTS: CONTRIBUTION of NITRIC OXIDE (NO) and INTRACELLULAR IRON IONS

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The universal signal molecule nitric oxide (NO) produced from L-arginine has been reported to impart the joint genotoxicity and mutagenicity. This study examined involvement of iron ions in the mechanisms of the above phenomena. The aims of our work were: to determine whether NO- activates the SOS regulon and whether iron ions modify the activation; elucidate the role of iron in NO-mediated signal transduction in soxRS [2Fe-2S] transcription activity; study the NO-induced mutagenicity. We first reported the ability of NO and NO producing agents- GSNO and dinitrosyl iron complexes with cysteine or glutathione (DNIC) to activate the SOS-response in E. coli PQ37 sfiA::lacZ. The most stable in vitro DNICglu was the weakest SOS-inducer. O-phenanthroline prevents sfiA gene expression by NO – and it reveals a leading role of iron in generation of the SOS signal. Oxidative stress regulon provides defense against NO. To elucidate mechanisms of signal transduction in soxR [2Fe-2S] transcriptional regulation we compared ability of NO, DNIC and GSNO to activate soxR sensor in E.coli soxS::lacZ. EPR spectroscopy has been used to monitor formation of inducible protein-DNIC complexes. DNIC, GSNO and NO were potent inducers of soxS expression. A high stability of DNICglu in vitro contrasts with low signal transducing activity. Chelating agents prevented the soxS-activation by NO-donors. Treatment of intact cells with DNIC, GSNO or NO in equal dose of 150 µM showed a formation of the single EPR-detectable DNIC-type species with g=2.03. So, in all that cases a formation of low molecular DNIC from NO took place as an initial step in NO-mediated activation of soxR and namely these DNIC (but not pure NO!) desintegrated the soxR [2Fe-2S] clusters and initiated the transcription of the soxRS regulon. S. typhimurium tester strain TA1535 provided an appropriate tool to study base-pair substitution in NO-mutagenicity. Reversion produced by DNIC, GSNO and NO was moderately enhanced. So, NO functions as a weak endogenous mutagen and potent genotoxicant, activating both SOS and soxRS regulons. Iron ions are absolutely indispensable for the NO-regulatory functions in the cell.

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L.VIII.7. DNA REPAIR in DEFENSE against GENOTOXIN-INDUCED APOPTOSIS

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Ultraviolet light, ionising radiation and chemical genotoxic agents induce a variety of DNA lesions that differently contribute to the formation of mutations, chromosomal breakage and cell death. In order to identify critical apoptotic DNA lesions and to clarify the role of receptor activation in genotoxin-provoked apoptosis, we compared various well-defined DNA repair proficient and deficient cell types as to their apoptotic response. Studies with MGMT deficient cells revealed that for alkylating agents inducing >12 different DNA alkylation products O\(^6\)-methylguanine (O\(^6\)-MeG) is an important apoptotic lesion. This lesion becomes converted via DNA replication and the mediation of mismatch repair (MMR) into critical proapoptotic secondary lesions, which are thought to be DNA double-strand breaks (DSBs) (1,2). Similar conversion of critical lesions has been shown to occur in cells deficient in DNA polymerase β (Polβ) which are hypersensitive to alkylating agents and display a high frequency of chromosomal breaks and apoptosis (3). Pol β hypersensitivity is due to incomplete base excision repair leaving un repaired patches in DNA that can become converted via DNA replication into DSBs (4). Indeed, DSBs are highly efficient in triggering apoptosis as demonstrated by electroporation experiments with restriction enzymes that cleave DNA in vivo (5). DSBs are also involved in UV-C and cisplatin-induced apoptosis, which was studied in NER deficient cell lines (6,7), as well as apoptosis provoked by the virostatic drug ganciclovir incorporated in DNA (8). In all these experimental systems a hallmark of DNA damage-triggered apoptosis was Bcl-2 decline which was followed by caspase-9/-3 activation and DNA degradation. Bcl-2 can also become degraded by active caspase-9 causing an amplification loop of activating the apoptotic pathway (8). A model is proposed for p53-independent DNA damage-triggered apoptosis suggesting DSBs to act as a critical common ultimate apoptotic lesion. Work was supported by German Research Fund Ka 724/10-1.

2. Ochs and Kaina, Cancer Res., 60, 5815-5824, 2000;
3. Ochs, K. et al., Cancer Res., 59, 1544-1551, 1999;
5. Lips and Kaina, Carcinogenesis, 22, 579-585, 2001;
L.VIII.8. PROGNOSTIC VALUE of STUDIES on DNA DAMAGE and REPAIR in TUMOR THERAPY

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The risk of developing cancer depends on a combination of exposure to carcinogenic factors and individual susceptibility that may be genetically conditioned. A similar interplay of genes and gene-damaging factors occurs during most cancer therapies. Normal tissue reactions frequently interfere with therapy, and patients vary in the severity and time of appearance of these reactions. If assays were available that could measure the intrinsic sensitivity of patient’s cells to therapeutic agents before beginning therapy, more individualized treatment of patients would be possible. The aims of the present work were to examine if cytogenetic assays could predict individual response to irradiation, and if the capacity of peripheral lymphocytes to repair radiation-induced damage of DNA may be related to risk for head and neck cancer. The studies were performed on lymphocytes from blood of healthy donors and of patients with head and neck cancer. Inter-individual differences in DNA damage, rates of DNA repair, and proliferation after irradiation were assessed using comet assays to measure background DNA damage and the kinetics of DNA repair, cytochalasin-blocked micronucleus assays to measure spontaneous and γ-ray-induced abnormalities indicating DNA repair problems during cell division, nuclear division indices to measure ability to proliferate, and apoptosis tests to measure programmed cell death. Background DNA damage and rate of DNA repair showed significant differences between the tumor patient and healthy donor groups as seen by comet assays. The time of appearance of acute reaction to radiotherapy was predictable on the basis of the kinetics of DNA repair. Micronucleus tests did not differentiate between healthy donors and patients and their results were not correlated with radiotherapy side effects, but their background frequency could probably estimate previous exposure to genotoxic agents. The level of patient’s acute reaction during radiotherapy was correlated with the nuclear division index of lymphocytes after irradiation in vitro with 4 Gy. Apoptotic tests also distinguished between healthy donors and patients but not between patients with a high or low level of side effects.

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Do genes differ in the rate at which they are repaired? Do people differ in their capacity to carry out DNA repair? These two questions, at opposite extremes of this field of research, have been addressed using modified versions of the comet assay (single cell alkaline gel electrophoresis). Our emphasis is on the repair of oxidative DNA damage.

To investigate repair at the level of genes, the comet assay has been combined with fluorescent in situ hybridisation (FISH). Oligonucleotides complementary to terminal exons of specific genes, tagged with either biotin or FITC, are hybridised to the DNA in comets from cells incubated for different times following the introduction of damage. Antibody amplification culminates in Texas red or FITC fluorescent labels at the two ends of the gene. Appearance of these labels in the comet tail indicates that damage is present in the DNA nearby, and its repair is monitored as 'retreat' of the gene-specific signals from the tail, compared with repair of total DNA. While strand breaks in the methylguanine methyl transferase gene (MGMT) are rejoined at the same rate as total DNA, p53 is repaired more rapidly.

The repair capacity of human lymphocytes can be measured (i) by incubating cells after treatment to induce damage and measuring the rate of removal of damage, or (ii) by preparing a cell extract and measuring repair in vitro on a defined substrate of DNA containing specific damage. There are substantial and reproducible differences in repair rates between normal healthy subjects. We are investigating whether repair capacity can be modulated by nutritional factors or by occupational exposure to genotoxins, and whether repair declines with age.
Bacteriophage RB69 encodes a replicative DNA polymerase with an associated 3′→5′ proofreading activity (Exo). Like T4 gp43 and the DNA polymerases of some archaeons, gp43 from bacteriophage RB69 is a member of the B-family (Pol-α like) polymerases that include the eukaryotic replicative polymerases α, δ, and ε. Crystal structures have been determined for RB69 DNA polymerase with and without its DNA substrate. Structures of recently reported B-family DNA polymerases from archaea show marked similarities to RB69 gp43, suggesting that the RB69 polymerase may be a good structural model for other B-family polymerases.

In our fidelity studies we have focused on RB69 residue Tyr567 which is highly conserved among B-family polymerases. In the RB69 crystal structure, this residue is located in the P helix within the finger domain at the polymerase active site (Pol).

Our recently published fidelity studies in vivo showed that the Y567A polymerase is a base-substitution mutator generating mostly transitions. Kinetic analyses of purified PolY567A Exo– and PolY567A Exo+ polymerases showed that the PolY567A polymerase generates mispairs much more frequently but extends mispairs substantially less efficiently than does the RB69 Pol+ Exo– polymerase. Thus, Tyr567 is a key determinant of the fidelity of base selection.

We have now extended these studies with an analysis in vitro. We measured the fidelities of the Pol+ Exo–, PolY567A Exo+ and PolY567A Exo– enzymes using the Kunkel M13mp2 LacZα gap-filling assay both with and without the accessory proteins gp32 (ssDNA-binding protein), gp44/62 (clamp loader), and gp45 (processivity clamp). Compared to their behavior in vivo, the three mutator polymerases exhibited modestly higher mutation rates in vitro and their mutational predilections were also somewhat different. Although the accessory proteins exerted little or no effect on total mutation rates in vitro, they strongly affected mutation rates at many specific sites, increasing some rates and decreasing others.
LACK of STRAND BIAS in ULTRAVIOLET-INDUCED MUTAGENESIS in *Escherichia coli*

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We have investigated whether UV-induced mutations are created with equal efficiency on the leading and the lagging strand of DNA replication. We employed an assay system that permits measurement of mutagenesis in the *lacZ* gene in pairs of near-identical strains (1, 2). Within each pair, the strains differ only in the orientation of the *lacZ* gene with respect to the origin of DNA replication. Depending on this orientation, any *lacZ* target sequence will be replicated in one orientation as a leading strand and as a lagging strand in the other orientation. In contrast to previous results obtained for mutations resulting from spontaneous replication errors or mutations resulting from the spontaneous SOS mutator effect (3), measurements of UV-induced mutagenesis in *uvrA* strains fail to show significant differences between the two target orientations. These data suggest that SOS-mediated mutagenic translesion synthesis on the *E. coli* chromosome may occur with equal, or similar, probability on leading and lagging strands.

I.P.3. INVOLVEMENT of DNA POLYMERASE II in CHROMOSOMAL DNA SYNTHESIS in *Escherichia coli*

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It was shown recently that *E. coli* cells possess 5 DNA polymerases. In contrast to Pol I, Pol III and Pol V, which carry out defined roles in DNA repair and replication, the physiological function of Pol II and Pol IV is poorly understood. It has been shown that the constitutive intracellular concentration of Pol II is \( \approx 50 \) molecules/cell, at least 5 times more than Pol III (the major DNA replicase of *E. coli*). *In vitro* studies have also shown that Pol II is fully competent to synthesize DNA with high fidelity and processivity. Therefore one may speculate that Pol II might serve as an enzyme that helps Pol III during DNA replication.

In the present study we have investigated the role of DNA Pol II in chromosomal DNA synthesis and tested whether Pol II of *E. coli* is able to replace temporarily Pol III during DNA replication process. We used in our studies an assay system, developed in our laboratory, which allows measuring a potential difference in replication fidelity between leading and lagging strand replication on the *E. coli* chromosome. To specifically determine the involvement of Pol II in chromosomal DNA synthesis we used *E. coli* strains carrying *polBex1* allele (encoding proofreading defective Pol II) and strains without functional Pol II. The experiments were performed with the use of strains carrying wild type Pol III and strains with impaired Pol III HE (*dnaE915*, *dnaE919*, *dnaQ930* and *dnaX36*).

Three aspects of the obtained data are particularly notable. First, Pol II is able to compete effectively with Pol III HE during the lagging strand synthesis. Second, Pol II can proofread or extend a primer with a mismatched terminus created by Pol III HE. Third, and most important, the role of Pol II in chromosomal DNA replication increases when Pol III HE is impaired.
I.P.4. The ROLE of τ SUBUNIT in MAINTAINING the FIDELITY of LEADING and LAGGING DNA STRANDS SYNTHESIS in Escherichia coli

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The mechanisms that control the fidelity of DNA replication are being investigated by a number of approaches. Important tools in these studies are mutant versions of DNA polymerases that affect the fidelity of DNA replication. Replication of *Escherichia coli* chromosome is performed by the DNA polymerase III holoenzyme (Pol III HE). Pol III HE is asymmetric complex containing a total of 18 subunits. It has been suggested that proper interactions within the Pol III HE could be essential for maintaining the optimal fidelity of DNA replication. We have been particularly interested in elucidating the physiological role of τ subunit (product of *dnaX* gene). The *E. coli* *dnaX* gene encodes both the τ and γ subunits of DNA Pol III HE. The τ protein plays a crucial role in functioning of the Pol III HE. The C-terminal portion of τ protein fulfils several functions within Pol III HE. It binds α subunit, affecting dimerization of the polymerase. It interacts also with the dnaB helicase, linking Pol III HE to the helicase/primase and activates the helicase for high-speed fork movement. τ may also contact β subunit and protects the leading strand β clamp from unloading by the γ-complex.

Thus, the τ protein plays essential role in effectively coupling all the replicative activities in the replication fork. Although genetic and biochemical studies have revealed insights into the organization and function of the τ protein, little is know about the role of τ in replication fidelity. To explain the role of τ subunit in maintaining the fidelity of leading and lagging DNA strands synthesis during replication of chromosomal DNA in *E.coli* we carried out a series of experiments measuring the level of mutagenesis in the *dnaX*³⁶ strains. We found that the *dnaX*³⁶ mutation decreases the fidelity of replications on both DNA strands. However, the effect of *dnaX*³⁶ was stronger on the lagging strand. Interestingly this difference in mutability of the lagging versus leading strand in *dnaX*³⁶ was abolished by deletion of the dinB gene.

Our results indicate that τ subunit have important function in determining the fidelity of chromosomal replication in *Escherichia coli*. 
I.P.5. ROLE of the \textit{DINB-ENCODED DNA POLYMERASE IV} in SPONTANEOUS MUTAGENESIS in \textit{Escherichia coli}

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The \textit{dinB}-encoded DNA polymerase IV from \textit{Escherichia coli} belongs to the recently identified Y-family of DNA polymerases. In addition to their poor fidelity on undamaged templates, these DNA polymerases manifest very low processivity \textit{in vitro}, and all lack identifiable 3’-5’ exonuclease activity. Although overexpression of the \textit{dinB} gene encoding DNA pol IV is known to result in enhancement of untargeted mutagenesis, it remains uncertain whether there is a strand specificity (leading or/and lagging DNA strand) for the synthesis catalyzed by this DNA polymerase. In our studies we made an attempt to get more information about the biological function of DNA polymerase IV in the cell, and how this polymerase is recruited to the replication fork. We previously constructed and described an experimental system that permits investigation of the potentially different replication fidelity during leading and lagging DNA synthesis on the \textit{E. coli} chromosome (1). In this system we determined the reversion frequency of certain defined \textit{lacZ} missense alleles (2) as an indicator of DNA replication fidelity. This system allowed us to determine that most of the mutations which occur in cells overproducing DinB are generated in one of the replicating DNA strand (lagging strand ?). To get information what is the role of DinB polymerase in spontaneous mutagenesis we used our chromosomal \textit{lacZ} system and performed a series of experiments with \textit{dinB} deletion mutants. We found that in wild type background deletion of the \textit{dinB} gene does not result in decrease in spontaneous frameshift and base substitution mutations. Only in case of -1G deletion we observe \textasciitilde25\% decrease of mutagenesis in \textit{dinB}-background. However, when \textit{E. coli} cells possess mutant form of Pol III HE (e.g. DnaX36) the higher mutability observed in such strains is decreased by the deletion of the \textit{dinB} gene.


I.P.O.6. CONTRIBUTION of POL II, POL IV and POL V to SPONTANEOUS MUTAGENESIS in E. coli AB1157 mutD5 STRAIN

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Some of the mutations in dnaQ gene encoding proofreading ε subunit of DNA pol III, the main replicational polymerase of E.coli, results in increased number of spontaneous mutations. Using argE3→Arg’ reversion to prototrophy system in AB1157 we have found that the level of Arg’ revertants in exponentially growing mutD5 strain can be modified by three SOS-inducible DNA polymerases: pol II, pol IV and pol V (Note: under starvation condition in mutD5 strain SOS response is chronically induced). Deletion of umuDC (pol V) or dinB (pol IV) appeared to decrease the number of Arg’ revertants (4 and 2 fold, respectively). On the contrary, the deletion of polB (pol II) appeared to increase the number of these revertants (about 2 fold). The above observations concern growth-dependent mutations. When mutD5 strain grows for several days on minimal E-Arg plate Arg’ starvation-associated (adaptive) revertants arise with the rate of about 8 colonies per day per 10^8 cells. Deletion of dinB (mutD5ΔdinB strain) reduced the rate of mutation 5 fold and umuDC (mutD5ΔumuDC strain) 10 fold. Deletion of dinB and umuDC (mutD5ΔdinBΔumuDC strain) resulted in adaptive mutation rate only 4 fold higher than in mutD5 strain. The pol II deficiency (polB deletion) in mutD5 and mutD5ΔdinB strains dramatically increased the rate of Arg’ adaptive revertants (60 fold) however, it did not influence the rate of mutations in mutD5ΔumuDC and mutD5ΔdinBΔumuDC strains. Summing up, under conditions of invalid ε subunit of pol III adaptive mutagenesis seems to be much more pol V than pol IV dependent, whereas in mutagenesis taking place in exponentially growing bacteria the role of these polymerases is equal. In both types of spontaneous mutagenesis pol II plays important role as an error free repair polymerase.
I.P.7. The EFFECT of \textit{E. coli} rec\textit{A} EXPRESSION on the SENSITIVITY of \textit{S. cerevisiae} rad\textit{51} and rad\textit{52} MUTANTS to DNA DAMAGING AGENTS

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DNA double-strand breaks (DSBs) are perhaps the most deleterious DNA lesions as they disrupt both DNA strands causing problems for all DNA transactions. If left unrepaired, they can cause chromosomal fragmentation, translocations and deletions, a consequence of which might be cell death, or in multicellular organism, cancer. DSBs are generated by a wide range of factors, including ionizing radiation (IR), free radicals – the products of oxidative metabolism and radiomimetic chemicals such as methyl methanesulfonate (MMS).

The RecA protein plays a pivotal role in homologous recombination (HR) in \textit{E. coli}. In \textit{S. cerevisiae}, the RAD52 epistasis group of genes is thought to represent a pathway for DSB repair by HR. Two genes of the group, RAD51 and RAD52, were shown to have key roles in this process. The product of the RAD51 gene has been found to have extensive structural homology with the \textit{E. coli} RecA protein. On the other hand, the RAD52 encoded product does not show obvious homology to any known recombination proteins in bacteria and therefore appears to be unique to eukaryotes. The importance of the RAD52 gene is underlined by the presence of homologues in all eukaryotic organisms investigated to date.

We have tested effect of the \textit{E. coli} rec\textit{A} expression on the sensitivity of the \textit{S. cerevisiae} rad\textit{51} and rad\textit{52} mutants to the agents creating the DNA DSBs such as MMS, IR and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). We have found that the RecA protein increases survival of the rad\textit{52} mutants to IR, MMS and H\textsubscript{2}O\textsubscript{2}. On the other hand, no effect of rec\textit{A} expression on sensitivity to these agents was observed in the rad\textit{51} mutant. Survival data are in good correlation with the increased efficiency to repair DSBs, as measured by pulsed field gel electrophoresis, in the rad\textit{52} and rad\textit{51} strains expressing RecA. Thus, it seems that the RecA protein can participate in DSB repair in yeast unless the Rad51 protein is present. As DSBs repair in yeast is mediated by the multiprotein complex, the two hybrid experiments are in progress in our laboratory to verify possible involvement of RecA in protein-protein interactions within this complex.

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I.P.8. BASE EXCISION REPAIR (BER) is INVOLVED in MUTAGENESIS INDUCED under CONDITIONS NONPERMISSIVE for DNA REPLICATION

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We have previously shown that UV- and MMS- induced mutations in λsusO8 phage may be generated in an *E. coli* 594su- host, nonpermissive for phage DNA replication. This mutagenic pathway differs from that in the permissive host, *E. coli* C600supE, in several aspects: (1) UV-induced reversion of λsusO8 → su+ in the 594su- strain requires either induced SOS function(s) or delivery of a high level of UmuD’ protein from the plasmid. (2) Deletion of the umuDC operon abolishes phage mutagenesis in a 594su-, but not C600supE, host. In the latter strain, induction of some other SOS function(s), possibly DinB – DNA polymerase IV, is required. (3) Efficiency of UV-induced mutagenesis in the 594su- host is about 40% that observed in C600supE. (4) UV- and MMS-induced mutations in the 594 su- host are refractory to mismatch repair, while in the C600supE refractory attains a value of 40%.

We have now studied the possible role of base excision repair (BER) in mutagenesis induced under conditions independent of DNA replication. We constructed *E. coli* strain su-, nonpermissive for phage λsusO8 DNA replication, with xthA or Δxth mutations, deficient in AP-endonuclease, as well as the triple mutant nth-1 nfo-1 Δxth deficient in endonuclease III and IV, recognizing pyrimidines with a saturated 5,6 bound, and AP-endonuclease.

The results show that deficiency of AP-endonuclease (Xth) leads to a 2-fold decrease of the reversion frequency of λsusO8 → su+ induced by UV-light, and a 2,5-fold decrease by MMS. On the other hand, deficiency in both endonucleases III (Nth) and IV (Nfo) and AP-endonuclease (Xth) leads to 3,5-fold and 10-fold reduction of mutation induced by UV-light and MMS, respectively.

The results indicate that, in mutagenesis induced under conditions nonpermissive for DNA replication, BER appears to be involved, and suggest that the base excision repair pathway may be a source of mutations.
LPO.9. SOS REPAIR INFLUENCES GENETIC INSTABILITY of DNA TRIPLET REPEATS

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Molecular mechanisms responsible for the genetic instability of the trinucleotide sequences (TRS) accountable for fourteen disorders including Myotonic Dystrophy, Huntington’s disease, Fragile X syndrome or Friedreich’s ataxia has been extensively studied for the last decade. Replication, transcription, repair processes, and recombination influence the frequency of deletions and expansions of such repeated motifs.

Herein, we demonstrate that expression of bacterial SOS repair dramatically decreases the stability of long (CTG/CAG)n tracts in plasmids. A frequency of deletions in E. coli strain, which has the SOS regulon genes constitutively expressed (recA441, lexA71::Tn5) was substantially higher than that observed in a strain with no SOS response (mal::Tn9 lexAind1). This observation strongly suggests that induction of SOS repair increases genetic instability of TRS. Topology analyses of reporter plasmids isolated from SOS+ and SOS- strains revealed substantially higher linking number difference in strain with constitutively expressed SOS regulon. Hence, in further studies we confirmed the relevance of the increased supercoiling in the genetic instability of the TRS using topoisomerase I and gyrase mutants.

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One of the most abundant products of lipid peroxidation is trans-4-hydroxy-2-nonenal (HNE), with concentration in human plasma ranging between 0.1 – 1 µM. We have previously shown that HNE produces adducts to all four DNA bases (poster I.P.12), which are characterised by the presence of long 6-7 carbon atom chains in addition to exocyclic etheno-rings and possibly propano-. DNA polymerase fingerprinting on HNE-modified templates suggests that the presence of these adducts in DNA stops replication by T7 DNA polymerase, and that reactivity of DNA bases with HNE follows the order: dG>dC>dA≥dT.

Mutagenicity studies in M13 phage system shows that modification of phage DNA with HNE decreases phage survival in JM105 strain and increases mutation frequency in M13 lacZ gene in a time and dose dependent manner. As much as 40-fold increase in mutation frequency (at 8% survival) was observed after 2 h of DNA modification with 2 mM HNE at pH 5.5. Sequence analysis of M13 lacZ mutants revealed that the most frequent mutations were recombination events with the lacZ gene of F’ factor, detected as simultaneous deletion of 93 (∆M15 deletion of F’lacZ) and 54 (M13 polylinker) nucleotide fragments of phage DNA. Similar numbers of base substitutions and frameshift mutations were found. The major base substitution was C→T transition. This mutation constituted 37% of all point mutations. Interestingly, 37% of point mutations were frameshifts, and this was 4-fold higher level than that observed in the spectrum of spontaneous mutations. Within frameshifts one nucleotide additions of cytidine and guanosine prevailed and to a lesser extent deletions, mainly A deletions, were found. Additions/deletions were found almost exclusively in runs of C, G or A. Thus, template damage probably evoked DNA polymerase “jump” over the lesion, during which the enzyme added one nucleotide to reach the template behind HNE adduct with a long side-chain. Such mechanism could also explain a high number of recombinations, where DNA polymerase could switch to undamaged homologous template of F’ factor.

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Exocyclic DNA adducts are formed by various bifunctional agents of exogenous and endogenous origin. We studied the excision by *E.coli* DNA-glycosylases of the simplest types of 3,N'-exocyclic cytosine adducts, i.e. α-hydroxyethano (HEC), etheno (EC) and α-hydroxypropano (HPC) adducts. Since thymidine does not react with chloroacetaldehyde (CAA) at wide range of pH, we employed reaction of CAA at pH 9 with 25-mer T₅C₄T₅C₄T₅ to generate HEC with ~10% admixture of EC, which in turn was converted to EC by prolonged incubation at pH 6.5. The pH 4.5 was found to be optimal for selective modification of C in 25-mer by acrolein to give HPC. The qualitative and quantitative analysis of modified 25-mers was done by HPLC after enzymatic digestion to deoxynucleosides. The 25-mers containing 30-50% of modified cytosines were chosen for adduct-excision studies. The modified 25-mers were ³²P-5'-labeled, annealed to complementary 25-mer, incubated with Mug or AlkA glycosylases, followed by Nth protein serving as endonuclease and then subjected to denaturing PAGE and autoradiography. Adducts excision was manifested by the appearance of 6-,12- and 18-mers bands on gel autoradiogram. Incubation at pH 13 can not be employed for making strand breaks at abasic sites since the formation of breaks at adduct sites occurred also in absence of enzyme under these conditions. We found that HEC and EC are excised by Mug, but not by AlkA glycosylase. The acrolein adduct, HPC, is excised by AlkA, and with higher efficiency by Mug. Since HEC and HPC are quaternary bases and at pH 7.5, which is standard pH for the assay, they are in protonated form (the pK of these adducts in deoxynucleoside form is 8.1 and 8.5, respectively), we have undertaken study on pH-dependence of their excision. We found that HPC is several-fold more efficient excised by Mug and Alk at pH 9 than at pH 7.5, what suggests that neutral form of this adduct is a better enzyme substrate than protonated form. The studies on pH-dependence of excision of HEC and EC are in progress.
I.P.12. REACTION of trans-4-HYDROXY-2-NONENAL with DEOXYNUCLEOSIDES

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Mutagenesis studies on M13 phage reacted with trans-4-hydroxy-2-nonenal (HNE) have revealed that all four DNA bases, A, C, G and T, are targets for HNE (Kowalczyk et al., Poster I.P.10). At present, only reaction of HNE with dG was described in literature and adducts were characterised. Since it is known that the simplest HNE homologue, acrolein, forms adducts with dA, dC, dG and dT, we have undertaken studies on reaction of HNE with these nucleosides. The HPLC analysis of reaction mixtures showed formation of several products in each case, and reactivity follows the order dG>dC>dT=dA. MS of the whole reaction mixtures showed mass peaks corresponding to HNE-dN 1:1 adducts, and in lower abundance also corresponding to 2:1 and 3:1 adducts. In dA, dC and dG reactions peaks corresponding to heptyl-substituted etheno-adducts were detected, what indicates that during reaction HNE undergoes oxidation to 2,3-epoxide, probably by oxygen from air. MS of reaction mixtures besides peaks of HNE-dN adducts shows also peaks corresponding to adducted bases as well as to the dehydrated derivatives, what is in conformity with expected fragmentation patterns. The four most abundant products of the HNE-dC reaction mixture were isolated by TLC and temporarily characterised on the basis of MS, UV and pKa evaluation: A is N3-substituted HNE-dC cyclic or linear, B and C are N4-substituted HNE-dC, whereas D is dehydrated heptyl-substituted etheno-dC adduct. The preparation of higher quantity of pure adducts for NMR analysis is in progress.
I.P.13. DEGRADATION of MODIFIED DEOXYNUCLEOSIDE-5'-TRIPHOSPHATES by HUMAN TISSUE HOMOGENATES

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Bases in deoxynucleotide-5'-triphosphate (dNTP) pool can be damaged by mutagens as bases in DNA. The level of bases damage in dNTP pool is much higher than in the DNA. Modified dNTPs can be mutagenic, because they can be incorporated into DNA by DNA polymerases. The E. coli MutT protein is an enzyme which sanitise dNTP pool of 8OH-dGTP by its hydrolysis to 8OH-dGMP and inorganic pyrophosphate. The human homologue of MutT, hMTH1 protein, hydrolyses also oxidised forms of dATP, 2OH- and 8OH-dATP. An important role in dephosphorylation of damaged dNTPs can also play other enzymatic activities, like phosphatases and nucleotidases, and final product of dephosphorylation, the modified dN can be exerted from cells.

We studied degradation of dNTPs formed during oxidative stress, 8-OH-dGTP and etheno derivatives - εdCTP and εdATP, by homogenates from human tissues (lung tumour and healthy surrounding). The HPLC profiles of degradation of the examined dNTPs showed the sequential formation of dNDPs, dNMPs and dNs, what suggests that various enzymatic activities are engaged in this process. We found that 8OH-dGTP and εdCTP are dephosphorylated much more efficiently than their unmodified equivalents, whereas εdATP is dephosphorylated nearly as efficiently as dATP. This suggests that degradation of at least some damaged dNTPs could involve enzymatic activities more specific for the damage than these which are involved in degradation of unmodified dNTPs. In all tested patients, the rate of dephosphorylation 8OH-dGTP and εdCTP by tumour tissue homogenates was always higher than by healthy tissue ones, whereas the rate of dephosphorylation of unmodified dGTP by both types of homogenates was similar. This indicates higher level of sanitising activities in tumor than in healthy tissues.

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1. N^6-ethenoadenine (εA) is an exocyclic DNA adduct formed by carcinogens such as vinyl chloride and the lipid peroxidation products. εA is mutagenic in bacteria and mammals and to some extent stops DNA replication. It has been shown that this modified base is a source of secondary lesions, which derive from water molecule addition or OH^- ions addition to C(2)-N(3) bond of εA pyrimidine ring (product B) (1). Product B undergoes further rearrangement, yielding 4-amino-5-(imidazol-2-yl) imidazole (product C) that has been found 20-fold more mutagenic in bacteria than parental εA (2).

The aim of this work was to investigate DNA synthesis past compounds B and C in the template by different DNA polymerases. We have tested several prokaryotic and mammalian DNA polymerases, namely: phage pol T7 exo-, bacterial Taq, Tli, pol I, Kf of pol I (large and exo-) and calf thymus pol β. For all polymerases studied, we have observed strong inhibition of DNA synthesis on templates containing mainly product B, in comparison with that containing εA. However, for T7, Tag and Tli DNA polymerases more efficient replication was found on templates containing product C, whereas for other DNA polymerases, compound C still consisted strong replication block. Molecular modeling was performed to interpret experimental data in the structural context. Modified bases were modelled in the active sites of DNA polymerases for which the structures of protein-DNA complexes are solved. Possible conformations of the modified bases in the polymerase active sites were analysed to study the effects of base modifications on reaction mechanism.

I.P.15. SYNTHESIS of OLIGONUCLEOTIDE ANALOGUE of ETHENOADENOSINE for DNA REPAIR

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DNA damage by toxic agents compromises the coding potential and strand integrity of the genetic material. DNA damage, mediated by \( \alpha, \beta \)-unsaturated aldehydes represents a major contributor to cellular DNA damage. The modification of DNA may occur at the nucleobase of DNA nucleotides. One class of the resulting lesions may labialize the otherwise stable DNA backbone and/or induce mutations during cellular nucleic acid synthesis. Thus, evolution has developed complex enzymatic pathways that recognize and repair DNA damage. The rate of cleavage was found to differ with the lesion and was also affected by neighbour sequences geometry and the resulting local conformational changes, which can be sequence-dependent. An understanding of the chemical mechanisms of DNA damage and repair is central to uncovering the biological effects of genomic lesions.

Therefore, oligonucleotides having adducts such as the highly mutagenic DNA lesion, ethenoderivatives are of particular interest because they are capable of Watson-Crick type base pairing, yet the presence of a substituent precludes Hoogsteen-type base pairing as might be found in triplex DNA. Also, these derivatives have long fluorescent lifetime, detectability at low concentration, and relatively long wavelength of excitation.

Sequence is an important factor in the thermodynamic stability of the duplex and influences the adduct-induced destabilization. The major objective of this study was to synthesize a building block and to incorporate it into a sequence context for a comparative study of the effect on duplex stability. This study is also directed toward the determination of the effect of different doublet neighbour base sequences flanking the lesions, and that of the opposite bases, and the fluorescent properties of the oligonucleotide.

I.P.16. URACIL-DNA GLYCOSYLASE KNOCKOUT MICE

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Uracil-DNA glycosylase (Ung) is an enzyme that removes uracil from DNA. Uracil may arise in DNA through either deamination of cytosine or by being misincorporated during replication. If left un repaired, uracil will cause mutations, directly or indirectly, and the need for effective repair systems is reflected in the high conservation of the Ung gene through evolution. However, also other enzymes have the capability to remove uracil, and in mammals 4 other uracil-DNA glycosylases have been characterised: TDG (thymine mismatch DNA-glycosylase), SMUG1 (single-strand-selective monofunctional uracil-DNA glycosylase), UDG2 (uracil-DNA glycosylase 2) and MBD4 (methyl-CpG binding thymine glycosylase).

In order to examine the biological role of Ung, knockout (ko) mice have been made where the gene encoding Ung is silenced. We have examined the uracil levels in organs from wild type (wt) and ko mice, and in general there is an accumulation of uracil in organs from Ung ko mice as compared to wt. Also, these ko mice show increased mortality from about the age of 15 months, and the most prevalent cause of death seems to be malign lymphoma.

The accumulation of uracil in Ung ko mice seems to be due to a reduced, but not eliminated, ability to remove uracil from DNA. There are several candidates for the residual uracil removing activity, and by the use of different uracil containing DNA substrates under varying conditions, the activity profile can give indications as to which enzymes that are active. Combined with immunoprecipitation and western analyses, preliminary results indicate that the ko mice have an, as yet, unidentified enzyme with properties somewhat similar to wt Ung protein. Also, SMUG1 seems to have a role in uracil removal in ko mice, as in wt mice. We do not, however, have indications that SMUG1 is upregulated in Ung ko-mice. There is no significant activity of either TDG or MBD4 neither in wt nor ko mice.
I.P.17. SUBSTRATE PREFERENCE and ENZYME KINETICS REVEAL DISTINCT PROPERTIES of UNG2 and SMUG1 in CELLULAR BASE-EXCISION REPAIR

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Uracil in DNA can be introduced via two major pathways. Deamination of cytosine appears at a rate of ~100-500 events per human cell per day to yield mutagenic U:G mismatches. In addition, uracil may appear in DNA as a consequence of misincorporation of dUMP opposite A-residues during replication. At least four human DNA glycosylases display uracil-excision activity, indicating a complex mechanism of DNA-uracil repair. The evolutionary conserved UNG gene encodes the major uracil-DNA glycosylase activity in replicating human cells. Ung knock out mice indicate, however, a role for SMUG1 in repair of deaminated cytosine residues. We have compared the subnuclear localisation of hUNG2 and hSMUG1. Furthermore, we have characterised biochemical and kinetic properties of recombinant hUNG2 and hSMUG1 on different substrates to elucidate their specific roles in uracil-DNA repair. In addition, we have measured the contribution of hUNG2 and hSMUG1 to initiate uracil-DNA repair in nuclear extracts prepared from human cell lines. hSMUG1 and hUNG2 are differentially localised within the nucleus. A fraction of UNG2 localises to replication foci in the S-phase cells. In cells outside the S-phase UNG2 seems to be evenly distributed in the nucleoplasm. Only hSMUG1 is observed in nucleoli where it appears to be especially abundant. Mg2+ strongly increases the affinity of UNG2 for uracil in single-stranded DNA. In the presence of Mg2+ the Kcat/Km value against Uss and U:G is ~1000 fold and ~300 fold higher respectively, for hUNG2 than for hSMUG1. In contrast to hUNG2, hSMUG1 has much broader substrate specificity. In addition to uracil, both HmU and εC but not 5OH-U are substrates for SMUG1. Our results indicate that UNG2, in addition to its role in post-replicative repair of U:A, has a major role in excision of deaminated cytosine from chromatin in freely cycling cells. This is supported by a strong reduction in in vitro BER of U:G substrate in nuclear extracts preincubated with neutralising antibodies against hUNG2.
I.P.18. DNA BASE EXCISION REPAIR in NUCLEOSOME CORE PARTICLES

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The organisation of DNA into nucleosomes and higher order chromatin structure in eukaryotic cells is thought to afford little protection against the spontaneous formation of DNA base damage through hydrolysis. Uracil in DNA is a major lesion arising either by hydrolytic deamination of cytosine or from misincorporation of dUMP during DNA replication. Base excision repair, the major pathway repairing these lesions, has not been studied in chromatin. As a first step towards this goal, we have utilised nucleosome core particles reconstituted from chicken erythrocyte histone octamers, and a 146bp fragment of the Lytechinus variegatus 5S rRNA gene containing site specific U:A base pairs to study base excision repair in nucleosome core particles in vitro. Uracil excision by the two major human uracil-DNA glycosylases, UNG2 and SMUG1, was analysed with both core particles and naked DNA. We have also addressed whether the rotational setting of the 5S rDNA on the core particles influences the accessibility of the damage to the DNA glycosylase. Finally, we have reconstituted short-patch base excision repair nucleosome core particles using purified human proteins.
Methylating agents generate cytotoxic and mutagenic DNA damage. Cells use 3-methyladenine-DNA glycosylases to excise some methylated bases from DNA and suicidal O6-methylguanine-DNA methyltransferases to transfer alkyl groups from other lesions onto a cysteine residue. We have discovered that the highly conserved AlkB protein repairs DNA alkylation damage by an unprecedented mechanism. AlkB has no detectable nuclease, DNA glycosylase or methyltransferase activity, yet Escherichia coli alkB mutants are defective in processing methylation damage generated in single-stranded DNA. Theoretical protein fold recognition had suggested that AlkB resembles the α-ketoglutarate-Fe(II) dependent dioxygenases which employ iron-oxo intermediates to oxidise chemically inert compounds. We have found that purified AlkB repairs the cytotoxic lesions 1-methyladenine and 3-methylcytosine in single- and double-stranded DNA in a reaction dependent on oxygen, α-ketoglutarate and Fe(II). The AlkB enzyme couples oxidative decarboxylation of α-ketoglutarate to the hydroxylation of these methylated bases in DNA, resulting in direct reversion to the unmodified base and the release of formaldehyde. We are now investigating the role of the human alkB homolog.
Apurinic/apyrimidinic (AP) sites are the most common DNA lesions generated by both spontaneous and induced base loss. The cytotoxicity and mutagenicity of this lesion was demonstrated unequivocally in both prokaryotic and eukaryotic systems. In bacterial cells molecular analysis of mutations revealed the preferential insertion of an adenine opposite to an AP site (“A rule”). In order to investigate the mutagenic effect of this lesion a single AP site-containing shuttle vector was constructed and after transfection in COS7 cells, the mutations were selected by RFLP/PCR. All mutations were targeted where the AP site was originally constructed. The preference for insertion opposite the AP site was: A(52%)>C(39%)>G(9%). A small proportion (17%) of deletions was also observed.

Translesion DNA synthesis is thought to be one of the major sources of mutagenesis in living cells. DNA polymerase eta (Pol eta) catalyses accurate translesion synthesis past induced cyclobutane dimers. In vitro studies suggest that Pol eta can be involved in the translesion synthesis of the abasic sites and other mutagenic lesions, such as O6methylguanine (O6MeGua) and 8-oxoguanine (8-oxoGua). In order to verify if Pol eta is involved in the processing of these lesions in vivo, human fibroblasts XPV (XP30RO) were treated with MNU, MMS, and KBrO3. The survival of the parental cell line was compared with that of the same cells stably transfected with an expressing Pol eta vector. The parental XPV cell line was found to be much more sensitive to killing by all three agents. However our preliminary data suggest that the hypersensitivity to methylating agents was unrelated to the activity of Pol eta itself. We found, unexpectedly, that it was the result of inactivation of O6MeGua methyltransferase in the parental XPV cell line, and reactivation in the transfectants. Thus our data suggest that Pol eta is not involved in the lethal effect mediated by O6MeGua, whereas it does affect the sensitivity to oxidative DNA damage. Experiments are in progress to clarify the role of Pol eta in 8-oxoGua and AP site-induced mutagenesis.
In mammalian cells, the activation of Poly(ADP-ribose)polymerase (PARP; EC 2.4.2.30) is one of the earliest cellular responses to DNA base damage caused by numerous endogenous and environmental genotoxic agents.

To investigate the function(s) of PARP in the repair of oxidative DNA modifications, we analysed stable transfectants (COM3 cells) of the SV40-transformed Chinese hamster cell line COR3, which conditionally (upon addition of dexamethasone) overexpress the PARP DNA-binding domain and thereby inhibit PARP activity. We studied the influence of this PARP inhibition on (i) the steady-state (background) levels of oxidative base modifications, (ii) the susceptibility of the cells to oxidative DNA damage by exogenous oxidants and (iii) the repair of oxidative DNA base modifications. COM3 cells without dexamethasone treatment and COR3 cells were used as comparisons. Oxidative DNA damage was quantified by means of the alkaline elution technique in combination with the repair glycosylase Fpg protein. Steady-state levels of oxidative DNA damage and the susceptibility to damage induction were found to be similar in all cell lines. However, the repair rate of Fpg-sensitive oxidative base modifications induced by the photosensitizer Ro 19-8022 plus visible light (mostly 8-hydroxyguanine) was significantly slower in PARP-inhibited cells ($t_{1/2} \sim 6$ h) than in COM3 and COR3 cells without dexamethasone ($t_{1/2} \sim 3$ h). The retardation of the repair was not associated with a significant accumulation of single-strand breaks (repair breaks). In addition, the repair of other types of DNA modification, i.e., single-strand breaks induced by t-butylhydroperoxide and pyrimidine dimers induced by UVB, was also affected by PARP inhibition.

The results indicate a role of PARP in the repair of oxidative DNA base damage.
Mitochondria are main source of endogenously generated reactive oxygen species (ROS), and these are held responsible for the background levels of oxidative DNA modification that are observed in all types of mammalian cells. To verify the contribution of mitochondria to the level of oxidative DNA modifications in the nucleus, we investigated ROS production and oxidative DNA damage in HeLa wild-type and HeLa rho0 cells. The latter have been depleted from mitochondrial DNA and are therefore unable to perform oxidative phosphorylation. As expected, HeLa rho0 cells produced less ROS, as measured by assays based on ROS-mediated oxidation of dihydrofluorescein diacetate and hydroethidine. Nevertheless, the background level of oxidative DNA modifications, detected by a modified alkaline elution assay using the repair glycosylase Fpg, was in HeLa rho0 cells as high as in HeLa wild-type cells. Moreover, we investigated the influence of the mitochondrial blocker antimycin A, the ATP-synthase-inhibitor oligomycin and the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) on HeLa wild-type cells. As a result of the incubation with these substances alone or in combination, ROS production increased up to the sixfold, whereas the level of oxidative DNA modifications remained unchanged. Even after depletion of the cellular antioxidant glutathione, the mitochondrial blockers / uncouplers did not induce additional oxidative DNA modifications in HeLa wild-type cells. In conclusion, the endogeneous mitochondrial ROS production seems not to be responsible for the background level of oxidative DNA modifications. Genotoxic effects of ROS generated by mitochondria might be limited to the mitochondrial DNA.
I.P.23. COORDINATION of the INITIAL STEPS of BASE EXCISION REPAIR of 8-oxoGUANINE in MAMMALIAN CELLS

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The generation of reactive oxygen species in the cell provokes in DNA, among other lesions, the formation of 8-oxo-7,8-dihydroguanine (8-oxoG) and abasic sites. Due to mispairing with adenine during replication, 8-oxoG is highly mutagenic. To prevent high rate of mutation, human cells have a specific 8-oxoG DNA glycosylase (hOGG1) that initiates the base excision repair (BER). hOGG1 excises the oxidized base and generates an abasic site that is cleaved by the major human AP endonuclease (APE1). We have previously shown that the glycosylase activity of hOGG1 is stimulated by APE1. This interaction of the first two enzymes sets the stage for a series of functional and physical protein-protein interactions that result in a highly coordinated process of repair. The initiation of AP sites repair by APE1 is stimulated by XRCC1 through a physical contact between the proteins. XRCC1 is an essential protein, first described as involved in repair of DNA single stranded breaks, as well as in ligation, the last step of BER. Aside from APE1, XRCC1 is known to interact with BER protein such as LIG3, Polß, PARP1 and PNK. We present here evidence for an XRCC1 interaction with hOGG1 at the first step of the BER of 8-oxoG. The interaction XRCC1-hOGG1 was found by yeast two-hybrid and confirmed by GST pull down assays. Results indicating that XRCC1 can stimulate hOGG1 activities will be presented.
Ionising radiation induces clustered DNA damage where two or more lesions are located proximal to each other on the same or opposite DNA strands. It was suggested that individual lesions within a cluster are removed sequentially and the presence of a vicinal lesion(s) may affect the rate and fidelity of DNA repair. In this study we addressed the question of how 8-oxoguanine located opposite to normal or reduced abasic sites would affect the repair of these sites by the base excision repair system. We find that 8-oxoguanine located opposite to an abasic site does not affect either the efficiency or fidelity of repair synthesis by DNA polymerase β. In contrast, 8-oxoguanine located one nucleotide 3'-downstream to the abasic site significantly reduces strand displacement synthesis supported by DNA polymerase β or δ, and cleavage of the generated flap by flap endonuclease, thus inhibiting the long-patch base excision repair pathway.
I.P.25. REMOVAL of DAMAGED DNA BASE is the RATE-DETERMINING STEP of BASE EXCISION REPAIR

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Base excision repair is one of the major pathways for repair of simple DNA base lesions, such as the products of deamination, oxidation and alkylation. Repair is initiated by DNA glycosylases, which cleave the N-glycosidic bond linking the base to the sugar. There are a number of different glycosylases in human cells, each with its own unique specificity. We have examined the repair of several DNA base lesions: uracil, 8-oxoguanine, hypoxanthine and 5,6-dihydrouracil, each of which is a substrate for a different glycosylase. Closed circular DNA substrates containing the lesions were constructed and incubated with cell-free extracts derived from human fibroblasts. Preliminary results show that the rates of removal of damaged bases in cell extract are: dihydrouracil > uracil > hypoxanthine > 8-oxoguanine. We also find that in each case after base removal and formation of an abasic site repair readily proceeds to completion. These data indicate that in cell-free extracts the rate-determining step of base excision repair is removal of the damaged DNA base.
I.P.26. CHARACTERISATION of BASE EXCISION REPAIR in an XRCC1 DEFICIENT CELL LINE

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XRCC1 has been implicated in short-patch base excision repair (BER) through its interaction with DNA ligase IIIα, DNA polymerase β, polynucleotide kinase and poly(ADP-ribose) polymerase. Whilst no enzymatic function has been attributed to XRCC1, cells that contain a mutated XRCC1 gene have been isolated and show increased chromosomal instability and sensitivity to alkylating agents (Zdzienicka, M.Z. et al. (1992) Mutagenesis, 7, 265-269). We investigated the efficiency of both short- and long-patch BER pathways using whole cell extracts derived from EM-C11 and the parental control CHO-9 cells, and closed circular DNA substrates containing either a normal or reduced abasic (AP) site at a predetermined position. The latter can only be repaired via the long-patch pathway and allows us to distinguish between the two BER pathways. We find a partial deficiency in the repair of normal AP sites in the EM-C11 cells when compared to the parental control cell line as previously shown by others (Cappelli, E., et al. (1997) J Biol Chem, 272, 23970-23975). However, we also observed a similar decrease in the ability of EM-C11 cells to repair a reduced AP site, suggesting that both short- and long-patch BER pathways are deficient in EM-C11 cells.
I.P.27. DNA REPAIR ENZYME ACTIVITY DETECTION USING MICRO SUPPORTS

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Cell exposure to different physical (ionising and UV radiations) or chemical agent is known to induce lesions in DNA. If not repaired these lesions are responsible for the occurrence of mutations in the genome. Damage in the DNA are eliminated by a complex network of repair mechanisms composed of enzymes and proteins. The Base Excision Repair system (BER) is in charge of « small lesions » such as strand breaks or oxidative damage and the Nucleotide Excision Repair (NER) pathway is in charge of the repair of bulky lesions, such as the UV-induced pyrimidine dimers. The release of the damage is followed by DNA synthesis to fill the excision gap.

The aim of this work is to set up a biochip based tool aimed at the quantification of damage repair by cell extracts, measured as excision/resynthesis rate of different DNA lesions. For this purpose we immobilize on definite area of Poly-L-Lysine covered glass microarray, plasmid DNA bearing range of specific damage. The spots are incubated with whole cell extract. During this reaction, enzymes excise the lesions and replace them with fluorescent nucleoside triphosphates. The damage repair is correlated to the fluorescent signal appearance on the micro spots.

The first step of the project consists in setting up the right parameters to obtain reproducible results. These parameters are the: plasmid concentration, damage quantity, repair medium composition. We also tested different fluorescent markers and controlled the reaction specificity.

This tool displays several advantages over the existing methods. It allows working with small amount of biological samples. It also permits to perform comparative studies between the different repair system activities contained in the same biological sample. The biochip technology should result in a gain of sensitivity and new applications could emerge from this progress.

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I.P.28. ROLE of MITOCHONDRIAL REPAIR of 8-oxoGUANINE in AGING

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Mitochondria are essential for cell viability, and oxidative damage to mitochondrial DNA has been implicated as a causative factor in a wide variety of degenerative diseases, and in cancer and aging. Reactive oxygen species (ROS) are formed in all living cells as a by-product of normal metabolism (endogenous sources) and following exposure to environmental compounds (exogenous sources). Endogenous ROS are largely formed during oxidative phosphorylation in the mitochondria of eukaryotic cells and, therefore, mitochondrial DNA is at particularly high risk of ROS-induced damage. One of the most common oxidative DNA lesions is 7,8-dihydroxyguanine (8-oxoG), which can introduce G:C to T:A transversions when replicated. 8-oxoG is repaired primarily by the base excision repair (BER) pathway where the 8-oxoguanine glycosylase incises the lesion in the first step of repair. We previously demonstrated that mammalian mitochondria efficiently remove 8-oxoG from their genome, yet 8-oxoG accumulates in mitochondrial DNA during aging.

Cockayne Syndrome (CS) is a segmental premature aging syndrome in humans that has two complementation groups, CSA and CSB. Clinical features of CS seem reminiscent of syndromes that involve mitochondrial dysfunction (i.e., neurological disease and dysfunction in heart, skeletal muscle and kidneys). One possible explanation of this observation is that mitochondrial DNA damage accumulates in CSB cells. Previous studies showed that CSB-deficient cells have reduced capacity to repair 8-oxoG. Here we have examined the role of the CSB gene in regulating repair of 8-oxoG in mitochondrial DNA in human and mouse cells. 8-oxoG repair was measured in liver cells from CSB-deficient mice and in CSB-deficient human cells carrying expression vectors for wild type or mutant forms of the human CSB gene. Evidence is presented to support the hypothesis that CSB regulates expression of the 8-oxoguanine glycosylase. Although we find that the ATPase domain of the CSB protein is particularly important in various functional assays it does not seem to be essential for its role in mitochondrial 8-oxoG repair.
1.PO.29. EFFECT of the DIN7 PROTEIN on the STABILITY of the MITOCHONDRIAL DNA in 
*Saccharomyces cerevisiae*

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We reported previously that the product of the DNA damage-inducible gene of *S. cerevisiae*, DIN7, belongs to the XPG family of proteins that are involved in DNA repair and replication (Mieczkowski et al. 1997). The family includes *S. cerevisiae* Rad2p and its human homolog XPGC, Rad27p and its mammalian homolog FEN-1, and Exonuclease I. All these proteins are endowed with DNA nuclease activity. Din7p is specifically located in mitochondria (Fikus et al. 2000). We have found that overproduction of Din7p from the DIN7 gene placed under control of the GAL1 promoter dramatically increases the frequency of the mitochondrial petite mutants and the frequency of mitochondrial mutations conferring resistance to erythromycin (E'). In order to get more insight into the nature of the effect of Din7p on metabolism of mtDNA, we asked the question whether the mitochondrial mutator phenotype caused by overexpression of DIN7 is related to the nucleolytic activity of Din7p. Sequence alignment of the proteins belonging to the XPG family reveals two regions of strong homology designated the N (N-terminal) and I (internal) blocks. Therefore, we constructed the din7-D173A allele altered in the sequence encoding I domain and examined the effect of the wild-type Din7p and Din7-D173Ap on the frequency of the mitochondrial mutations. It appeared that in contrast to Din7p, overproduction of the Din7-D173Ap fails to increase the frequency of petite formation and the frequency of generation of E' mutants. We also investigated the effect of Din7p on the stability of mitochondrial microsatellite sequences. It appeared that overproduction of Din7p but not of Din7-D173Ap considerably increases the frequency of 2 bp deletions within poly GT tract inserted into the arg8mut gene. Taken together, these results strongly suggest that elimination of the nucleolytic activity of Din7p abolishes mitochondrial mutator phenotype conferred by the elevated level of the protein. Interestingly, the mitochondrial mutator phenotype observed in cells overproducing Din7p is counteracted by concomitant overexpression of the MSH1 gene encoding a homolog of bacterial MutSp and involved in yeast mitochondrial DNA error avoidance. Finally, mtDNA isolated from petite mutants induced by enhanced production of Din7p was subjected to restriction analysis. It appeared that the restriction patterns of mtDNA isolated from all tested clones were clearly different from the rho+ control indicating that the clones are rho-. Since it is generally believed that processes involving recombination are responsible for the production of mtDNA deletions and/or repetitions of the conserved DNA during formation of rho- mutants, we are testing the possibility that the elevated level of Din7p results in the enhanced frequency of mtDNA recombination. Possible mechanisms by which Din7p interferes with metabolism of mtDNA in *S. cerevisiae* will be discussed.

Stationary-phase mutation (also called adaptive) occurs in non-dividing cells during prolonged non-lethal selective pressure, e.g. starvation for an essential amino acid. Because in such conditions no DNA replication is observed, mutations arise probably as a result of non-efficient DNA repair. In order to understand the role of the yeast mismatch repair (MMR) system in the mutagenesis in stationary-phase cells, we studied the effects of deletions in genes encoding MutS- and MutL-related proteins on the reversion frequency of the $lys2\Delta Bgl$ frameshift mutation. We found that the level of Lys$^+$ reversion was increased in all MMR mutants. The strongest effect was observed in strains with deletion of the $MSH2$, $MSH3$ or $MSH6$ genes ($MUTS$ homologs). MutL-related proteins were also required for mutation avoidance in stationary-phase cells, but to a lesser extent than the MutS homologs. Among MutS homologs Mlh1 seems to play the major role in this process, while Pms1 and Mlh3 are partially redundant and can substitute for each other. These data suggest that all MMR proteins, particularly the MutS homologs, are involved in the control of mutability in stationary-phase cells.
I.P.31. POST – TRANSLATIONAL MODIFICATION of MISMATCH REPAIR PROTEINS MSH2 and MSH6

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Mammalian mismatch repair (MMR) is involved in the removal of mispaired bases, the prevention of mutations and cancer and, for various agents including anticancer drugs, the induction of genotoxicity and apoptosis. Despite the importance of mismatch repair for preventing mutagenic and carcinogenic effects of agents inducing mispairing lesions, only limited data is available concerning the post-translational regulation of mismatch repair. Previously we have shown that treatment of cells with O6-methylguanine generating agents results in increased GT mismatch binding of MutSα and translocation of the repair complex from the cytoplasm into the nucleus. Extending this work we report here that MutSα mismatch binding activity is modulated by phosphorylation. We demonstrate that MutSα composed of MSH2 and MSH6 is subject to phosphorylation in vitro by protein kinase C (PKC) and casein kinase II (CKII), but not by protein kinase A. Phosphorylation of MSH2 and MSH6 was also observed under in vivo conditions and was found to be reduced by incubation with CKII-specific inhibitor quercetin. Phosphorylation of MutSα impacts the GT binding activity of the repair complex. Thus, lack of MSH2 and MSH6 phosphorylation within the cell due to phosphate depletion or kinase inhibition resulted in abrogation of nuclear MutSα translocation and significantly reduced GT binding activity of the complex. In addition, treatment of cellular and recombinant MutSα with different phosphatases (λ-PPase, CIP and SAP) abrogated its GT binding activity. Phosphorylation of MSH2/MSH6 may be considered to be part of the response of cells to genotoxic stress. This work was supported by DFG, SFB519/B4.

I.P.32. **SPONTANEOUS and BLEOMYCIN INDUCED GENOMIC ALTERATIONS in the PROGENY of a MISMATCH REPAIR MUTANT of *Drosophila***

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Deficiency in DNA mismatch repair (MMR) confers instability of simple repeated sequences (MSI) and has been linked to cancer development. Although the fundamental role of the MMR genes is to correct replication errors, some of these genes are also implicated in other repair and cellular processes related to DNA damage response. The *Msh2* gene is essential for MMR and has also been implicated in double-strand break repair, transcription-couple repair, meiosis and apoptosis. Therefore, lack of Msh2 function can lead to a global genomic instability, besides MSI. In addition, this instability might depend on the *Msh2* status (homozygous versus heterozygous) and might be modulated by environmental factors.

We studied the spontaneous and induced genomic instability in germ cells of *Drosophila* that are deficient to one or two copies of the *Msh2* gene. The genomic instability in the germline was estimated by analysing the DNA alterations in the progeny of individual crosses of flies with different genotypes: *Msh2*−/−, *Msh2*+/− and *Msh2*++ , using the arbitrarily primed polymerase chain reaction (AP-PCR) as a genomic fingerprint assay. The progeny of the *Msh2*− and *Msh2*++ parents shown higher frequency of spontaneous genomic alterations than the *Msh2*+/− progeny. In addition, the DNA damage transmitted to the progeny, after exposure of adult parental males to bleomycin, indicates that whereas the induction of mutations related to microsatellite instability depends on the lack of the *Msh2* function, the induction of other mutational events may require at least one functional *Msh2* allele.

In the present study, the results obtained with heterozygous individuals may have special relevance for cancer development since they show that a disrupted *Msh2* allele is sufficient to generate inherited genomic damage, being this effect enhanced by mutagenic stress.
Trinucleotide repeats associated to human diseases, such as myotonic dystrophy (CTG repeat) and fragile-X syndrome (CGG repeat), are unstable both in somatic and germ cells. This instability is modulated by different factors, including length and purity of the repeated sequence, mitotic drive and replication and repair processes, although no data exist on the possible role of mutagenic stress on such instability.

By analysing single cell clones derived from the SW480 cell line, which have normal length alleles for trinucleotide repeats associated to myotonic dystrophy and fragile-X syndrome, we have show that mitomycin C and, to a lesser extent, bleomycin induce instability in these trinucleotide repeats. However, no alterations were found when a mononucleotide repeat was analysed (BAT-25). In addition, a low level or a lack of instability in these trinucleotide repeats was found in cell lines deficient in mismatch repair (LoVo and HTC116) and in their functionally corrected by chromosome transfer counterparts, respectively. This would indicate that the induced instability is basically independent of this repair process. We have also found that the majority of the SW480 clones identified as unstable after the exposure to the selected mutagens (bleomycin or mitomycin C), presented mutations in both trinucleotide repeats, which would suggest a common and specific trans-acting mechanism of induced instability for trinucleotide sequences.

Therefore, our data would indicate that the mutagenic stress might contribute to generate new alleles variants within the normal length range of disease-associated trinucleotide repeats.
I.P.34. RECRUITMENT of NER FACTORS in the NUCLEUS of MAMMALIAN CELLS FOLLOWING LOCAL EXPOSURE to UV LIGHT

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We have analysed the assembly of the nucleotide excision repair (NER) complex in normal and repair-deficient (xeroderma pigmentosum) human cells, employing a novel technique of local UV-irradiation combined with fluorescent antibody labelling. Local exposure of small parts of the nucleus of normal human cells causes rapid accumulation of NER factors (XPC-hHR23B, XPA-RPA, XPF-ERCC1, TFIH (XPB and XPD)) to the sites of local damage. This accumulation disappears after about 2 hours. However, when locally irradiated cells are incubated in medium containing inhibitors of the DNA synthesis step of NER, i.e. hydroxy urea plus araC, the accumulation of NER factors at local damage sites persists at least for 16 hours suggesting that the NER factors stay at the damage sites until the repair process is finished.

Analysis of the accumulation of NER factors in various NER deficient cells obtained from patients belonging to various complementation groups of the genetic disease xeroderma pigmentosum showed that in XP-D, XP-F and XP-G cells the NER complex is still formed at local sites of UV exposure. This was shown using antibodies against XPA, XPB, XPC, XPD, ERCC1, RPA and XPG. In contrast XP-C cells did not show any accumulation of NER factors at locally damaged sites, suggesting that the XPC-hHR23B complex is involved in damage recognition and is essential for the accumulation of all other NER factors. In XP-A cells most NER factors investigated (XPC, XPB, XPG, RPA) were still able to accumulate at locally damaged sites with the exception of ERCC1. Apparently XPA is not responsible for recruiting of other NER factors to the repair complex except ERCC1. Interestingly, RPA which normally forms a complex with XPA, is still recruited to damage sites in XP-A cells. This observation suggests that XPA associates relatively late, is required for anchoring of ERCC1-XPF and may be essential for activation of the endonuclease activity of XPG. These findings support a concept of sequential assembly of repair proteins at the site of the damage rather then a preassembled repairosome.
I.P.35. SEQUENCE-SPECIFIC REPAIR of UV DAMAGE in HUMAN PERICENTROMERIC HETEROCHROMATIN

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Nucleotide excision repair is responsible for the removal of UV photoproducts from DNA. One of its two subpathways, the global genome repair system (GGR), removes damaged nucleotides from transcriptionally inactive and relatively condensed parts of the genome, which constitute the majority of sequences; these are not affected by the other subpathway, transcription coupled repair (TCR). The most condensed chromatin is found in pericentromeric regions, which is involved in a disproportionate number of chromosome aberrations. We have developed a multiplex, quantitative PCR based method to monitor UV damage and repair in the human pericentromeric heterochromatin sequence, chAB4. We have analysed repair kinetics of 10 human (primary human fibroblast, HeLa, A375, RVH421 and melanomas, HT1080 fibrosarcoma, TPC1 thyroid papillary carcinoma, EJ30 bladder carcinoma, two XPA (GM01630 and GM02994) and an XPC (GM02096) and 2 human/rodent hybrid cell lines containing one human chromosome 15 (A9+15, 15A). Rates of repair of the heterochromatin sequence vary between cell lines. In some lines (primary human fibroblast, HT1080, A375, TPC1) rates were comparable to TCR, with 90% of lesions removed within 12 hours. Remarkably, heterochromatin repair was rapid even in human/rodent cell lines where overall repair is known to be slow. The Xeroderma pigmentosum A and C lines, and RVH421, HeLa and EJ30 showed little or no repair in this region. Treatment of HeLa with butyrate, an inhibitor of histone deacetylase which causes chromatin decondensation and increases overall rates of UV repair, does not affect the repair of the heterochromatic chAB4 sequence.
PhIP (2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), a carcinogenic heterocyclic amine formed during cooking, is believed to be involved in the etiology of colon cancer. Metabolically activated PhIP (N-OH-PhIP) forms bulky adducts mainly at C8-guanine. We aimed at investigating the involvement of NER in the repair of PhIP DNA adducts, and the possible influence of the tumour suppressor Adenomatous polyposis coli (Apc) gene on this process. For this purpose, we examined the repair of DNA lesions induced by N-OH-PhIP in wild-type (WT) and NER-deficient lymphoblastoid cell lines (XPA−/−, XPC−/−, CSB−/−) as well as in Apc+/− and Apc+/+ mouse colonic cell lines. Cells were cultivated for repair in the presence of repair inhibitors (RI) for the accumulation of sites of DNA repair incision, and the resulting DNA single-strand breaks (ssb) were measured by alkaline elution. At low concentrations of N-OH-PhIP (0.3-3µM), WT lymphoblastoid cells exhibited a time-dependent accumulation of ssb. In XPA−/− cells no accumulation of ssb in the presence of RI was observed, whereas in XPC−/− and CSB−/− cells the accumulated levels were very low. The induced DNA-adducts were partly alkali labile. Repair was hence suppressed in the three NER-deficient cell lines. We conclude that PhIP forms DNA adducts that are repaired via NER in WT lymphoblastoid cells and that both the global genomic and transcription-coupled repair seem to be involved. In Apc−/− mouse colonic cells the incision of PhIP DNA adducts was lower than in Apc+/+ cells. This suggests that mutations in the Apc gene may be involved in the increased susceptibility for colon cancer induced by, e.g., heterocyclic amines.
I.P.O.37. UBIQUITINATION DETERMINES BALANCE BETWEEN HOMOLOGOUS RECOMBINATION (HR) and ENDJOINING (NHEJ) in YEAST

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The Ubiquitin Proteasome Pathway is required for the regulation of various cellular processes. It has been proven for Saccharomyces cerevisiae that postreplication repair is also assigned to ubiquitination (1). The RAD6, UBC13, MMS2 genes encode ubiquitin-conjugating enzymes (E2), and the RAD18 and RAD5 genes code for RING finger proteins which function as ubiquitin ligases (E3) and mediate physical contacts between the members of the RAD6 pathway. Rad5p recruits the Ubc13p-Mms2p complex, and Rad5p association with Rad18p brings it into contact with the Rad6p/Rad18p complex. The Rad6p/Rad18p complex and the Rad5p/Ubc13/Mms2 complex differ in the type of ubiquitination. Rad5p has a regulatory function for the repair of restriction enzyme–mediated deletions in plasmids. In RAD5-proficient cells, correct DSB and gap repair of plasmids occur by (HR) only. In rad5 mutants, 75% of gap repair is performed by NHEJ (2).

In order to study the roles of the RAD6 group proteins for the regulated HR/NHEJ balance, we analysed rad6, rad18, ubc13, mms2 mutants and the respective rad5 double mutants using a two-marker plasmid. ubc13, mms2 and the double mutants show 30% correct repair by HR while the remaining 70% were repaired by NHEJ yielding ura’ clones. rad6 and rad18 mutants, however, restore the gap by HR nearly as efficient as wildtype. In rad5/18 and rad5/6 double mutants, the rad5 phenotype is fully suppressed which supports the biochemical findings that Rad5p, Rad6p and Rad18p act in a complex (1).

We propose that the regulatory decision whether gap repair of plasmid DNA is performed by HR or NHEJ depends on the Lys48- or Lys63-type of ubiquitination controlled by the different members of the RAD6 epistasis group. These differences in ubiquitination may have differential impact on the degradation or conformational changes of chromatin proteins. Chromatin conformation in turn may determine whether gap repair of the plasmid DNA is done by HR. As E2 and E3 homologues of the RAD6 group exist in mammalian cells we speculate that their specific HR/NHEJ balance may be as well dependent on differential ubiquitination.

EC grant FIGH-CT-1999-00010

2. Ahne F. et al., NAR 25, 743, 1997. ()
I.P.38.  DEFICIENCY in 20S PROTEASOME ACTIVITY CAUSES SPONTANEOUS MUTATOR PHENOTYPE of *S. cerevisiae*

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Ump1 is a small chaperone-like protein specifically engaged in assembly of 20S proteasome. Dysfunction of Ump1, the 20S proteasome maturase, leads to reduction of the proteasomal peptidase activities and causes a defect of ubiquitin mediated proteolysis. Here we show that deletion of *UMP1* causes spontaneous mutator phenotype in *S. cerevisiae*. The frequency of spontaneous mutations occurring in the strains carrying deletion of UMP1 increases 2-7 times depending on the genetic background and marker analyzed. The analysis of the spectrum of mutations in the *SUP4-o* gene reveals, that mutations of GC base pairs predominate in strains carrying *∆ump1*. The mutator phenotype is suppressed by *rev3* mutation impairing the activity of polξ. These results implicate the role of 20S proteasome in the maintenance of genetic stability of yeast cells. To verify this hypothesis we determined the frequency of spontaneous mutations occurring in strains carrying mutations in active centers of proteasomal peptidases. It has been established that mutations affecting chymotrypsin-like or trypsin-like activity of 20S proteasome causes an increase of spontaneous mutagenesis. The results point to the 20S proteasome as a new modulator of the level of spontaneous mutagenesis in *S. cerevisiae*. The mechanism of the mutator effect will be discussed.

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DNA polymerase ε is a distinctive replicative polymerase found first in *Saccharomyces cerevisiae*. The largest subunit, Pol2, has polymerase activity; however, the non-catalytic C-terminus is the only part of Pol2 essential for viability. The C-terminus is also essential for the S/M checkpoint. To help identify the essential function of the non-catalytic domain of POL2, we carried out a yeast two-hybrid screen using the C-terminus as bait. The screen revealed a strong interaction between the C-terminus of Pol2 and Trf5. Further two-hybrid analysis revealed that a TRF5 paralog, TRF4 (72% similar and 52% identical to TRF5), also interacts with POL2. TRF4 has recently been shown to encode DNA polymerase κ and is essential for DNA replication in the absence of TRF5, suggesting that TRF4 and TRF5 play redundant roles. Pol kappa falls in the UmuC/Din1/Rev1/Rad30 family. Like other family members, it may carry out a limited amount of synthesis to bypass obstructions to pol ε in the template. We are currently studying the influence of purified pol κ on pol ε using in vitro polymerase assays. Both trf4 and pol2 are defective in both DNA repair and in the S/M checkpoint. Also, Trf4 is required for establishment and maintenance of sister chromatid cohesion. The interaction between Trf4/5 and Pol2 suggests that pol ε in conjunction with pol κ is involved not only in elongation during replication, but also in establishment of sister chromatid cohesion, which is essential for faithful segregation of chromosomes at mitosis. We used C-terminal pol2 mutants to directly test if pol ε might play a role in sister chromatid cohesion. Using centromeres tagged with GFP, we find that a mutant strain pol2-12 is defective in sister chromatid cohesion. Furthermore, mcd1/scc1 is synthetically lethal with a pol2-12 mutation (Mcd1/Scc1 is a component of cohesin).
I.PO.40. STUDIES on MECHANISMS INVOLVED in MAMMALIAN MUTAGENESIS

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By applying new methodology, we have challenged the interplay between the processes involved in bypass mechanisms during replication stress and their importance for establishing gene mutations. When DNA replication is stalled due to non-coding DNA lesions, mechanisms are triggered that can bypass the lesions during replication. During the bypass process, the so-called translesion synthesis (TLS), double strand breaks (DSB) may be formed, which trigger repair by homologous recombination (HR) or non-homologous end-joining (NHEJ). We used the alkaline DNA unwinding technique (Ahnström and Erixon, 1981) to study replication gaps from different lesions and at the same time, investigated the level of mutations induced in the hprt gene (Jenssen, 1984). In parallel, we apply studies on homologous recombination by using the SPD8 cell line, which is a mutant exhibiting a partial tandem copy of the hprt gene found to revert by a rad51-supported homologous recombination mechanism (Arnaudeau et al., 1999). We found earlier a good correlation between the level of replication gaps per loci and the number of mutations induced in the hprt gene (Jenssen et al, 2002). The results indicated that the level of mutations induced by alkylating agents was in agreement with expectation of the low fidelity DNA polymerases, e.g., about 1 per 10⁴. Furthermore, our recent findings were that when recombination pathways are blocked, increased levels of mutations were obtained. We suggest, as a working hypothesis, that TLS might be interplay between pathways involving low fidelity DNA polymerases and recombination mechanisms, the former pathway being more error-prone. Thus, the level of mutations formed might be a matter of competition between the type of substrate formed during replication stress and the proteins involved in different alternative bypass mechanisms.

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The INFLUENCE of X-RADIATION on the EXPRESSION of DNA-PK SUBUNITS in TWO L5178Y SUBLINES DIFFERING in RADIOSENSITIVITY

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Of the two murine lymphoma L5178Y (LY) sublines, LY-S is more sensitive to ionising radiation than LY-R due to impaired repair of DNA double-strand breaks (DSB) (Włodek D and Hittelman WN, 1987, Radiat.Res. 112, 146-155). The defect appears to be limited to G₁ phase of the cell cycle as the survival of LY-S cells irradiated in G₁ phase was several hundred-fold lower than in LY-R. The recovery after irradiation in G₂/M phase was equal in both sublines (Włodek D and Hittelman WN, 1988, Radiat.Res. 115, 550-565) and DSB inflicted in S-phase by camptothecin, a specific topoisomerase I poison, were rejoined even faster in LY-S than in LY-R cells (Grądzka I et al., 1998, Cell Biochem.Funct., 16, 239-252). In mammalian G₁ cells the predominant way of DSB repair is non-homologous end joining (NHEJ), greatly accelerated in the presence of DNA-dependent protein kinase (DNA-PK). PCR analysis of genes encoding DNA-PK components, i.e. a regulatory heterodimer Ku70/Ku80 and a DNA-PK catalytic subunit (DNA-PKcs) showed identical DNA patterns for both LY sublines. Also, no differences were found in the enzymatic activities, as measured by an in vitro ‘pulldown’ kinase assay. Yet, a specific DNA-PK inhibitor, OK-1035, slowed down DNA repair only in LY-R cells, with no effect in LY-S cells (Kruszewski M et al., 1998, Mutat.Res., 409, 31-36). RT-PCR analysis revealed lower constitutive expression of Ku70 and Ku80 genes in LY-S cells. X-irradiation (2 Gy) of LY-S cells induced continuous increase in the levels of all three mRNAs for DNA-PK subunits for at least 1 hour post-treatment, possibly as a response to the sustained DNA damage. On the contrary, LY-R cells exhibited only a transient increase in Ku70 mRNA level at 15 min after irradiation. No changes in protein levels, detectable by Western blotting, followed the mRNA level increase in LY-S cells, although in LY-R cells a slight accumulation of Ku70 protein in cytosol at 1 hour was observed. The data point to a link between efficiency of NHEJ and transcription stimulation of genes that code DNA-PK subunits, whereas the nature of the repair defect in LY-S cells remains obscure.
DNA topoisomerases are nuclear enzymes capable of sensing and changing the topology of DNA. They are therefore very attractive components of the pathways for repair of different DNA lesions. A possible involvement of DNA topoisomerase I has been suggested in repair of X-ray induced DNA damage. A common, but not universal, finding in x-ray sensitive mutants is that of an abnormality in the rate of DNA strand break repair.

X-ray sensitive Chinese hamster V79 cells mutant V-C8 of the complementation group XRCC11 shows slow repair of double strand breaks (dsb) as described by Zdzienicka (1). Recently, it has been shown (2) that the mutant is defective in the breast cancer susceptibility gene BRCA2. The very complex phenotype of V-C8 cells was complemented by a single human chromosome 13 providing the BRCA2 gene, as well as by the murine Brca2 (2). All cells used in present study were constructed and kindly supplied by Prof. M.Z. Zdzienicka (Leiden University, Dept of Radiation Genetics and Chemical Mutagenesis).

The enhanced sensitivity to camptothecin, the inhibitor of topoisomerase I, correlated with the decreased activity of the enzyme, was previously observed in the cell mutant V-C8. V-C8 shows 10-fold higher sensitivity to camptothecin, as compared to parental cell line V79. Topoisomerase I was extracted from nuclei of parental cell line, as well as from mutant V-C8 and mutant cells complemented by human chromosome 13 providing the BRCA2 gene or by the murine Brca2. The sensitivity of the enzyme to the inhibitor was measured in vitro using pBR322 as a substrate. The most sensitive was topoisomerase I from the mutant V-C8 cells (65% of inhibition at 50 µM camptothecin). After introduction of human chromosome 13 or murine Brca2 gene the decrease of sensitivity to camptothecin was observed (18% and 13% inhibition at 25 µM camptothecin, respectively). The enzyme from parental cells was not sensitive to 50 µM. camptothecin.

UV-C light is a potent inducer of DNA damage causing genotoxic effects, receptor activation (e.g. EGFR) and cell killing due to apoptosis. Both DNA lesions and activation of death receptors may contribute to apoptosis. To analyse the role of UV-C induced DNA damage in apoptosis, we compared nucleotide excision repair (NER) deficient and proficient Chinese hamster (CHO) cells as to their apoptotic response. We show that hypersensitivity of NER deficient cells (the mutants 43-3B and 27-1 lacking ERCC1 and ERCC3 respectively) to UV-C is due to elevation in the frequency of apoptosis. This clearly indicates that in fibroblasts non-repaired DNA damage is the main trigger of apoptosis following UV-C irradiation whereas damage-independent receptor activation plays a minor role. Apoptosis in NER deficient cells was initiated by proteosomal degradation of Bcl-2 followed by release of cytochrome c from mitochondria and caspase-3,-9 and –8 activation. Transfection of Bcl-2 protected against UV-C induced apoptosis, indicating Bcl-2 to be causally involved. Inhibitors of caspase-3 and –8 blocked UV-C induced apoptosis in NER deficient cells. Caspase-8 activation upon UV-C irradiation results from caspase-3 activity rather than death receptor activation since i) transfection of dominant-negative FADD failed to block apoptosis and ii) caspase-3 expressing but not caspase-3 mutated cells activate caspase-8 upon UV-C treatment. Overall the data provide evidence that UV-C induced unrepaired DNA lesions trigger the mitochondrial damage pathway of apoptosis. However, the lesions do not trigger the response per se. Cells require replication in order to become apoptotic. Therefore we propose that UV-C induced primary DNA lesions become converted into DNA double-strand breaks (DSBs) via replication. These breaks which were shown to arise following UV-C irradiation in replicating cells may act as the ultimate apoptotic genotoxic stimulus finally causing Bcl-2 degradation. p53 appears not to be involved in this process since the cells do not express functional p53 and p53 ko mouse fibroblasts proved to be hypersensitive to UV-C.
I.P.44. INHIBITION of PARP ACTIVITY PREVENTS UV-C-INDUCED RNA POLYMERASE ARREST and APOPTOSIS

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Poly (ADP-ribose) polymerase (PARP) is a nuclear protein that recognizes and binds to DNA strand breaks, using nicotinamide adenine dinucleotide (NAD⁺) as substrate in a mechanism of “nick protection”. The (ADP-ribosyl)ation of histones (“histone shuttle”) and other nuclear proteins has been reported to be involved in chromatin stability, DNA repair and apoptosis. In order to investigate the role of PARP in UV-induced apoptosis, we used a competitive inhibitor (3-aminobenzamide, 3AB) in normal and NER deficient (XPB mutated) CHO cells. This analog of NAD⁺ inhibits the (ADP)ribosylation reaction, but PARP still remains bound to DNA strand breaks. Cells maintained in the presence of 3AB had a decreased apoptotic response after UV-C irradiation. In the same experimental conditions, inhibition of PARP activity prevents the UV-induced transcription arrest after DNA damage. These results were further confirmed by increased clonogenic survival of UV-C irradiated cell when PARP activity is inhibited by 3AB. It was reported that PARP is associated with regions actively transcribed by RNA polymerase II, participates in the assembly of pre-initiation complex and recognizes the template and also could prevent transcription initiation of damaged DNA binding to nicked DNA. The enzyme could in fact co-localize with transcripts and sense DNA damage in actively transcribed regions. It could also participate in transcription arrest simply by decreasing the ATP levels inside the nuclear context after DNA damage and impairing the activity of helicases like XPB and XPD during TCR. CHO cells defective in the XPB gene, which participates in NER (TCR and GGR) and transcription, display decreased effects of 3AB in apoptosis prevention and RNA synthesis recovery in comparison to DNA repair proficient cells. Since PARP stimulation is dependent on DNA strand breaks, these results are consistent with XPB deficient cells generating fewer DNA strand breaks after DNA repair processes, and, consequently, (ADP-ribosyl)ation would not be important for apoptosis induction in this context. Another possibility to be investigated is a possible dependency of PARP on normal XPB activity to signal for apoptosis.

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Fusion tyrosine kinases (FTKs) such as BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFB and NPM/ALK arise from reciprocal chromosomal translocations and cause acute and chronic myelogenous leukemias and non-Hodgkin's lymphoma. Murine hematopoietic growth factor dependent BaF3 cells and cells transformed by FTK (BaF3-FTK) were used to investigate the role of FTKs in response to DNA damage. FTK-transformed cells displayed resistance to genotoxic treatment including γ-radiation and cytostatic agents such as idarubicin and MNNG. More FTK-transformed cells survived genotoxic treatment and were able to proliferate in comparison to parental non-transformed cells. Similar or higher levels of DNA damage was detected in γ-irradiated BaF3-FTK cells in comparison to BaF3 parental cells. Idarubicin induced different amounts of DNA damage in various BaF3-FTK cells. All BaF3-FTK cells treated with MNNG displayed significantly more DNA damage in comparison to BaF3 cells. Despite the extent of genotoxic effect BaF3-FTK cells were able to repair damaged DNA more efficiently than the non-transformed counterparts. We hypothesize that facilitation of the DNA repair in FTK-positive cells may contribute to their resistance to genotoxic treatment.
I.P.46. ROLES of PCNA and ATM in NON-TARGET MUTATION EFFECTS of GAMMA-IRRADIATION in DROSOPHILA X-CHROMOSOME

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The non-target irradiation effects are widespread in low-dose and include bystander effect and induced genetic instability. These effects may be observed in cells that are not irradiated directly but have a contact with irradiated cells or are their progenies. Also non-target radiation effects may be found at the chromosomal level, when irradiated chromosomes induce mutations in unirradiated ones. In primary study on Drosophila, it was found that irradiated male X-chromosomes induce recessive lethals in unirradiated female homologues (Abeleva et al., 1961, Radiobiologiya. 1:123-126). We assume that this effect may be a result of activation of error prone repair in response to preliminary DNA lesions in irradiated chromosome and this mechanism may be responsible for the non-linear effects of low-dose irradiation. In this research we analyse the frequencies of the recessive lethal mutations in the X-chromosome of Drosophila females after the crossing with irradiated males. We used two irradiation conditions: chronic low-dose irradiation during one generation with a dose rate of 60 sGy and acute irradiation a with a dose rate of 10 Gy. For testing our hypothesis we use the mus209 and mei-41 mutant females. Mus209 is a PCNA gene homologue and mei-41 is a homologue of ATM gene. These genes are involved in post-replication DNA repair which may be error prone repair in Drosophila. Results of experimental testing PCNA and ATM roles in non-target effects of low and acute irradiation will be reported.
I.P.47. SOME MECHANISMS of the ADAPTIVE RESPONSE to ALKYLATED AGENTS in Drosophila

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The protective effects of low dose pre-treatment against high dose mutagenesis, e.g. the adaptive response, have been found in bacteria, plant, human and mammalian cells. Nevertheless, this mechanism has been imperfectly studied in vivo (especially in germ cells), although it appears to play the key role in individual fitness to environmental contamination as well as can prevent mutation dissemination in populations. In present work, ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) were applied for modelling processes studied, wild type and Basc strains being used. Males were exposed to high EMS doses by feeding of adults or larvae. To induce the adaptive response, pre-treatment of adult males or larvae with low mutagen doses was carried out. Some physiological parameters (male sterility and fertility), embryonic and postembryonic lethality in F1 as well as frequency of sex-linked recessive lethals (SLRLs) in F2 were analyzed. In adults, the brooding technique was used for fractionation of germ cells treated at different spermatogenesis studies. The response of mature sperm cells to low, high and combined mutagen doses was compared to those in premeiotic cells. MMS at the dose of 0.1mM did not affect male fertility and sterility as well as SLRL rates in both post- and pre-meiotic cells. Nevertheless, pre-treatment of adults with this MMS dose slightly worsened physiological status and significantly increased SLRL frequency as compared to the EMS challenge dose (10mM). Thus, under condition tested we failed in revealing adaptive response of germ cells to alkylating agents. In the second experimental set, larvae were pre-treated with the low EMS dose (0.05mM) whereas next larval stage or adults were exposed to challenge EMS dose (1–5mM). Larvae were more mutagen sensitive, since in this case male sterility and dominant lethality much exceeded those following challenge treatment of adults. However, larval pre-adaptation led to significant reduction in all analysed parameters including SLRL frequency as compared to larval challenge exposure. The positive effects were less evident if adults were exposed to challenge EMS doses. Thus, the adaptive response was revealed in larvae rather than in adults indicating involvement of detoxifying mechanisms extra inducible DNA repair pathways.
I.PO.48. CELLULAR RESPONSES to LOW DOSE IONISING RADIATION and ENVIRONMENTAL CARCINOGENS

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Low dose radiation and pollution are cellular stress agents believed to threat human health. The cellular responses to this low dose DNA damaging agents cannot be directly extrapolated from high, single dose data. The discovery of radioadaptation processes in cells (i.e.: increased resistance to the clastogenic effect of a "challenge dose" administered after a lower "adapting dose") has fuelled the debate on possible cellular processes relevant for low dose exposures. Little is currently known about the precise nature of this underlying mechanism, but there is evidence that it operates by increasing the rate of the DNA repair, resulting in enhanced repair capacity of adapted cells. The mutagenic potential of neutrons and alpha particles on hprt-locus was studied to demonstrate radio-adaptive response (RAR). The number of induced mutants was to be reduced by 33-57% in adapted cells compared to single dose of 2-4 Gy. The most appropriate priming dose was 2 mGy and five hours were essential between the two irradiation for the full development of RAR. The cytotoxic effect of different pollutants was also studied.

Purposes: To verify the hypothesis that the induction of a novel, efficient repair mechanism for DNA damage may be involved in RAR, the DNA damage and repair has been studied in cultured mammalian cells with comet-assay. To approach the contribution of the radical repair processes to RAR, the antioxidant potential of cells was measured in adapted and non-adapted cells. The involvement of radiation induced proteins in enhanced repair capacity of cells was studied with PAGE.

Our results indicate that the increased rate of DNA repair, the enhanced activity of some antioxidant enzymes (superoxide dismutase, catalase etc.) and the synthesis of specific stress proteins (18, 36-38, 60, 70 kDa) are involved in the mutagenic adaptive response induced by low dose high LET radiation.

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I.P.O.49. REPARATION KINETICS of CHROMOSOMAL ABERRATIONS in HUMAN LYMPHOCYTES in EARLY G1-PHASE of the CELL CYCLE

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Chromosomal aberrations (CA) are the results of two independent but inter-acting processes of DNA damage and repair. Ionizing radiation produces promptly, if not directly DNA double strand breaks (DSB). Single strand breaks (SSB) and base damages (BD) can also give rise to indirectly induced DSBs with subsequent formation of CAs. However, the nature of lesions, and the time available for the rejoining of breaks is not well known in the early G1 stage of the cell cycle. Following X-ray irradiation we previously found a considerable increase of exchanges between 0-6 hrs after PHA stimulation, but no changes in the frequency of deletions for the same intervals up-to 12 hrs. This observation suggested a hypothesis about the existence of different repair mechanisms: one of them leading to exchange- and another one to deletion type of aberrations. The present study offers further evidence that the lesions and their illegitimate repair responsible for dicentrics differ from those responsible for acentric fragments. Human lymphocytes were irradiated to 2 Gy of X-rays and then post-treated for 2 hrs with a repair-inhibitor of BDs and SSBs Ara-C, up-to 8 hrs of G1. Both, exchange- and deletion-type aberrations reached the maximum values at 4-6 hrs following the PHA-stimulation, however Ara-C resulted in a smaller increase of exchanges than deletions. We suppose that more BDs and SSBs are repaired and not converted into DSBs in the case of deletions (intra-chromosomal damages) than in the case of dicentrics (inter-chromosomal damages) when cells are only irradiated. Results of a split dose experiment support the same suggestion. When the first 2 Gy-fraction of irradiation was followed by the second 2 Gy fraction up-to 8 hrs intervals, the yield of all aberrations was lower than following single dose administration. However, exchanges increased up-to 4-6 hrs and then declined, but the frequency of deletions remained the same irrespective of the time-intervals between the fractions. It seems that in the formation of exchanges a great proportion of primary breaks induced by the first fraction is available for an interaction with primary breaks of the second fraction, and this process can be prolonged up-to 6 hrs. Deletion formation is probably connected with a rapid repair, which takes less than 2 hrs after PHA stimulation in G1 phase of the cell cycle.
I.P.50. GENOTOXIC POTENTIAL of MCLR in HUMAN HEPATOMA CELL LINE

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Microcystins (MCs) are hepatotoxic cyclic heptapeptides produced by freshwater cyanobacterial species. Their target organ is the liver as they are preferentially taken up by hepatocytes via bile acid transport system. The well-known mechanism of MCs is the inhibition of protein phosphatases. MCs are well known tumor promoters (1). They were suggested to act also as tumor initiators (2). Recently, it has been demonstrated that cyanobacterial extract and MCLR induced the radical oxygen species (ROS) formation (3). This indicates that MCs can also act as oxidative agents and oxidative damage might be involved in the hepatotoxicity and tumorigenicity of microcystins.

In our in vitro study the genotoxic activity of MCLR on human hepatoma cell line (HepG2) was evaluated by using a single cell gel electrophoresis, also called the comet assay. We observed the induction of DNA damage by MCLR. After four hours of treatment a maximal level of DNA breaks was detected. DNA breaks declined with further incubation. In the presence of DNA repair inhibitors cytosine arabinoside (AraC) and hydroxyurea (HU), DNA damage did not decrease, indicating that MCLR induced DNA damage was repaired.

Furthermore, the modified version of classical comet assay, which uses specific DNA repair enzymes for detection of specific types of oxidative DNA damage, endonuclease III (endoIII) and formamidopyrimidine-DNA glycosylase (fpg), for quantifying the levels of oxidised pyrimidines and purines in DNA, respectively, was applied. After the addition of endoIII or fpg, enhanced level of DNA breaks was observed, indicating the presence of oxidised DNA bases. The amount of oxidative DNA damage induced by MCLR was significantly decreased when the free radical scavenger DMSO was added simultaneously with MCLR to the incubation mixture. This confirms the hypothesis that MCLR acts as an inducer of oxidative DNA damage and reactive oxygen species such as superoxide radicals, hydrogen peroxide and hydroxyl radicals are involved in hepatotoxicity of MCs. The reactive oxygen species are probably involved also in MCLR induced cancer initiation and promotion.

I.P.51. GENOTOXICITY of ARSENITE and its METHYLATED METABOLITES

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Chronic exposure towards low concentrations of arsenite via drinking water appears to be one of the largest public health problems and has been associated with increased risk of skin, lung, liver, kidney and bladder cancer. For arsenite, proposed mechanisms are the interference with DNA repair processes and an increase in reactive oxygen species. However, much less is known about the genotoxic potential of the two methylated metabolites monomethylarsonic (MMA(V)) and dimethylarsinic (DMA(V)) acid, probably because in vivo biomethylation is thought to be the main detoxification process. The aim of this study was to investigate the induction of oxidative DNA damage by arsenite, MMA(V) and DMA(V) in HeLa cells by the alkaline unwinding technique. Surprisingly, short term incubations (0.5-3 h) with very low doses of arsenite induced a high frequency of Fpg-sensitive sites, yielding up to about 1.2 Fpg-sensitive sites/10^6 base pairs at a concentration as low as 10 nM. In contrast, the induction of oxidative DNA damage after 18 h incubation with arsenite was rather low even at high concentrations. With respect to the methylated metabolites, both MMA(V) and DMA(V) showed a pronounced dose-dependent increase in Fpg-sensitive sites after short term incubations with 10–250 µM and the induced lesions persisted after 18 h incubation. The induction of DNA strand breaks was rather weak for all incubation times and compounds. In contrast neither arsenite nor pentavalent or trivalent methylated metabolites induced DNA strand breaks or Fpg-sensitive sites in isolated PM2 DNA. However the trivalent methylated metabolites showed a pronounced inhibition of the Fpg-protein. Taken together our results show that very low physiologically relevant doses of arsenite induce a high level of oxidative DNA damage in cultured human cells. Furthermore we demonstrate for the first time a genotoxic potential also of the methylated pentavalent metabolites generating Fpg-sensitive sites at non-cytotoxic concentrations. Therefore it seems questionable whether biomethylation is merely a process of detoxification; potential mechanisms of DNA damage induction are currently investigated.
I.P.52. ETHENO DNA ADDUCTS: KINETICS of FORMATION and PERSISTENCE in DNA REPAIR DEFICIENT MICE EXPOSED to VINYL CARBAMATE

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Ethenobases are promutagenic DNA lesions generated by exposure to carcinogenic agents such as urethane or through endogenous processes. It has been shown that ethenobases are cleaved from DNA by the base excision repair (BER) pathway. However, the role of other repair systems has not been clarified. To examine whether nucleotide excision repair (NER) is involved, we measured the formation and persistence of 1,N⁶-ethenoadenine (εA) and 3,N⁴-ethenocytosine (εC) in hepatic DNA from wild type mice and from NER-deficient mice, following a treatment with vinyl carbamate (Vcar), the proximal metabolite of urethane. 10-day old XPC-/- mice, deficient in global NER, XPA-/- mice, deficient in both global and transcription-coupled NER, and wildtype mice received a single i.p. injection of 250 nmol/g bw of Vcar. Mice were sacrificed from 1 to 16 h after treatment, and εA and εC residues in DNA were analysed by immunoaffinity/³²P-postlabelling. Levels of both etheno adducts increased rapidly in the first two hours following exposure, reached a quasi-plateau between 2 and 8 h, and decreased rapidly thereafter. This complex kinetics of formation/persistence of εA and εC in liver DNA results from the kinetics of: i) distribution of Vcar within the body, ii) metabolic activation of Vcar into Vcar epoxide, iii) repair of etheno adducts. Levels of εA induced by Vcar were roughly 80 times higher than levels of εC. Under these experimental conditions, and within the time-frame investigated, mice deficient in NER did not exhibit higher levels of εA and εC in hepatic DNA than wild type mice, possibly reflecting a redundancy of the repair pathways of etheno adducts in vivo and a rapid repair through the BER system. Experiments are underway to determine whether NER deficiency affects the formation/persistence of etheno DNA adducts under different experimental conditions (e.g. repeated exposure).

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SITE-DIRECTED MUTAGENESIS of Arabidopsis thaliana thi1 GENE: DISTINCT ACTIVITIES for MITOCHONDRIAL DNA DAMAGE TOLERANCE and THIAMIN BIOSYNTHESIS

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Arabidopsis thaliana thi1 gene was cloned in a functional assay for DNA repair deficiency complementation in E. coli. This gene, as well as its Saccharomyces cerevisiae homologue THI4, codes for a mitochondrial targeted protein involved both in the biosynthesis of the thiazole moiety of the vitamin thiamin, and in mitochondrial DNA damage repair/tolerance. In the present study, we performed site-directed mutagenesis experiments to ascertain if the two activities of THI1 are distinguishable. thi1 mutant cDNAs were produced by PCR, and cloned in a yeast constitutive expression vector to test for both activities. Thiamin biosynthetic activity was assayed through complementation of thiamin auxotrophy of the thi4 yeast strain, and mitochondrial DNA repair/tolerance was assayed by determination of induction of petite cells after treatment with MMS or UV light. Curiously, the yeast thi4 strain also present increased petite induction after growth at 37°C. This may also reflect inability to maintain mitochondrial genome stability. The results obtained point to distinct activities of the THI1 protein. The mutant cDNA G121V (replacement of the glycine residue in the position 121 of the encoded protein by a valine) is not functional in the thiamin auxotrophy complementation test, but is able to reduce the level of petite cells after genotoxic treatments to wild-type levels, thus encoding a functional protein for mitochondrial DNA damage tolerance. Other mutant cDNAs are being tested to confirm the separation of the THI1 activities. During the testing of the mutant cDNAs, we found an intriguing phenotype for the thi4 yeast strain, sensitivity to the genotoxic agent 4NQO. Preliminary results with mutant cDNAs suggest that this phenotype is caused by a protective effect of endogenous thiamin in wild-type cells.

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I.P.54. MOLECULAR CLONING of an Arabidopsis thaliana ORTHOLOG of the Escherichia coli mutT GENE

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One of the major mutagenic base lesions in DNA caused by exposure to reactive oxygen species is 8-oxo-7,8 dihydro-2'-deoxyguanine (8oxodG). During DNA replication, it can pair with adenine and cytosine with almost equal efficiencies. E. coli MutT protein hydrolyzes 8-oxodGTP to the monophosphate, thus avoiding the incorporation of 8-oxodG into nascent DNA.

Lack of the mutT gene increases occurrence not only A:T → C:G transversion mutations in E. coli genome (1000 fold over the wild type level), it also causes transcriptional errors (non-genomic mutations). Genes for MutT homolog proteins were identified in various bacteria and mammals. These proteins share the common, conserved phosphohydrolase signature: GX_EX7REVXEEXGU (U = Leu, Val, Ile).

Here we report cloning and preliminary characterization of AtNUDT2 gene of Arabidopsis thaliana, a possible ortholog of E. coli mutT gene. Follow the search of an A. thaliana homolog of mutT gene in the NCBI data base, for further analysis gene MLJ15.8 from chromosome 3 was selected.

The gene encodes 202 amino acids protein with a predicted molecular mass of 23186.95 Da and pI 4.66. Mitochondrial targeting signal is present at the N-terminus of this protein.

A cDNA clone of MLJ15.8 gene (designated as AtNUDT2) was obtained from A. thaliana cDNA library and was integrated into pQE80L vector.

Plasmids: pQE80L with no insert and pQE80L with AtNUDT2 were transformed in to E. coli mutT strain EC5976 [pro, thi Δ(lac, ara) F’ lacZ101, pro] mutT and tested for complementation of E. coli mutT mutator phenotype. The complementation experiments shown that AtNUDT2 protein decreased the elevated spontaneous mutation rate in E. coli mutT strain for about 45% indicating that AtNUDT2 protein might be a functional homolog of the E. coli MutT protein. The further characterization of AtNUDT2 is in progress.
I.P.55. A PLANT-SPECIFIC FAMILY of DNA REPAIR ENZYMES: CHARACTERIZATION of TWO PUTATIVE DNA GLYCOSYLASES from *Arabidopsis thaliana*

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All living organisms must face the task of maintaining their genome integrity, continuously challenged by spontaneous chemical changes and by genotoxic agents of environmental origin. Although our knowledge about DNA repair mechanisms is well advanced in microorganisms and mammals, it is still underdeveloped in plants. The genome sequence of the model plant *Arabidopsis thaliana* provides a powerful tool for the identification of evolutionary conserved as well as plant-specific DNA repair mechanisms. Isolating full-length cDNAs for the correct annotation of two genomic sequences, we have identified in *A. thaliana* two genes encoding proteins that define a new family of DNA repair enzymes. The proteins are 1987 and 1365 amino acids long, and both show a C-terminal domain (240 aa) with extensive sequence similarity to DNA glycosylases from the HhH-GPD superfamily, including a conserved lysine residue and the FCL region. Similar protein sequences are encoded in the genome of other plants but not in archaea, bacteria, fungi or animals. This suggests that they may represent a plant-specific family of DNA repair enzymes. Both genes are actively expressed in different plant tissues, including leaves, flowers, shoots and roots. Overexpression studies are underway in order to determine the substrate specificity of these proteins.
I.P.56  TRANSLESION SYNTHESIS DNA POLYMERASES in *Arabidopsis thaliana*


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Translesion synthesis is an important mechanism used by cells to tolerate the presence of non-repaired DNA damage and overcome replication blockage. Recent studies in microorganisms and mammalian cells suggest that the key participants in this process are specialized DNA polymerases able to copy damaged DNA templates with different degrees of accuracy. These enzymes define a new family of structurally-related DNA polymerases typified by *E. coli* Pol V (UmuC) and Pol IV (DinB), and *S. cerevisiae* Pol η and Rev1. We have identified in *A. thaliana* three genes (AtPOLK, AtPOLH and AtREV1) encoding putative homologues of Pol IV, Pol η and Rev1, respectively. AtPOLK encodes a protein of 672 aa and it is expressed in a wide range of plant tissues. Its transcript undergoes alternative splicing generating at least 3 different mRNAs. Overexpression of AtPOLK cDNA in *E. coli* cells causes a significant increase in their spontaneous frameshift mutation frequency. Thus, like its bacterial and mammalian counterparts, AtPOLK may play a role in the extension of mismatched base pairs during normal DNA replication. AtPOLH encodes a protein of 672 aa and it is expressed in aerial plant tissues, but not in roots. Overexpression of AtPOLH in bacteria protects against the cytotoxic and mutagenic effect of UV light. Finally, AtREV1 transcript undergoes alternative splicing generating at least 4 different mRNAs. Overexpression studies are underway in order to characterize the enzymatic activities of all three proteins.
I.P.57. HUMAN DNA GLYCOSYLASES of the BACTERIAL FPG/MUTM SUPERFAMILY: An ALTERNATIVE PATHWAY for the REPAIR of 8-oxoGUANINE and other OXIDATION PRODUCTS in DNA

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The mild phenotype associated with targeted disruption of the mouse OGG1 and NTH1 genes has been attributed to the existence of backup activities and/or alternative pathways for the removal of oxidised DNA bases. We have characterised two new genes in human cells that encode DNA glycosylases, homologous to the bacterial Fpg (MutM)/Nei class of enzymes, capable of removing lesions that are substrates for both hOGG1 and hNTH1. One gene designated HFPG1, showed ubiquitous expression in all tissues examined whereas the second gene, HFPG2, was only expressed at detectable levels in the thymus and testis. Transient transfections of HeLa cells with fusions of the cDNAs to EGFP revealed intracellular sorting to the nucleus with accumulation in the nucleoli for hFPG1, while hFPG2 colocalised with RPA in replication foci. These results suggest that hFPG2 is involved in the removal of damage originating at the replication fork. Human FPG1 was purified and shown to act on DNA substrates containing 8-oxoguanine, 5-hydroxycytosine, and abasic sites. Removal of 8-oxoguanine, but not cleavage at abasic sites, was opposite base-dependent, with 8-oxoG:C being the preferred substrate and negligible activity towards 8-oxoG:A. It thus appears that hFPG1 has properties similar to mammalian OGG1 in preventing mutations arising from misincorporation of A across 8-oxoG and could function as a backup repair activity for OGG1 in ogg1⁻ mice.
I.P. 58. The *in vitro* REPAIR of DNA DOUBLE-STRAND BREAKS by HUMAN HELA CELL EXTRACT is END-GROUP DEPENDENT

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DNA double-strand breaks (DSBs) are caused, directly or indirectly, by a variety of DNA-damaging agents, including ionizing irradiation and oxidative metabolism. Mammalian cells primarily repair these breaks by nonhomologous end-joining.

Although genetic studies have identified several proteins involved in DSB repair, the exact biochemical mechanism of repair remains unknown. To address this question, we have developed an *in vitro* double-strand-break repair assay using plasmid DNA linearized by the radiomimetic drug bleomycin. Bleomycin damage produces DSBs blocked with 3’-phosphoglycolate (3’-PG). This complex unligatable lesion is similar to those caused by ionizing radiation and other oxidative DNA-damaging agents.

We show that human HeLa cell extracts support end joining of complex DSBs and form multimeric plasmid products. End joining reactions required only 100 ng or less of substrate DNA, and greater than 50% conversion of substrate to product was achieved. Several parameters affecting the repair reaction were examined and optimized. Optimum reaction conditions were found to be DSB end-group dependent.

The initial rate of repair for complex bleomycin-induced DSBs (non-ligateable) was 6 fold slower than for similarly configured (blunt ended), but less complex restriction enzyme-induced DSBs (ligateable). Also, repair of DSBs produced by gamma radiation or 125I was much less efficient than for DSBs produced by bleomycin, or restriction enzymes.

These data indicate that as DSB lesion complexity increases, the ability to repair damage by the end joining pathway decreases. This assay can provide a means for predicting the repairability, and thus the potential cytotoxicity, of DSBs produced by DNA-damaging agents.
Mismatch repair (MMR)-deficient cells can be hypersensitive, both in vitro and in vivo, to killing induced by chemotherapeutic agents that introduce interstrand DNA crosslinks (ICLs). These drugs include cyclohexylchloroethylnitrosourea (CCNU) and mitomycin C. Recent data indicate that double strand breaks (DSBs) are formed as intermediates during the repair of ICLs. Several proteins (XRCC1, XRCC2, XPA and XPF), which are known to have a role in nucleotide excision repair (NER) or in homologous recombination, have been proposed to be involved in the repair of ICLs. We have investigated whether MMR plays a role in the processing of DSBs induced by CCNU. As a model system we used the MMR-proficient Raji cells, derived from a Burkitt lymphoma, and an hMLH1-defective derivative Clone R10. DSBs, as monitored by Field Inversion Gel Electrophoresis (FIGE), appeared 3-6 hr after exposure to CCNU. No significant difference in DSBs formation was observed in the two cell lines suggesting that loss of MMR does not affect the initial steps of ICLs repair. Twelve hours after 1 hr exposure to CCNU MMR-proficient and –deficient cells showed a similar delay in the progression through the S phase of the cell cycle, as determined by cytofluorimetric analyses. Exposure to CCNU induced, however, increased levels of apoptosis in the MMR-defective variant compared to the MMR-proficient Raji cells. The critical event in this differential induction of apoptosis resulted to be the transition through the S phase of the cell cycle. Inhibition of S phase progression by the use of aphidicolin abolished the MMR-dependent apoptosis induced by CCNU. By using this experimental approach we were also able to show that MMR-deficient cells display slow kinetics in the rejoining of these DSBs. Immunofluorescence experiments with specific antibodies against the MRE11 and RAD51 proteins identified alterations in the kinetics of nuclear foci localization at sites of recombination associated with loss of MMR. These data strongly suggest that MMR is required for the correct processing of DNA damage induced by CCNU.
II.PO.1. DNA DAMAGE after EXPOSURE on CYANOBACTERIAL EXTRACT and MICROCYSTIN-LR or MICROCYSTIN-RR

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Microcystins (cyclic heptapeptides) are the most common group of cyanobacterial toxins. The toxicity of microcystins has been well documented but knowledge on their genotoxicity is still relatively insufficient. It was found that microcystins could induce DNA damage in mouse liver cells and in primary rat hepatocytes.

In the present study, DNA damage induced by microcystic cyanobacterial extract (MCE) and pure microcystin-LR (MC-LR) or microcystin-RR (MC-RR) in human lymphocytes in vitro was examined using single cell gel electrophoresis (SCGE) and pulsed field gel electrophoresis (PFGE).

The alkaline version of the SCGE is specific mainly for detecting single DNA strand breaks and alkali-labile sites. The highest value of DNA damage, which could be described in arbitrary units (U), is equal to 400 U. MCE caused significant DNA damage. The highest value of DNA damage (279 U) was noticed after 12 h of MCE exposure. MC-LR was less genotoxic, the highest value of DNA damage being around 103 U. A decrease in DNA damage observed after 24 h exposure could be linked to progressive DNA degradation. Fragments of DNA smaller than 40 kb (described by this method) seem to be lost during alkaline lysis and electrophoresis.

DNA degradation to mega base (mb) and kilo base (kb) fragments was analysed using PFGE. MCE after 24 and 48 h caused a wide range of DNA damage, with fragments in the range of 50-2200 kb, and below 50 kb being detected. MC-LR and MC-RR after 24 and 48 h induced DNA damage with a smaller fragment size range, between 50-1125 kb. One of the hallmarks of apoptosis is the breakdown of chromatin, usually first into 300 kb and 50 kb fragments; the detection of 50 kb DNA fragments could be indicative of the apoptotic process.

Summarising, MCE and pure MC-LR and MC-RR showed DNA damage in SCGE as well as PFGE. The DNA damage after exposure to MC-LR or MC–RR was less pronounced in comparison with MCE. The genotoxicity of MCE depends on the action of the different types of microcystins in the extract. Further studies on the genotoxic effect of the different types of microcystin, not only MC-LR and MC-RR will be needed to evaluate the human health hazard connected with exposure to microcystins in recreational and drinking water reservoirs.
II.P.2. The GENOTOXICITY of TIAMULIN S on CULTURED HUMAN LYMPHOCYTES

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The genotoxic effects of antimicrobial preparation Tiamulin S (Hemovet, Vrsac, Pharmaceutical Company, Yugoslavia) has been investigated in vitro on human peripheral blood lymphocytes by following the capability of Tiamulin S to induce numerical and structural chromosome changes. To test a possible effect of the investigated substance on DNA we used the sister chromatid exchange (SCE) assay in vitro. The investigated substance Tiamulin S was tested at three experimental concentrations: 25 µM, 50µM and 100µM. Negative control groups were treated with physiological saline. Our results show that all experimental doses of the investigated substance Tiamulin S show the potential for transformation of the karyotype of human lymphocytes by inducing numerical aberrations type aneuploidy and polyploidy and structural aberrations type gaps and lesions. The overall cytogenetic changes induced by Tiamulin S at all three doses clearly show a high statistically significant increase in respect to the control group. We have also estimated a correlation between an increase in dosage and cytogenetic changes. Cytogenetic changes and a dose-effect dependency clearly show a genotoxic potential of Tiamulin S on lymphocytes of human peripheral blood. All three doses of Tiamulin S induced a highly statistically significant increase in the frequency of SCE with respect to the untreated control groups. High SCE frequencies show the possibility that the investigated substance generates changes in the DNA structure. Our results classify Tiamulin S a klasogenic agent.
II.P.3. STUDY of the MUTAGENIC ACTIVITY of YUCCAOLS from the BARK of Yucca schidigera Roezl.

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The aim of the present study was to estimate the mutagenic potential of purified phenolic constituents isolated from Yucca schidigera bark: yuccaols A, B, and C, trans-resveratrol, trans-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene in the Ames test.

Yucca schidigera Roezl. (Agavaceae) is a commercially used plant, due to the high (10% in dry matter) concentration of steroidal saponins. This has a GRAS (Generally Recognised As Safe) label approved by the Food and Drug Administration (FDA), which allows usage of this plant and its extracts as food additives and in cosmetics and pharmaceutical industries.

Studies of chemical constituents of yucca revealed also that the bark of this plant contains considerable amount of phenolics. One of them, trans-resveratrol, has been known as one of the strongest natural antioxidants, occurring also in red wines. The bark contains also a high concentration of the trans-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene and three very specific compounds, yuccaols A, B, and C. Yuccaols A, B and C are unique compounds, not reported previously in any other plant species and possess spiro-structures rarely occurring in the plant kingdom.

The mutagenic activity of isolated phenolic constituents was tested by the Ames test with S. typhimurium TA97, TA98, TA100, TA102 in the presence and absence of metabolic activation (fraction S9). Studies on the mutagenicity of phenolics were performed with non-toxic concentrations up to 10 to 100 µg/plate.

The results presented in this study indicated the lack of mutagenic activity of yuccaols A, B, and C, trans-resveratrol, and trans-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene in the Ames test. The above observations suggest that yuccaols could be used in numerous medical treatments without creating any health hazards.
ii.P.4. POTENTIAL GENOTOXICITY in SURFACE DRINKING WATER TREATED with CONVENTIONAL and ALTERNATIVE DISINFECTANTS

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Drinking water disinfection may produce toxic compounds, particularly when water is obtained from surface supplies and then chlorinated. Water chlorination is known to induce mutagenic/carcinogenic by-products formation from reaction of chlorine with organic compounds (humic and fulvic acids) naturally present in water. It is therefore important to test disinfectants alternative to chlorine in order to reduce these potential health risks. The aim of this research was the monitoring of the genotoxicity in surface drinking water treated with three different disinfectants, sodium hypochlorite (NaClO), chlorine dioxide (ClO₂), and peracetic acid (PAA), a possible alternative disinfectant. Comparison of water genotoxic by-product formation by peracetic acid and chlorine based disinfectants was performed to provide useful data to waterworks managers and health authorities. A pilot plant was built and an integrated approach, using in vitro and in vivo genotoxicity tests, chemical analyses and microbiological tests, was followed. The study was carried out during four different seasonal periods (July 2000, October 2000, February 2001, June 2001) in order to assess the treatments at different physical and chemical lake water conditions. In this part of the research, the findings on in vitro genotoxicity of water extracts were reported. Gene conversion, reversion, and mitochondrial mutability were assessed in the Saccharomyces cerevisiae D7 strain, with and without endogenous metabolic activation; DNA damage was evaluated in fresh human leukocytes by the comet assay. In the yeast, P450 resulted as a significant factor (P=0.008), also when associated with the various end-points (P=0.009) and sampling times (P<0.001). The association end-point sampling time also resulted highly significant (P<0.001). A high variability of genotoxic compounds with respect to sampling time rather than to water treatment was found. These conclusions were confirmed in human leukocytes: for example, genotoxic potency of extracts showed an increase (July 2000) or decrease (February 2001) with respect to raw water by disinfection processes. Each disinfectant showed a peculiar activity depending on seasonal variability of raw water quality.
II.PT.5. DETECTION of GENOTOXICITY of ATMOSPHERIC PARTICLES with a HIGHER THROUGHPUT MICROPLATE SOS/UMU TEST SYSTEM

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A sensitive umu-microplate test system with nitroreductase- and O-acetyltransferase-overproducing strain Salmonella typhimurium NM3009 and O-acetyltransferase-overproducing strain S. typhimurium NM2009 has been previously developed for the detection of genotoxic activities of nitroarenes and aromatic amines. The new test system was applied for the detection of genotoxic activity of atmospheric particles in the urban area with quite a small sample load. The environmental particulate samples were collected every month for a year in Osaka City using an Andersen Cascade Impactor, which can fractionate particles size-separately on 9 filter sheets. The monthly samples were extracted by ultrasonication with dichloromethane for 30 min. The sample solution was concentrated to almost dryness and thoroughly dissolved in 20 µl of DMSO, then each 4 µl of the solution was carefully diluted on a PTFE 96-well microplate. After incubation with the exponential bacterial culture for 4 hrs with shaking, a part of the culture was transferred to new plate and 0.1 % SDS plus Z-buffer solution was added. The relative β-galactosidase activity, which reflects umuC gene induction, was determined colorimetrically with CPRG. In this method, the particulate size distribution curve of genotoxic activity was obtained in the presence and absence of metabolic activation for the test strains NM3009 and NM2009, respectively. The results showed that the test system was able to detect slight increase of induced genotoxic activity in the atmospheric particles with quite a small sample load (0.2–0.5 mg of particulate sample) and the genotoxicity was mainly detected in the fine fraction but was partly in coarse fraction. It may be supposed that the genotoxicities of the particles are mainly contributed from direct sources such as fine diesel exhaust particles but are partly from indirect effects of re-suspension such as coarse road dust. These results suggest that the sensitive microplate test system has a capacity to be available for the screening of various other environmental samples.
II.P.6. ALUMINUM INDUCES CHROMOSOMAL ABERRATIONS in the ROOT MERISTEM CELLS of WHEAT SEED

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Comprising 8.8% of the earth’s crust, aluminum (Al) is locked in minerals that conceal its status as the third element after oxygen and silicon. Acid rains release Al ions and compounds from soils and minerals into water where it is accessible to living organisms. Al has been found to cause bone and neurological disorders and Al toxicity has been recognized as a major factor limiting crop productivity on acid soil.

The purpose of the present work was to find out whether structural chromosome aberrations appear in wheat apical meristem cells due to Al and, if they do, to investigate their range and concentration dependence of their yield. Potassium salts and gamma – irradiation (50 Gy) were chosen as control agents.

Spring wheat has been sprouted in solutions of aluminum nitrate and sulfate. Samples of 10 roots of germinated seeds reaching the length of 5-10 mm were fixed in ethanol – acetic acid (3:1) at 4 °C for 2-12 h. Squashed preparations were obtained from apical root meristem and stained with acetic orcein.

After treating the wheat sprouts with aluminum salts the maximum number of cells with aberrations was detected at a concentration of 0.5 – 1.0 mg/l. Further increase in aluminum concentration decreased the yield of structural chromosome aberrations. This is probably related to the cytotoxic effect of the metal, which suppresses cell division. Potassium salts at corresponding concentrations had no cytogenetic effect on wheat cells.

Aluminum ions induced all types of chromosome damage: genomic, chromatid and chromosome aberrations.

We have investigated the mutagenic effect of water boiled for 2 h in an aluminum pot; for control water was boiled in an enameled pot. An insignificant cytogenetic effect of water boiled in an aluminum but not in an enameled pot was demonstrated.

The damage of wheat cells by aluminum is most likely mediated by damage of membrane structures where DNA replication initiation sites are located.
II.P.7. DNA-ADDUCTS INDUCED by VEHICLE EMITTED INHALABLE PARTICLES CONTAINING PLATINUM-GROUP ELEMENTS (PGE)

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There is growing evidence for the detrimental health effects of very small particles in environmental air, although their mechanisms of action still remain unclear. One major source are catalytic converters in car exhaust systems which emit aluminium oxide particles coated with platinum group metals into the environment. The particles can be inhaled and subsequently taken up by lung cells. Thus, elevated concentrations of Pt in various organs after intratracheal instillation of synthetic Al₂O₃–particles coated with Pt have been shown previously in rats [1]. Nevertheless, the resulting species and intracellular distribution are unknown.

Within the present study we aimed to investigate whether PGE bind to DNA after incubation of A549 human lung cells with metal powders of pure platinum and palladium as well as synthetic Al₂O₃ particles coated with Pt. After incubation, cellular DNA was gently extracted and its metal content was determined with a high resolution ICP-MS-System ("Axiom", ThermoElemental). Concentrations of Pt and Pd between 0.5 and 10 µg/cm² caused a dose- and time-dependent increase in DNA adducts in the non-cytotoxic concentration range. The level of platination was comparable to incubation with 10 µM of cis-Platin or 100 µM of PtCl₄ for 24 h. Synthetic Al₂O₃–particles coated with Pt caused a dose-dependant increase in Pt-DNA adducts.

These results point towards the bioavailability of inhalable metallic platinum and palladium. Further investigations are needed to specify the nature and biological consequences of the adducts.

II.P.8. EFFECTS of DI(2-ETHYLHEXYL) PHTHALATE on the SOMATIC and GERM CELLS of MALE MICE

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Phthalate esters are among the most extensively used industrial chemicals and are widely distributed in the environment due to their extensive use as industrial solvents and plasticizers in the manufacturing of plastics. Di(2-ethylhexyl)phthalate is a well-known agent used in polyvinylchloride (PCV) industry for its plasticizing capacities.

This pollutant was detected in lipophilic solutions in some blood preparations in plastic bags, and a case of inhibition of human plateled function by DEHP contaminant has been reported after transfusion[1].

Phthalates, in particular DEHP despite low acute toxicity, produce liver tumours in rodents. DEHP is listed in special class of carcinogens: peroxisome proliferator tumor promoters (PPs).

Mutagenicity of the esters has been investigated by various authors. The clastogenicity of MEHP (hydrolysis product of DEHP) suggested a contributory role for this compound in the observed carcinogenicity of DEHP [2]. This compound induced testicular atrophy and biochemical changes in Sertoli cells. A number of carcinogens and mutagens are known to induce abnormal sperm morphology, and some abnormalities are inherited by the offspring of treated males.

In these studies the mice morphology test, micronucleus test and comet assay were used to determine the effects of investigated compound in germ and somatic cells. Sperm morphology assay is said to provide method for locating genetic damage in male germ line. For estimation of DNA damage comet assay was preferred. The micronucleus test has been widely used, in vivo, as a cytogenetic test for detection of environmental chemicals of clastogenic activity.

Experiments were carried out on Swiss male mice, which received 5 succesive daily doses of DEHP (800, 6000, 8000 mg/kg b.w.) which were administered at 24 h intervals. Controls received the solvent only. The animals were sacrificed 35 days after the first injection. Abnormalities were categorised following Wyrobek and Bruce [3]. DNA damage (in comet assay) were analysed in germ cells using fluorescence microscope. Cells were graded by eye into 5 categories. In other studies we investigated frequencies of micronuclei in reticulocytes from peripheral blood. Experiments were carried out on Swiss male mice 8-10 weeks old. The animals were killed 24 and 48 hours after the last treatment. The smeared and fixed cells were stained with AO. Micronuclei with green fluorescence were scored [4].

The results show that highest doses DEHP (8000 and 9000 mg/kg b.w.) decreased the testes weight and induced sperm abnormalities. After treatment with 9000 mg/kg b.w DEHP slight biological effect in induction of DNA in germ cells was observed.

II.P.9. GENOTOXIC EFFECTS in GLAUCOUS GULLS at SVALBARD

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Glaucous gull (Larus hyperboreus) which is one of the top-predators in the arctic marine ecosystem, at Svalbard has contact with very high concentrations of polychlorinated biphenyls (PCBs). Previous studies suggest that PCBs have a genotoxic effect in birds.

Material for analysis was taken from juvenile gulls of the same age reared from eggs collected in Isfjorden and Kongsfjorden. The chicks were divided into two groups, one was the “clean” (control) and one was supposed to be polluted through the food. All the chicks had a basic diet consisting of polar cod, water and vitamins. The “polluted” group was also fed with eggs of gulls. Seabirds and seabird eggs are suggested to be one of the most polluted ingredients in the diet of glaucous gull. To ensure a diet as similar as possible, the “clean” group had hen eggs when the other group had gull eggs.

The sampling of the birds took place in August 1999. Samples were taken of a total of 39 individuals, 19 birds in the control and 20 in the persistent organic pollutant (POP) exposed group. For the analysis of chromosome aberrations, purified and metaphase enriched peripheral blood lymphocyte and bone marrow spreads were used, and for analysis of DNA strand breaks small samples of whole blood were used. DNA adduct analysis was carried out with liver tissue, which was frozen down immediately after killing the gulls. The results of genotoxic analysis of the two groups of gulls will be compared and discussed.

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II.P.10. GENOTOXICITY of 7H-DIBENZO[c,g]CARBAZOLE and its ORGAN SPECIFIC DERIVATIVE 5,9-DIMETHYLDIBENZO[c,g]CARBAZOLE

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The objective of this study was to assess the role of cytochrome P4501A subfamily in biotransformation of 7H-dibenzo[c,g]carbazole (DBC), a potent liver and skin carcinogen, and its synthetic methyl derivative 5,9-dimethyl dibenzo[c,g]carbazole (diMeDBC), a strict organ specific hepatocarcinogen. Genetically engineered Chinese hamster V79 cell lines with stable expression of human cytochrome P4501A1 (CYP1A1) or cytochrome P4501A2 (CYP1A2) was used to characterize the particular form of P450 isoenzyme involved in biotransformation of DBC and diMeDBC. In addition, a V79 cell line with co-expression of CYP1A2 together with a phase II enzyme, N-acetyltransferase (NAT), was utilized to study the role of an entire metabolic activation system in activation of both carbazoles. The rise of 6-thioguanine resistant (6-TG) mutations and micronucleus formation were followed as endpoints for the assessment of biological activity. Antikinetochore antibodies and immunofluorescence staining were applied to identify centromere-containing micronuclei. Neither carbazole elevated significantly the frequency of mutations or micronuclei in the parental V79MZ cell line lacking any cytochrome P450 activity or in the V79NH cells expressing NAT activity. A variable genotoxicity was found in the cells expressing CYP activity. DBC induced gene mutations as well as micronuclei in the both V79MZh1A1 and V79MZh1A2 cells. In contrast, diMeDBC devoid any genotoxicity in the V79MZh1A1 cells; however, a significant increase of gene mutations and micronuclei were detected in the V79MZh1A2 cells. While DBC induced approximately equal level of kinetochore positive (C+) and kinetochore negative (C-) micronuclei in both cell lines, diMeDBC manifested rather aneugenic than clastogenic activity in the V79MZh1A2 cells. Co-expression of CYP1A2 together with NAT activity significantly reduced or totally eliminated the mutagenicity of DBC and diMeDBC. These data suggested that CYP1A1 is probably involved in the activation of sarcomagenic DBC derivatives, whereas CYP1A2 is included in biotransformation of both DBC and diMeDBC. Reactive intermediates formed due to CYP1A2 activation are, however, substrate for conjugation reaction mediated by N-acetyltransferase.
II.P.11. GENOTOXICITY EVALUATION of the SARNO RIVER POLLUTANTS in MOSQUITOFISH

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The aim of our research was to assess the biological damage caused by exposure of the teleost *Gambusia holbrooki* (Cyprinodontiformes, Poeciliidae) to various mutagenic agents present in the polluted waters of the Sarno river (Campania, Italy).

The first evident effect has been identified on *Gambusia* chromosomes. In fact, the karyotype showed the presence of a centric fusion involving two acrocentric elements and, in several metaphase plates, a gap on chromosome arms.

Subsequently, the presence of micronuclei and DNA migration into an electrophoretic field (comet) were evaluated in erythrocytes from the Sarno river specimens; the results were compared to the individuals of *G. holbrooki* coming from uncontaminated waters. At the same time some *G. holbrooki* specimens from the Sarno river were transferred into the aquaculture laboratory for 100 days.

Finally the randomly amplified polymorphic DNA (RAPD) technique was applied to each specimen of *G. holbrooki* to evaluate polymorphisms that were caused by environmental pollution.

The obtained data showed a statistically significant difference between the groups of *G. holbrooki* examined. In fact the specimens coming from the Sarno waters showed an increase both of micronuclei numbers and of DNA damage at the cell level.

Moreover, the specimens of *G. holbrooki* coming from the aquaculture laboratory presented a decrease of DNA damage after 100 days in the clean water.

Randomly amplified polymorphic DNA analysis showed differences between contaminated and reference populations of *G. holbrooki* in terms of band frequencies.

A more likely explanation of this result, based on literature data, could be that the differences between examined populations are an indication of perturbations of population-level processes as a result of multigenerational exposure to pollutants.

Finally our work represents an integration study on genotoxic somatic effects (DNA damage) and population genetic effects (changes in genetic diversity or gene frequencies).
II.P.12. APPLY of Pica pica L. for ESTIMATION CONTAMINATION of ENVIRONMENT

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Under conditions of intensified anthropogenic influence, the problem of bioestimation of environmental pollution requires increasing attention. One of the most genetically dangerous technical undertakings is a Burshtyn thermal electric power station situated midstream of the river Dniester. Its major pollutants are SO2, NO2, CO and coal ashes that include whole spectrum of dangerous metals’ combinations: Fe, Mn, I, Ni, As, Pb, Sn, Co, Cr, Sb, Al, Ba, Sc, Be and other. Ni, As, Pb, Sn, Co, Cr and Be are regarded as most genetically dangerous and potentially mutagenic in the investigated region.

Taking into account local species accessible for control, we considered a bird community as a test unit. We chose young magpies (P. pica) as the most suitable bioindicator of the degree of pollution in closed and opened landscapes. This is connected with the bird’s restricted radius of flights and features of nourishment that enable us to study local pollution of environment.

We tested chromosomal aberrations in cells of pulps of feathers and the contents of metals in eggshells of birds living in areas of 4-35 km from the Burshtyn thermal electric power station. As a control, we investigated magpies living in a “healthy” area, that is in the district of Bystrytsa river inflows.

The maximum cytogenetic effect was observed in birds living 10-12 km from the contamination source (highest percentage of aberrant anaphases). It exceeded the adopted spontaneous level 3.7-5.4-fold. In these test-objects there was considerable, in comparison with the controls, chromosome aberration spectrum displacement towards chromatic aberrations. This fact confirms the chemical nature of increased mutagenic background in the region of air borne technogenic influence of the thermal electric power stations. Besides, the degree of damage of the P. pica chromosome apparatus positively correlates with the level of biological concentration of metals in the eggshell and a series of morphometric parameters (eggshell thickness and area).

Hence, the magpie (P. pica) population reacts adequately to metal pressure and can be used for estimation of environmental pollution.
II.P.13. MONITORING of TOXICITY and GENOTOXICITY in URBAN AIR with TRADESCANTIA-MCN BIOSSAY and PASSIVE SAMPLING (SPMDs)


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Several epidemiological studies revealed that the exposition to polluted air cause adverse health effects and risks to urban populations. On the basis of these observations, this study was designed to assess the presence of toxic/genotoxic compounds in urban air of Caserta, Southern Italy. Ten different urban sites with different volumes of vehicular traffic were chosen for monitoring gaseous pollutants in two seasons of the year. The bioindicator plant *Tradescantia* (clone 4430) was exposed to the field to detect genotoxic substances and determine differences in the air pollution levels. Genotoxicity was evaluated registering the formation of micronuclei in the meiotic pollen mother cells (Trad-MCN assay). At the five sampling points showing the highest values of micronuclei, semipermeable membrane devices (SPMDs) containing the neutral lipid triolein that mimics the bioconcentration organic contaminants in fatty tissues of organisms were deployed. The dialysates and their extracts were analyzed by GC-MS and HPLC to identify trace levels of organic pollutants while toxicity and mutagenesis were performed on the bacterium *Vibrio fischeri* with the system Microtox and Mutatox respectively. Significant increases in the frequency of micronuclei were observed in different points of the town grid while mutagenesis with Mutatox system did not show the same sensitivity of Trad-MCN assay. SPMDs were shown to be an efficient tool for *in situ* biomimetic concentration of chemicals and assess toxic contaminants. Also the relationship between what was present in the air as sampled by SPMDs and what was the frequency in micronuclei was explored.

II.P.14. *In vivo* MN TEST WITH FLOW CYTOMETRY in ACUTE and CHRONIC EXPOSURES of RATS to CHEMICALS

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The use of flow cytometry with rat peripheral blood erythrocytes *in vivo* micronucleus assay is expected to increase the sensitivity of the test and allow assessment of the genotoxic effects at doses which may be equal or close to those that are relevant to human exposure. The major advantage of incorporating the MN analysis into conventional chronic toxicology studies is the potentially significant conservation of resources, especially with respect to reduction in animal numbers as well as compound requirements. Such a combination would also allow kinetic aspects of genotoxicity of compounds to be addressed and permits other data to be extrapolated for the interpretation of those studies. However, there was a limitation in using rat peripheral blood erythrocytes since the spleen selectively removes MN erythrocytes from circulation. The selective analysis of young reticulocytes by using anti-CD71 antibodies can compensate for the spleenic clearance of micronucleated erythrocytes. In this project several compounds were tested in the two treatment regimens (acute and chronic) for their potential to induce MN in the rat peripheral blood reticulocytes using anti-CD71 antibody (FITC conjugated) and compared for some compounds with rat bone marrow MN test for acute exposure studies. Furthermore, with some classic genotoxic compounds, we performed kinetic studies of MN induction in peripheral blood samples obtained at various times after a single treatment. Such experiments gave important supplementary information about the time course of MN induction. All classic genotoxic compounds analysed (Mitomycin C, Ethyl methanesulfonate, Cyclophosphamide) were consistently positive in the acute (two treatments with an interval of 24 hours between the administrations) and chronic studies. From our data it seems that rat peripheral blood MN test in reticulocytes represents an appropriate test system for evaluating the genotoxic effect of compounds as part of the classic rat chronic toxicity studies and are at least as sensitive as for the assessment of MN in acute bone marrow MN test. However, further validation should be performed with other compounds that may be weakly inducing MN.
II.P.15. GENOTOXIC EXPOSURE of COKE OVEN WORKERS

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Coke oven workers are regularly exposed to many types of coke oven emissions, which are comprised mainly of polycyclic aromatic hydrocarbons (PAH). Many PAH have been identified as cancer-inducing chemicals for animals and/or humans and many biomarkers have been developed to estimate exposure and to assess in an early phase the risk of adverse health effects.

The objective of the study was to assess whether the exposure to PAH of coke oven workers from Polish plants induced some early biological effects in peripheral blood lymphocytes, such as sister chromatid exchanges (SCE), cells of high frequency of SCE (HFC), micronuclei (MN), chromosomal aberrations (CA) and DNA damage measured by the comet assay. Internal dose of exposure to PAH was measured by the level of urinary 1-hydroxypyrene (HpU) with HPLC and fluorescence detection. The exposure to complex mixtures was assessed by the mutagenic index using the Salmonella test with the strains: TA98 (MI98) and YG1024 (MI24), both with S9mix. Blood and urine samples were obtained from 50 coke oven workers after a shift, at the end of working week, and from 50 controls not exposed to PAH, employed at a big Polish steel factory. Occupational exposure to PAH resulted in statistically significant increased HpU levels. The median concentration of HpU in coke oven workers was 9.0 and in controls 0.6 µmol/mol creatinine. MI was also significantly higher in coke oven workers than in the control group: 2.2 vs. 1.2 for TA98 and 16.0 vs. 5.3 for YG1024. Occupational exposure caused significant induction of SCE, HFC and MN in coke oven workers: median SCE=5.9, HFC=12%, MN=6 compared to the controls: 3.9, 5% and 3.0, respectively. No effect of occupational exposure was found in relation to CA and DNA damage measured with the comet assay. The multivariable linear regression analysis was performed on the total population including exposure parameters (HpU, MI98, MI24) and various determinants and confounders (age, plant index: coke-oven plant and control, smoking, etc.). It revealed that HpU concentration was a significant positive determinant of SCE and HFC, and mutagenic index--of MN. Plant index was significantly associated with all three biomarkers. The results of the study revealed that the occupational exposure to PAH resulted in measurable early biological effects.
II.PO.16. MOLECULAR EPIDEMIOLOGICAL STUDY in POPULATIONS EXPOSED to ASBESTOS and MINERAL FIBRES


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A molecular epidemiological study was conducted in 3 factories in Slovakia (asbestos, glass fibres and rockwool [RW]). Altogether 387 subjects (239 exposed, 148 controls) were investigated. Biomarkers of exposure, effect and individual susceptibility were measured, including DNA damage (strand breaks [SBs], base oxidation and alkylation, using the comet assay); micronuclei and chromosome aberrations; genetic polymorphisms of xenobiotic-metabolising and DNA repair enzymes; individual DNA repair capacity in lymphocyte extracts; cellular defences (intrinsic antioxidants, antioxidant enzymes); humoral and cellular immune markers, growth factors and proinflammatory mediators.

Exposed asbestos workers had significantly higher numbers of chromosomal aberrations (P=0.005) compared with controls. Associations between DNA damage, exposure, smoking and sex and interaction of exposure with smoking habit were found in all three factories. Asbestos-exposed non-smoking (NS) men had higher number of DNA damage (SBs) compared with exposed NS women (P=0.03). The RW-exposed workers had higher level of oxidative DNA damage (P=0.029) compared with controls. Exposed NS had higher SBs than control NS (P=0.004). Control smokers had higher DNA damage than NS (P=0.043). Associations of DNA damage with exposure and age were also seen; level of oxidative DNA damage is dependent on age and exposure (P<0.0001). The group exposed to glass fibres had elevated numbers of DNA breaks (P=0.014), and oxidative DNA damage (P=0.001, or 0.008, resp.).

Associations between DNA damage, exposure, smoking, sex and genotype were found in all three populations. In the asbestos-exposed group, people with variant GSTP1 allele have higher levels of DNA damage (P=0.001). Interactions between GSTT1, exposure and SBs were also found (P=0.024). In the RW factory, associations of SBs with exposure, EPHX4 and smoking (P=0.035), and also between exposure and smoking (P=0.035) were detected.

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II.P.17. EFFECT of DICLOFOP on SUPPRESSION and/or INDUCTION APOPTOSIS of HEPATOCYTE in WISTAR RATS

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Peroxisome proliferators (PPs) are a class of rodent non-genotoxic hepatocarcinogens. The mechanisms by which PPs induce cancer in rodent are not explained although many hypotheses have been advanced. One of them is that PPs can suppress apoptosis that would normally act to remove damaged or initiated cells from the liver.

Our preliminary studies demonstrated that the herbicide diclofop (2-[4-(2,4-dichlorophenoxy)phenyloxy]propionic acid) exhibits the properties of rodent PPs [1].

The aim of this study was to determine whether diclofop (D) affects spontaneous and thioacetamide (TA) induced apoptosis of rat hepatocytes.

Male Wistar rats (Pzh:WIS) weighing 200 ± 10g were used. The studies included three series of experiments. In a first series, 15 rats (5 per group) received: • a single dose of TA (25 mg/kg b.w.) • simultaneously TA and D (56 mg/kg b.w.) • a single dose of D (56 mg/kg b.w.). In a second series, 15 rats were injected intraperitoneally with TA (40 mg/kg b.w) and 12 h later the rats were divided into 3 groups, • group I of animals received TA only • group II of rats were given D orally in daily dose of 56 mg/kg b.w. per day for 2 days and • group III of rats, after treatment with TA, received olive oil vehicle for 2 days. In a third series of experiments, the model of Roberts et al. [2] was used. In each series of experiments, control groups were given an equivalent amount of NaCl or olive oil vehicle.

After specified time of exposure the animals were anaesthetised with ether, livers were removed, rinsed with saline and weighed. Liver samples (right lobe) were fixed in 10% buffered formalin, embedded in paraffin and stained with H & E for histological examinations. Apoptotic bodies (ABs) were counted in field to correspond to about 50,000 hepatocytes and expressed per 1,000 hepatocytes. In addition, the increase in the relative liver weight (RLW), total hepatic DNA content and DNA synthesis were analysed.

The study demonstrated that a single administration of diclofop (56 mg/kg b.w per day) had no effect on the spontaneous basal rate of apoptosis. However, after prolonged administration of diclofop (10 doses) the number of ABs increased up to 450% of control value. Withdrawal of diclofop for 2 days and the re-administration of compound resulted in decrease in the number of cells undergoing apoptosis from 0.012 ± 0.005 to 0.005 ± 0.004. The induced-TA apoptosis was suppressed by diclofop; a 17- fold decrease in the number of ABs was noted. However, ABs analysis after administration of TA and olive oil vehicle for the next 2 days shows that conclusion regarding an inhibitory effect of diclofop on formation of ABs can be premature. After TA withdrawal for 2 days, a decrease in the number of cells simultaneously undergoing apoptosis from 2.24 ± 0.3 to 0.05 ± 0.03 was observed. TA and diclofop given stimulated an increase (about 60%) in the number of ABs, when compared to animals receiving only TA.

Research on the influence of gamma-ray low doses radiation on the processing of apoptosis of thymocytes of radiosensitive mice of BALB/c line has been performed. Fifteen animals 6-7 days old have been totally irradiated with the single dose of 0.05Gr and another fifteen – with the dose of 0.2Gr. The tested group was comprised of nine animals. The research was done on the first and a half and the tenth day post irradiation. Apoptosised bodies of various form and composition have been identified in the cerebral cortex and brain substances of the mice thymus irradiated with the 0.05Gr dose; the apoptosised bodies have been found among intact thymocytes at a considerable distance from macrophages. Besides, some thymocytes have been found at all phases of apoptosised transformation in the group of animals irradiated with the dose of 0.2Gr.

A frequent feature of apoptosised changes are queer nuclear forms with simultaneous supercondensation of chromatin. At later stages of the experiment groups of apoptosised thymocytes have not been affected by phagocytosis, nor frequently fragmented into apoptosised bodies, being surrounded with intact thymocytes or monospheres of epithelioreticulocytes and forming cyst-like structures. The most stable of these formations are nuclear, cellular and organelle membranes. The data obtained prove peculiarities in the structural mechanisms of apoptosised thymocytes proceeding under the influence of gamma-ray low doses radiation used in the experiment.
II.P.19. STUDIES on APOPTOSIS and NECROSIS in LYMPHOCYTES CAUSED by Pt (II) COMPLEX of 3-AMINOFLAVONE in COMPARISON WITH \textit{cis}-DDP

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\textit{Cis}-diamminodichloroplatinum (\textit{cis}-DDP) is an effective chemotherapeutic agent whose antineoplastic activity is connected with binding to DNA and disrupting its functions. The influence of \textit{cis}-DDP and its analogue – complex of Pt (II) 3-aminoflavone was investigated to evaluate the induction of apoptosis in human peripheral blood lymphocytes. It was carried out at the individual cell level using the TUNEL (TdT-mediated dUTP nick end labeling) assay, which detects the 3'-hydroxyl ends of fragmented DNA. Results of two other methods (fluorescence analysis of nuclear morphology by ethidium bromide/acridine orange and Hoechst 33258/propidium iodide double staining) were compared in order to estimate the percentage of viable, early apoptotic, late apoptotic and necrotic cells. The number of early apoptotic cells (identified by the TUNEL assay) caused by the treatment with complex of Pt (II) 3-aminoflavone was the highest at 24h and by \textit{cis}-DDP at 48h of incubation. However, the percent of apoptotic cells was higher for \textit{cis}-DDP in comparison with its analogue. The results obtained with fluorescent staining show a decreasing number of viable cells after incubation with \textit{cis}-DDP as compared to complex of Pt (II) 3-aminoflavone. These results could be explained by protective effects of flavonoids (complex of Pt (II) 3-aminoflavone is a synthetic flavone derivative), which have antioxidant properties and could inhibit free radical damages of DNA (\textit{cis}-DDP could cause oxidative damage of deoxyribose).

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II.P.20. ADAPTIVE RESPONSE of HUMAN BLOOD LYMPHOCYTES to GAMMA IRRADIATION and its CORRELATION with STRESS EXPRESSION

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The variability of blood lymphocyte reaction upon the adaptive irradiation (0.05 Gy 24 hours after PGA stimulation + 1.0 Gy 5 h later) was investigated by micronuclei assay with cytokinetic block by cytochalasin B. In addition, the mitotic index, the number of cells with 3, 4 and more nuclei, as well as the number of binucleated cells with 1, 2, 3 and more micronuclei were monitored.

Blood samples were obtained from 58 men (35-70 years old) exposed to different types of industrial pollutants. Just before blood sampling all donors were tested by block of standard psychological questionnaires for chronic stress evaluation. This block included determination of psychological depression by Cholms-Ray’s scale, anxiety by Taylor’s scale, overfatigue by Akkles’ scale and interpersonal relations by Kiselev’s method. All of the scales were adapted for Russia.

It was shown that the main part of donors had enhanced radiosensitivity – "radiosensitivity syndrome" after exposure to adaptive low dose of radiation or equal response to 1.0 Gy and (0.05+1.0) Gy irradiation by X² criterion and practically all of them were in a state of chronic stress (P ≤ 0.05). Moreover, significant correlation between the frequency of binucleated cells with micronuclei after 1.0 Gy irradiation and summarized estimation of stress expression were detected. However, this phenomenon was not observed for spontaneous level of binucleated cells with micronuclei.

All of the data will be discussed with respect to usefulness of psychological testing for the prognosis of genome radiosensitivity.
II.P21. WORTMANNIN ENHANCES the INDUCTION of MICRONUCLEI by LOW and HIGH LET RADIATION in V79 CHINESE HAMSTER CELLS

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In mammalian cells, the repair of DNA double strand breaks (DSBs) is mainly mediated by DNA non-homologous end-joining (NHEJ). DNA dependent protein kinase (DNA-PK), a nuclear serine-threonine kinase that is activated by DSBs is a key component of this pathway. Wortmannin (WM) is known to be an irreversible and potent inhibitor of DNA-PK, and has thus been proposed an effective sensitizer for ionizing radiation and for radiomimetic compounds. Although WM is not a specific inhibitor of DNA-PK there is growing evidence that this enzyme is most likely the target for WM radiosensitizer effects. The present study, using the cytokinesis-block micronucleus assay, reports on the differential involvement of DNA-PK in the repair of DNA damage in V79 Chinese hamster cells by low LET (60Co γ-radiation) and high LET (boron neutron capture reaction, α and Li particles) radiation. Significant increases in the frequency of micronucleated binucleated cells (%MNBN) as well as of the number of micronuclei per binucleated cell (MN/BN) were observed in the presence of different concentrations of WM for high LET radiation from the BNC reaction. The increases observed reached a maximum of about 2-fold in comparison to the respective controls. WM, however, had a more pronounced effect on 60Co γ-radiation-induced micronuclei, increasing the genotoxic damage from this radiation about 3-4 fold. These results are in general agreement with the concept that DSBs from high LET radiation may not be the more suitable substrate for these end-joining processes mediated by DNA-PK, yet they do not preclude a role for DNA-PK in high LET-induced damage repair.
II.PO.22. MODULATOR EFFECT of SMOKING HABITS and GSTM1 NULL GENOTYPE on DNA SENSITIVITY to GAMMA RADIATION in a HEALTHY POPULATION

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The variability between individuals observed in human response to carcinogens may be due to genetically determined variations in carcinogen metabolism and DNA repair capacity as well as to differences in personal exposure, nutritional status and/or immune response. In the present study in order to disclose the relative role of the genetic and environmental factors of the individual susceptibility to genotoxic agents, a mutagen sensitivity assay was performed on 31 healthy subjects and the results evaluated with respect to metabolic genotype, nutritional status, smoking habits and chemical exposure. Peripheral blood lymphocytes were irradiated in G0 with 2 Gy of gamma rays and used to analyse the kinetics of DNA repair by the comet assay (at 0, 15, 30 minutes after irradiation) and the amount of misrepaired DNA damage by the frequency of chromosomal aberrations. The design of the study allowed a direct comparison between these biomarkers measuring the global repair phenotype of each individual.

The frequency of chromosomal aberrations after irradiation was significantly higher only in GSTM1+/+ subjects compared to GSTM1-/- subjects (33.4±9.35 vs 26.45±6.02, respectively; p=0.025) and in non-smokers compared to heavy smokers (32.7±8.83 vs 24.3±6.83, respectively; p=0.05). Comparable results were obtained with the comet assay, analysing the residual DNA damage (%RD) shortly after irradiation (15 min); in fact, non smokers showed a higher amount of RD than heavy smokers (86.04±49.98 vs 29.93±36.07, respectively; p=0.016) as well as GSTM1+/+ individuals compared with GSTM1-/- subjects (76.13±56.61 vs 33.36±36.47, respectively). No intergroup difference was detected at later time (30 min). These data suggest a modulator effect of smoking habits and GSTM1 null genotype on the individual repair capacity. In this respect, the higher expression of enzymes involved in the repair of oxidative DNA damage in heavy smokers and GSTM1 null subjects may be hypothesized.

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II.P.23. RADIATION SENSITIVITY and TELOMERE BIOLOGY: PROTECTING the END?

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Recent findings have shown that telomere biology can play a crucial role in determining the final outcome after radiation damage. Telomeres are the complex nucleoprotein structures that comprise and protect the natural ends of linear Eukaryotic chromosomes. In a number of studies telomere biology has been analysed as a function of age under normal conditions and after radiation damage. Yet, very little information is available on both telomerase activity and telomere length dynamics early in development. We are currently investigating the impact of radiation damage on embryonic mouse development both in normal and p53 mutant mice. Recent evidence exists for the relation between telomere length and radiation sensitivity in mouse. Our goal is to further investigate this apparent link between telomere biology and outcome of radiation exposure in mice with different p53 gene status after low dose X-irradiation at various stages of prenatal development. Preliminary data show that observed external anomalies (exencephaly, gastrochisis, polydactyly, cleft palate, dwarfism) are linked to p53 genotypic status and to gender differences¹. To gain maximal information on telomere biology we are establishing a holistic methodology to assess both telomere length (average and individual) and telomerase activity (in cell extracts and in situ). We have recently described a method to measure individual telomere lengths using digital imaging and confocal microscopy². The use of confocal microscopy enables the acquisition of 3D images. Hence, possible variations in telomeric signal intensities due to wrong focussing are eliminated. In addition we are establishing a fluorescence resonance energy transfer (FRET)-based method to measure in situ telomerase activity. This latter technique must enable more accurate and quantitative assessment of telomerase activity in situ in individual living cells.

II.P.24. DNA-PKcs FUNCTIONALLY INTERACTS with TELOMERASE in MAINTAINING TELOMERE LENGTH in the MOUSE

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DNA-PKcs is the catalytic subunit of the DNAPK complex for non-homologous end-joining (NHEJ) of double strand breaks (DSBs), and also has a role in telomere capping. DNA-PKcs deficient mice show long but dysfunctional telomeres, as indicated by the occurrence of telomeric fusions in the absence of significant telomere loss. To further dissect the role of DNA-PKcs at the telomere, we have generated mice that lack both DNA-PKcs and telomerase. Primary cells from Terc−/−/DNA-PKcs−/− mice, show a faster rate of telomere shortening and an earlier loss of viability than the corresponding Terc−/−/DNA-PKcs+/+ controls. These results indicate a functional interaction between DNA-PKcs and telomerase in maintaining telomere length, thus providing a mechanism by which DNA-PKcs could influence telomeric capping. Loss of viability of Terc−/−/DNA-PKcs−/− cells is not concomitant with increased end-to-end fusions. In summary, the results presented here unravel functional links between DNA-PKcs, telomerase and telomeres, which in turn could be relevant to understand the mechanisms that govern cancer and aging in mammals.
II.P.25. FREE RADICAL SCAVENGERS CAN MODULATE the DNA-DAMAGING ACTION of DIABETOGENIC ALLOXAN

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Type 2 diabetes mellitus can be associated with many complications including atherosclerosis and cancer, which may account for premature death. Elevated oxidative stress in diabetes can contribute to the appearance of these diseases. To have a closer look at the possible link between diabetes and cancer it is reasonable to investigate the changes in DNA of normal cells in a diabetes-affected organism. Because alloxan is a diabetogenic substance, its interaction with normal cells may show the character of the changes generated in these cells in the course of diabetes. Using the alkaline comet assay we showed that alloxan at concentrations from the range 0.01-50 µM induced DNA damage in normal human lymphocytes in a dose-dependent manner. Treated cells were able to recover within a 120-min incubation. Vitamins C and E at 10 and 50 µM diminished the extent of DNA damage induced by 50 µM alloxan. Pretreatment of the lymphocytes with the nitrone spin trap, α-(4-pyridil-1-oxide)-N-tert-butylnitrone (POBN) or ebselen, which mimics glutathione peroxidase, reduced the extent of DNA damage evoked by alloxan. The cells exposed to alloxan and treated with formamidopyrimidine-DNA glycosylase (Fpg) and 3-methyladenine-DNA glycosylase II (AlkA), the enzymes recognizing oxidized and alkylated bases, respectively, displayed greater extent of DNA damage than those not treated with these enzymes. These results suggest that free radicals may be involved in the formation of DNA lesions induced by alloxan. The drug can also methylate DNA bases. These changes in DNA may contributed to cancer associated with diabetes.

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II.P.26. ASSESSMENT of DNA DAMAGE by COMET ASSAY in WORKERS OCCUPATIONALLY EXPOSED to ARSENIC

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Chronic exposure to inorganic arsenic compounds (i-As) is responsible for the prevalence of various types of cancer, including lung, skin, liver, kidney and urinary bladder cancers, as well as of other diseases. Studies of people exposed to high concentrations of i-As in drinking water show its genotoxicity. The various studies suggest that it does not affect DNA directly but may intensify the toxic effects of other physical and chemical agents, especially by DNA repair inhibition. The aim of this study was to assess the extent of DNA damage in leukocytes of workers occupationally exposed to i-As by inhalation at the copper smelters. Arsenic group consisted of 72 men employed at the Depts where high temperature processes have been used. Their mean age was 42.2±7.3 years and the mean duration to As exposure was 18.0±7.3 years. About half workers in the group are smokers. The control group included 80 volunteers from the health services. Most of them are men and smokers, at the mean age of 37.9±11.6 years. Blood samples were collected in the morning after night shifts, transported to the lab within 8 hrs and processed. DNA damage, including single strand breaks (SSB), alkali labile sites (ALS) and oxidative DNA damage (using Fpg enzyme), were measured by the comet assay. An image analysis system (LUCIA comet assay) was used to determine DNA damage. Two parameters were applied to indicate DNA fragmentation: mean tail length (TL) and mean tail moment (TM). The mean TL and TM values were compared among groups by the two-way analysis of variance. Overall differences were considered significant when p<0.05. Statistically significant differences in TL and TM were observed between the exposed and control groups. DNA migration increased significantly in the exposed group after using Fpg enzyme compared to the control group. Significant differences in oxidative DNA damage existed between smokers and nonsmokers of As group and respective controls when assessed both by TL and TM and in DNA-ssb these differences were significant only by TM. In view of these results, we can suggest that the occupational exposure to arsenic by inhalation at the copper smelters may induce the genotoxic effects in worker leukocytes and cause the harmful effects on the health of workers.

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II.P.27. INDUCTION of DNA STRAND BREAKS and DNA-PROTEIN CROSSLINKS by LEAD

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Lead acetate at 1-100 µM induced DNA damage in human lymphocytes as evaluated by the alkaline comet assay. At low lead concentrations an increase in the comet length was observed indicating production of strand breaks and/or alkali labile sites. At higher lead concentrations a decrease in the comet length was observed. This could follow from the formation of DNA-DNA or DNA-protein crosslinks. Pretreatment of lymphocytes with nitroprone spin traps, 5,5-dimethyl-pyrroline N-oxide (DMPO) and N-tert-butyl-α-phenyl nitronitrotrone (PBN) did not affect the extent of DNA damage evoked by lead, indicating that free radicals could not be involved in the observed effects. Moreover, lymphocytes exposed to lead acetate and treated with endonuclease III, formamidopyrimidine-DNA glycosylase and 3-methyladenine-DNA glycosylase II, enzymes recognizing oxidized and alkylated bases, displayed the same extent of DNA damage as those not treated with these enzymes. Proteinase K reduced or even abolished the decrease in the comet length induced by lead, suggesting that the metal might form crosslinks with nuclear proteins. Vitamin C and calcium potentiated the DNA-damaging effect of lead. Obtained results suggest that non-radical-based induction of DNA strand breaks and DNA-protein crosslinks may contribute to the genotoxicity of lead.

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Nickel is recognized to be genotoxic but the mechanisms underlying this feature are largely unknown and its interfering with DNA repair processes may contribute to its reported genotoxicity. We studied the effect of nickel chloride on the repair of DNA damaged by UV radiation or N-methyl-N-nitro-N-nitrosoguanidine (MNNG) in human lymphocytes using alkaline comet assay. Hydroxyurea at 10 mM inhibited the polymerization step of the excision repair of the UV-damaged DNA and transient DNA breaks introduced in repair process remained open. The presence of nickel chloride at 1 µM caused accumulation of the DNA breaks, which could follow from the inhibition of polymerization/ligation step of UV-damaged DNA repair. On the other hand nickel inhibited the formation of transient DNA breaks brought by the repair process after incubation with MNNG at 5 µM, what might follow from the interfering with recognition/incision step of the excision repair. Additionally, nickel chloride at 1 µM inhibited the activity of bacterial enzymes involved in the excision repair: formamidopyrimidine-DNA glycosylase (Fpg) and 3-methyladenine-DNA glycosylase II (AlkA). Our results suggest that nickel at a non-cytotoxic concentration can inhibit various steps of DNA excision repair and this may contribute to its genotoxicity.

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II.PO.29. VERY LOW CONCENTRATIONS of ARSENITE and other TOXIC METAL COMPOUNDS SUPPRESS Poly(ADP-Ribosyl)ation in CULTURED MAMMALIAN CELLS

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Poly(ADP-ribosyl)ation of various proteins is one of the earliest nuclear events following DNA strand break induction; it is believed to promote changes in the nuclear structure and to direct repair enzymes to sites of damage. Furthermore, PARP seems to play a complex role in drug-induced and spontaneous apoptosis, which, however, is not yet fully understood. It contains two zinc finger structures of the Cys3His1-type, which are involved in the recognition of DNA breaks and the subsequent formation of poly(ADP)ribose. Within the present study, we investigated the effects of toxic metal ions on H2O2-induced poly(ADP-ribosyl)ation. Poly(ADP)ribose was quantified by immuno-cytochemical detection applying a mouse monoclonal antibody (10H) and a FITC-conjugated goat anti-mouse IgG antibody. Among the carcinogenic metal compounds, H2O2-induced poly(ADP-ribosyl)ation was inhibited by Ni(II), Co(II), Cd(II) and concentrations as low as 10 nM As(III) in HeLa S3 cells. Furthermore, poly(ADP-ribosyl)ation was suppressed by Cu(II), while Hg(II) and Pb(II) were ineffective. Potential targets within the PARP molecule may be the zinc finger structures; mechanisms of inactivation may be either the displacement of zinc by other transition metals as well as redox reactions leading to thiol/disulfide interchange. Especially the results obtained with arsenite deserve special attention since poly(ADP-ribosyl)ation is the first reaction related to DNA repair found to be particularly sensitive towards As(III) and inhibitory concentrations resemble closely those found in blood and urine of the general population. Therefore, the suppression of poly(ADP-ribosyl)ation may contribute to increased cancer risks associated with chronic exposure to arsenic via drinking water.
In vitro INTERACTION of ADRIAMYCIN METABOLITES GENERATED by NADPH CYTOCHROME P450 REDUCTASE with NUCLEIC ACIDS

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Previous studies demonstrated that metabolic activation of adriamycin by NADPH cytochrome P450 reductase (P450red) potentiated cytotoxicity of this drug towards cultured tumour cells. Further experiments carried out with the aid of isotopically labelled adriamycin indicated that this potentiation results from the formation of metabolites capable of covalent binding with macromolecules rather than from stimulation of active oxygen species formation as a result of so called redox cycling. During these investigations, we observed that metabolic conversion by P450red was associated with the changes of the drug UV-VIS spectrum. This enabled us to develop the method of monitoring the kinetics of adriamycin reduction by this enzyme.

The application of the this method in combination with EPR measurements made possible to relate the appearance of particular adriamycin metabolites to the generation of superoxide radical formed as a result of the reaction of adriamycin semiquinone radical with molecular oxygen. Based on results obtained and the data available in literature, we proposed the route of adriamycin metabolic conversion by P450red. We suggest, that this process involves five stages, two of which are accompanied by intensive redox cycling (stages 2 and 3).

Most recently, we used the same approach to study the interaction (possibly also involving covalent modification) of adriamycin metabolites with nucleic acids and their monomers in cell-free system. The assumption was that the formation of adriamycin DNA adducts will in a way preserve the structure of the active metabolite(s) which will be reflected by its spectral properties. We tested the influence of different nucleic acids (ds-DNA, ss-oligonucleotide, RNA and their monomers) on the kinetics of adriamycin reduction by P450red. The greatest impact was observed for DNA and oligodeoxy-nucleotide, both of which blocked this process at the stage 3 of metabolism corresponding to the tautomerization of leuko form of adriamycin to methide. In the case of monomers, the modulation of the drug reduction was clearly seen only in the case of deoxycytidine and deoxyguanosine. The formation of adriamycin species capable of covalent bonding with DNA as a result of enzymatic reduction by P450red was confirmed by the newly developed in our laboratory method exploiting restriction enzymes for the detection of covalent DNA modification. Also this approach suggested that the metabolite arising during stage 3 of adriamycin reduction can covalently modify DNA, GC base pairs in particular.
II.PO.31. CADMIUM INDUCED MICRONUCLEI FORMATION in HepG2 CELLS

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Cadmium is a frequent environmental pollutant, classified as a human carcinogen by IARC, but the mechanisms of its genotoxic activities have yet to be elucidated. For non-occupationally exposed people food and cigarette smoke are the main sources of exposure to this metal. Cadmium has a weak genotoxic and mutagenic potential, but enhances DNA damaging activity of several genotoxic agents by inhibiting DNA repair mechanisms¹.

Cadmium accumulates in the body, mostly in the liver, kidneys and lungs. Therefore, we determined the genotoxic activity of low concentrations of cadmium on human hepatoma cell line HepG2. In cells exposed to 0.5 µM–5 µM CdCl₂ dose-dependent increase in micronuclei (MN) formation was observed. Weaker genotoxicity of CdCl₂ was observed using the comet assay. The reason for this may be that the micronucleus assay reveals also the mutations fixed in the chromosomes, which are not detected by the comet assay².

In order to determine whether cadmium exerts clastogenic or aneugenic potential, the combination of micronucleus assay and fluorescence in situ hybridization (FISH) with DNA centromeric probe was used. Centromere-positive and centromere-negative micronuclei were scored, representing aneugenic and clastogenic potential, respectively. Clastogenic activity of cadmium was observed in exposed cells and we conclude that increased formation of MN is the result of chromosome breaks.

As people are mostly exposed to mixtures of environmental contaminants, we exposed HepG2 cells to a combination of ubiquitous polycyclic aromatic hydrocarbon pollutant benzo(a)pyrene (B(a)P) (100 µM) and CdCl₂. In cells exposed to both agents, fewer MN were formed than in cells exposed to B(a)P alone. The decrease in number of MN can be explained by the fact that cadmium impaired the endogenous metabolic activation of B(a)P in HepG2 cells by inhibiting CYP enzyme activities, thereby lowering B(a)P genotoxicity³.

We conclude, that cadmium has strong clastogenic activity, that it induces MN formation as a result of chromosome breaks and that it lowers genotoxicity of the pollutants, which require metabolic activation.

II.P.32. INTERACTION of SELENIUM COMPOUNDS with the ISOLATED ZINC FINGER DNA REPAIR PROTEINS XPA and FPG

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Selenium is thought to exert anticarcinogenic properties. One mode of action may consist in the oxidation of thiol groups in proteins. For example certain selenium compounds are able to oxidize thiol groups in metallothionein and thereby release zinc and mediate zinc homeostasis. However, similar thiol complex formation like in metallothionein is present in transcription factors and other so called zinc finger proteins. Within the present study we investigated the effect of selenium compounds on the activity of the formamidopyrimidine-DNA glycosylase (Fpg), a zinc finger protein involved in base excision repair, and on the DNA binding capacity and structural integrity of XPA, a zinc finger protein essential for nucleotide excision repair.

The Fpg was inhibited in a concentration dependent manner by ≥ 75 nM ebselen (0), ≥ 1 µM phenylselenyl chloride (0), ≥ 1.5 µM 2-nitrophenylselenocyanate, ≥ 75 µM selenocystine (-I) and ≥ 100 µM benzeneseleninic acid (+II). No effects were observed in the presence of selenomethionine (-II) or methylselenocysteine (-II) or with the sulfur containing analogs methionine, cystine and benzenesulfinic acid. DNA binding of the XPA protein was prevented by ≥ 15 µM benzeneseleninic acid and ≥ 50 µM phenylselenyl chloride. As potential mechanism of protein inactivation some selenium compounds were able to release zinc from the zinc finger of the XPA protein in the lower micromolar range (2-nitrophenylselenocyanate > selenocystine > benzeneseleninic acid > phenylselenyl chloride > ebselen).

The observed effects could result from the oxidation of thiol groups within the zinc finger structure since ebselen, 2-nitrophenylselenocyanate, phenylselenyl chloride, selenocystine and benzeneseleninic acid are still reducible, while fully reduced selenomethionine or methylselenocysteine were inactive. Remarkably, the investigated zinc finger proteins differ in their reactivity, although zinc is complexed by four cysteines in both proteins. Our results indicate that certain selenium compounds may interfere with the function of zinc finger proteins involved in DNA repair and other processes essential for maintaining genomic stability.
II.P.33. EXPRESSION PROFILES of MURINE THIOREDOXIN and GLUTATHIONE REDOX SYSTEMS


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Mammalian redox homeostasis is regulated by thioredoxin- and glutathione-dependent systems. These pathways supply electrons for the redox control of a great variety of biological processes, reducing equivalents coming ultimately from NADPH via specific pyridinenucleotide disulfide oxidoreductases. The Trx system is composed of Trx, Trx reductase (TR) and Trx peroxidase. The GSH system consists of GSH, GSSG reductase (GR), glutaredoxin (Grx) and GSH peroxidase. Recently, a protein (TGR) that reduces components of both systems has been identified. Cytosolic and mitochondrial forms of some of these proteins have been described. Besides, some of the genes display alternative splicing patterns for proteins of different molecular mass. The main general objective of our study is the absolute quantification (mRNA molec/ng total RNA) of the expression profiles of relevant components of Trx and GSH systems in mouse in order: i) to investigate the physiological meaning of the existence of multiple isoforms, ii) to discover putative compensations among the systems under study, iii) to evaluate their relative importance upon oxidative stress conditions and their implication in the molecular cascade of redox regulation, iv) to distinguish among alternative expression patterns, as a function of different tissues (brain, heart, liver, lung, spleen, kidney and testis), and v) to identify novel oxidative stress biomarkers and to evaluate their utility in environmental pollution studies.

Basal expression levels and the response to paraquat stress of some components of these redox systems, TRX1, TRX2, TR1, TR2, GRX1, GRX2, GR, TGR and TR1-alt (coding a protein ~10kDa larger than TR1), are being quantified in the brain, heart, liver, lung, spleen, testis, and kidney of mouse. Kidney showed high basal expression levels of the genes studied, in particular GRX1 (3x10^5 molec/ng). In contrast, testis had the highest expression level of mitochondrial GRX2 (5x10^5 molec/ng) and of TGR (7.6x10^5 molec/ng). TR1-alt also displayed the highest level (239 molec/ng) in testis. However, even in testis, the TR1-alt transcript level was only 1.4 % of that of TR1.

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II.P.34. CYTOTOXIC and GENOTOXIC EFFECTS of TWO CATALYTIC INHIBITORS of TOPOISOMERASE II in CHO CELLS

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In contrast to topo II poisons, true catalytic inhibitors of the mammalian enzyme that do not stabilize the cleavable complex can become cytotoxic through their interference with topo II function by mechanisms that are only partly understood. Nevertheless, catalytic inhibitors of the enzyme are currently being thoroughly investigated, given their possible clinical implications. In the present work, we compared the cytotoxic and genotoxic effects of two topo II-targeted drugs that do not act as cleavable complex stabilizers, namely bis(dioxopiperazine) ICRF-193, considered the most potent topo II catalytic inhibitor, and bufalin, one of the components of the bufadienolides in the traditional Chinese medicine. DNA repair-proficient AA8 Chinese hamster cells as well as repair-defective EM9 have been treated with these anti-topo II drugs, in order to assess any possible influence of DNA repair on the outcome of treatments.

While both ICRF-193 and bufalin suppress cell growth and result in a clear inhibition of topoisomerase II catalytic activity, only ICRF-193 has been shown as able to induce both chromosome and DNA damage, with a more pronounced effect in the CHO mutant EM9 than in the repair-proficient line AA8.

Our results seem to indicate that important differences do exist between both drugs as to their possible effects and the cell capability to efficiently carry out repair does not seem to be a decisive factor, in contrast with that reported for topoisomerase poisons.
II.P.35. HIGH LEVEL of ENDOREDUPLICATION in CELLS TREATED with ICRF-193, a TOPOISOMERASE II CATALYTIC INHIBITOR

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A variety of agents either by disturbing cytoskeleton assembly, such as the spindle poisons colcemid, colchicin or concanavalin A, or by damaging DNA have been reported to induce endoreduplication to different degrees. More recently, agents that interfere with topo II have been used to provide further evidence that the enzyme is required for separation of daughter chromosomes. As a result of these studies, it has been shown that topoisomerase II poisons as well as specific catalytic inhibitors of the enzyme are able to induce endoreduplication due to prevention of decatenation of replicated chromosomes by topo II with subsequent failure to complete normal mitosis.

In the present investigation we have made use of bisdioxopiperazine ICRF-193, a topo II catalytic inhibitor that interferes with the normal turnover of the enzyme, in order to see whether both EM9 cells and its parental cell line AA8, which show differences in the spontaneous frequency of endoreduplicated cells, are equally sensitive to this particular topo II catalytic inhibitor. For this purpose, both cell lines were treated with a wide range of doses of bisdioxopiperazine. Our results show that both AA8 and EM9 cells respond to the treatment entering in an endoreduplication cycle, but the EM9 cells are extremely sensitive to the inhibition of topo II.
II.P.36. The INDUCTION of DNA BREAKS in LYMPHOCYTES and LIVER CELLS of MICE and CHINESE HAMSTERS TREATED with MMS or MNU: A COMPARISON with the INDUCTION of MICRONUCLEI

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MNRI male mice and males of Chinese hamsters were administered i.p. with methylmetanesulfonate (MMS) or with N- methyl-N-nitrosourea (MNU). Animals were sacrificed 2 hours later. Lymphocytes were isolated on ficoll gradients and liver cells were obtained by trypsinisation of liver tissue. DNA breaks were measured by the comet assay (alkaline version). In mice the frequency of micronuclei in bone marrow cells was analysed 24 hour after the administration of chemicals. In mice there was a dose-related increase in the amount of DNA breaks (from 0.5 to 3.5 SSB/10^9 daltons) within the range of doses 30-240 mg/kg of MMS. The dose response was linear in liver cells. The amount of SSB induced by MNU showed a linear increase of SSB from 0.5 to 4.5/ SSB/10^9 daltons both in mice and Chinese hamsters within the range of doses from 1.25 to 160 mg/kg. The lowest efficient dose was 2.5 mg/kg for liver cells, and 20 mg for lymphocytes. The induction of micronuclei in polychromatophilic erythrocytes in bone marrow of mice was also linear and numbered 22 MN/1000 cells at the highest dose tested (200 mg/kg). There exists a good correlation between the DNA damage induced in liver and lymphocytes, and the induction of micronuclei in bone marrow, provided that MN are measured 24 hours after administration. DNA breaks seem to be a more sensitive marker compared to MN, when measured 2 hours after the administration.
II.P.37. LEVEL of the INDUCIBLE NITRIC OXIDE (NO) in the TISSUES is an "AGING RISK MARKER" in MICE

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NO generated in vivo by NO-synthase (NOS) from L-arginine has been proven to posses cyto- and genotoxicity associated with the immune response. In the initial stage a high toxic NO is generated, thus decreasing resistance of bacteria, viruses and other invasive organisms. The present study examined the changes in NO-inducible (by LPS injection) level in two organs of mouse as a result of the single hr mutation. The experiments have been done in two isogenic lines of mice of the same age of 5 months: B10 (wild type) and "Rhino" with hr mutation. The mutant animals have some defects in immune system due to over expression of hr ("hairless") gene. They are characterized by a specific phenotype, suffer from erythroleukaemia and normally die at the age of 6-8 months. There is information that the hr mutation imparts a DNA-repair deficiency, as well. Additionally, we studied mice of NZB line at the ages of 5 and 8.5 month which are frequently used as a model of autoimmune hemolytic anemia, developing with age. To control the level of NO the EPR – spectroscopy method has been used. When injected into the animal, diethyl dithiocarbamate (DDC) formed [Fe^{2+}−DDC] complex with endogenous or exogenous iron. Acting as NO scavenger the complex binds NO giving rise to paramagnetic mononitrosyl iron complex with DDC (MNIC−DDC). The last one was characterized by the EPR signal with $g_1=0.35$ and $g_{11}=2.02$. Initially, until 2 months of age, the mice resembled each other in phenotype and inducible NO levels were more or less the same. During aging they started to diverge significantly in phenotype and differ greatly in NO levels from organ to organ. This was the case for NZB line at the age of 5 and 8.5 months when we observed a significant decreasing in NO levels in liver and intestines of the elder suffering animals. NO was found to accumulate in B10 mice (w.t.): 4-and 10-fold over the initial level in the liver and intestines, respectively. But this was not the case for isogenic Rhino line: only 0 and 3.5 fold accumulation, respectively. These differences in NO inducible level were due to the single hr mutation in the Rhino mouse. Hence, the level of inducible NOS (iNOS) activity is a specific "aging risk marker" in mice.

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II.P.38. SYNTHESIS and GENOTOXIC CHARACTERIZATION of MULTIFUNCTIONAL NO DONORS PROMISING for MEDICINE

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Nitrogen monoxide synthesized in cells has been proven to be the earliest evolutionary signal molecule that normally participates in different physiological–biochemical processes, immunity formation being the most important among them. NO is also involved in the regulation of the genetic system on transcription and transmission levels. Accumulation of NO, superoxide and the product of their reaction, peroxinitrite, increases drastically in the cell upon infectious diseases and tumor growth. For the latter case NO role is ambiguous. Due to the enormous biological significance of NO, the prospects of its application in medicine are being studied. Russian scientists have advanced results in this field. However, low stability of available monoxide donors complicates investigations with NO participation.

This investigation was aimed at the development of stable NO donors, and comparative study of their physical and chemical characteristics and biological activity. We have first obtained iron nitrosyl complexes (synthetic analogs of nitrosyl adducts of [2Fe-2S] ferredoxine active sites) of compositions [Fe2(S2O3)2(NO)4]2-, [Fe2(SR)2(NO)4]⋅nH2O with n=0.5-2 and different ligands R (mercapto-substituted pyridine, pyrimidine, benzimidazole, triazole). Using X-Ray diffraction, IR-, EPR-, NMR- and mass-spectroscopy, the properties of all synthesized complexes (in particular, stability in solid state and in solutions, ability to release NO, redox- properties) were shown to depend on their structure, i.e., the way of coordination of the iron atoms by the sulfur and nitrogen atoms of heterocyclic ligands.

Using the E.coli models with combined operons (sfiA::lacZ) and (soxR::lacZ) and in the experiments with S.typhimurium TA100 the SOS-, soxRS-inducible and mutagenic activity of the new NO donors was studied in comparison with DNIC, GSNO complexes and sodium nitroprussid. For newly synthesized compounds the maximal SOS-inducible activity was registered for the sodium salt with dianion: It was compatible with the activity of the strongest of SOS-inductors, DNICcys, and essentially exceeded nitroprussid. In the investigated test- system the activity was not observed for any of synthesized NO presently planning to investigate the new monoxide clinical trials.

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II.P.39. The INFLUENCE of VIRAL PROTEINS on GENETICAL STABILITY of MAMMALIAN CELLS in vitro

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Adenoviruses belong to the most widespread DNA-containing viruses and are used as vectors for the development of approaches in gene therapy of hereditary diseases. This explains the interest of the researchers in this group of viruses.

The purpose of this work was to investigate the biological activity of early adenoviral genes and their influence on the genetical stability of mammalian cells in vitro. In this work we have used Chinese hamster cells Bld-FAF28Cl237-A cultivated in Eagle's medium with antibiotics and 5-10% bovine serum. We have studied the DNA restriction fragments of standard high-oncogenic clone BAV3-1, and low-oncogenic clone BAV3-3. The transfection of viral DNA was carried out by the Ca-phosphate method, at DNA concentration of 1 мг/мл and the expression time of 3 days. Subsequently, 6-mercaptopurine resistant mutants (hprt-locus) were registered and counted.

It has been shown that all early viral genes of high- and low-oncogenic clones of BAV3 are capable of inducing genetic instability. The mutagenic effect of adenoviruses was determined by effective expression of their regulator operons. This observation is in agreement with the results of virus-induced carcinogenesis where the products of early virus genes play an initiating role. It is possible that mutagenic activity occurs through the interactions between viral proteins and cell cycle controlling proteins. Oncogenic protein 55R coded by early adenovirus operon E1B is capable of associating with nuclear protein р53 and inhibiting its transcription activity. Similarly, the other oncogenic protein coded by early Е1А operon forms complexes with cell protein рRb which is associated with transcription factor Е2F in G1 stage of cell cycle. In general comparison of our results with literature data permits the conclusion that proteins coded by operons Е1А and Е1В are capable of displaying mutagenic activity. We have found the difference in oncogenic and mutagenic activity of Е1 transforming region from low- and high-oncogenic adenoviruses. It is possible to explain this effect by the different numbers of DNA-repeats which are localized between Е1А and Е1В operons and influence their expression.
II.P.40. PHOTOGENOTOXICITY and PHOTOTOXICITY INVESTIGATIONS on PYRIDONE DERIVATIVES

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Nine structurally related pyridone derivatives were tested for photogenotoxicity (Ames test and chromosomal aberration test in V79 cells) and for phototoxicity (neutral red uptake in 3T3 cells). All nine compounds absorb light to a comparable degree at wavelengths between 380 and 430 nm. Seven of the 9 compounds were found to produce high quantities of singlet oxygen (1O2) upon irradiation in the presence of oxygen. These 7 compounds were highly phototoxic in the NRU test, 3 were clearly and 2 were marginally photomutagenic in the Ames test, 5 were assessed as clearly and 2 as equivocally photoclastogenic in the chromosomal aberration test. Of the two compounds which showed substantially lower 1O2 yield, one was distinctly less phototoxic and did not induce photogenotoxic effects. The other, however, was similarly phototoxic as the seven compounds with high 1O2 quantum yield and was also clearly photogenotoxic. The pyridone ring of these two compounds is condensed to a non aromatic ring, while for the 7 other compounds the chromophore system with the pyridone ring consists of two or three aromatic rings.

Overall, there was little correlation between the phototoxic and the photogenotoxic potencies and only limited parallelism between the potencies in the two photogenotoxicity assays. Light induced generation of ROS (Reactive Oxygen Species) is thought to be the underlying cause of the photo(geno)toxic activity of these compounds. However, the results with the strongly phototoxic compound possessing a low 1O2 quantum yield indicates that either a uniform pathway involving 1O2 generation cannot be implicated or singlet oxygen does not play a significant role in the photo(geno)toxicity of these pyridone derivatives.
II.P.41. GENETICAL ACTIVITY of SOME BIOLOGICALLY ACTIVE PEPTIDES

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The aim of this work was to investigate some biologically active peptides (S. nigra bark and inflorescence lectins) genetical activity and mutual modifying effects of those lectins and inorganic nickel salt (NiCl₂) on their mutagenic/cytotoxic properties. The permissive line of Chinese hamster fibroblasts BIIId-ii-FAF28C127 with semi-quantitative test on single-strand breaks in individual cells (comet test) and test on cytotoxicity in cell microcultures were used.

It was shown that the character of cytotoxic/mutagenic activity depends on the method of treatment of cells by those agents, especially on the nutrient medium composition. The dependence of S. nigra lectins cytotoxic/mutagenic activity on their concentration under our experimental conditions was observed. The influence of different concentrations of those agents on cell chromatin was investigated using the comet method.

The study involved the character of NiCl₂ cytotoxic/mutagenic activity per se and in combination with biologically active lectins in different variants of treatment and under different experimental conditions. The data obtained using microcultures and comet test were compared. The dependence of NiCl₂ genetical activity on nutrient medium composition was also described.

These data allowed some conclusions regarding the availability and character of genetical activity of those lectin preparations and the mutual modifying activity of lectins and NiCl₂.
II.P.42. PATHWAYS for INCREASING PRODUCTION of DOXORUBICIN in the STRAIN *Streptomyces peucetius subsp. caesius* ACC 27952-2

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The anthracycline family of antibiotics is an important source for therapeutically useful antitumor agents. Doxorubicin and its precursor, daunorubicin, are chemotherapeutic antitumor antibiotics produced by *S. peucetius*. These anthracyclines are currently obtained at high cost in low titers by submerged fermentation in complex media. Therefore, it is very important to create a strain – producers of DXR, that are characterized by a high level of DXR production. The strains *S. peucetius subsp. caesius* ATCC 27952, anthracycline producer, was characterized by resistance to antibiotics and antibacterial activities.

The collection of 27952 mutants resistant to chloramphenicol (CML), daunorubicin (DNR) and doxorubicin (DXR) was created and characterized. Cmlr- mutants demonstrate decreased total antibiotic activity in comparison to strain 27952-2. However, 92.8% cmlr-mutants, obtained after N-methyl-N/-nitro-N-nitrozoguanidine treatment of strain 27952-2 synthesized DXR and its intermediates. Two mutants among them are characterized by increased DXR production level.

It has been found that strain *S. peucetius subsp. caesius* ATCC 27952-2 is much more resistant to DXR than to DNR. Resistance to DXR characterized the majority of DNRr-mutants (76.9%), while among the DXRr-mutants only below 30% are resistant to DNR. The mean value of antibiotic activity was the highest among DXRr-mutants induced by NG and sampled in the medium with 30 mg/ml of DXR. The majority of the tested DNRr- and DXRr- mutants (13 of 15) synthesized 1.8-10.9- fold more of anthracycline compounds as compared with the parental strain.

All the mutants synthesized 2.6-28.3 and 1.6-20.7 -fold more of DNR and DXR, respectively, than the parental strain. As a result we obtained proof that it is worth searching among DNRr- mutants for strains with high production of DXR while DXRr- mutants are promising for choosing the producers of both antibiotics. 48-hours old cultures of DNRr-mutants of strain 27952-2 synthesize DXR from exogenously added DNR. In these mutants export of DXR was also increased as compared with the parental strain.

Active export of DXR would be important not only as a resistance mechanism but also from a metabolic flux standpoint. If DXR were allowed to accumulate, it might interfere with the biosynthesis of DNR. Thus, export of DXR from the cells allows the organism to produce more DNR and DXR. Resistance of strain *S. peucetius subsp. caesius* ATCC 27952-2 and it's DNRr- mutants to DNR depends not only on functioning of the resistance gene but also on the conversion from the more toxic (DNR) to the less toxic (DXR) compound.
II.P.43. METABOLIC ACTIVATION of the SUSPECTED HUMAN CARCINOGEN 3-NITROBENZANTHRONE by HUMAN ACETYLTRANSFERASES and HUMAN SULFOTRANSFERASES

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3-Nitrobenzanthrone (3-NBA) an extremely potent mutagen and suspected human carcinogen identified in diesel exhaust was shown to form multiple DNA adducts in vitro and in vivo in rats. In order to investigate whether human N,O-acetyltransferases (NATs) and sulfotransferases (SULTs) contribute to the metabolic activation of 3-NBA, we used a panel of newly constructed Chinese hamster V79MZ derived cell lines expressing human NAT1, human NAT2 or human SULT1A1, respectively, as well as TA1538-derived Salmonella typhimurium strains expressing human NAT1 or human NAT2 and determined DNA binding and mutagenicity. The formation of 3-NBA-derived DNA adducts was analysed by 32P-postlabelling after exposing V79 cells to 0.01 µM 3-NBA or 0.1 µM N-acetyl-N-hydroxy-3-aminobenzanthrone (N-Ac-N-OH-ABA), a potential metabolite of 3-NBA. Similarly up to 4 major and 2 minor adducts were detectable with both compounds, the major ones being identical to those detected previously in DNA from rats treated with 3-NBA. Comparison of DNA binding between different V79MZ derived cells revealed that human NAT2 and, to a lesser extent, human NAT1 and human SULT1A1, contribute to the genotoxic potential of 3-NBA and N-Ac-N-OH-ABA to form DNA adducts. However, the extent of DNA binding by 3-NBA was higher in almost all V79 cells at a 10-fold lower dose than by N-Ac-N-OH-ABA, suggesting that N-Ac-N-OH-ABA is not a major intermediate in the formation of 3-NBA-derived adducts. 3-NBA showed a 3.8-fold and 16.8-fold higher mutagenic activity in Salmonella strains expressing human NAT1 and NAT2, respectively, than in the acetyltransferase-deficient strain, whereas N-Ac-N-OH-ABA was only weakly mutagenic in the Salmonella strain expressing human NAT2. Our results indicate that O-acetylation and O-sulfonation by human NATs and SULTs may contribute significantly to the high mutagenic and genotoxic potential of 3-NBA.
II.P.44. METABOLIC ACTIVATION of the SUSPECTED HUMAN CARCINOGEN 3-NITROBENZANTHRONE by HUMAN LIVER MICROSONES and by HUMAN RECOMBINANT CYTOCHROMES P450

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Determining the capability of humans to metabolise the suspected carcinogen 3-nitrobenzanthrone (3-NBA) and understanding which human cytochrome P450 (CYP) enzymes are involved in its activation are important in the assessment of individual susceptibility to this environmental contaminant found in diesel exhaust. We compared the ability of eight human hepatic microsomal samples to catalyse 3-NBA-DNA adduct formation. Using the ³²P-postlabelling method we found that all hepatic microsomes were competent to activate 3-NBA. Qualitatively similar DNA adduct pattern with multiple adducts were observed, as found recently in vivo in rats. All 3-NBA-adducts were derived from reductive metabolites bound to purine bases; we found no indication for ring oxidation. The role of specific CYPs and NADPH:CYP reductase in the human hepatic microsomal samples in 3-NBA activation was investigated by correlating the P450-linked catalytic activities in each microsomal sample with the level of DNA adducts formed by the same microsomes. On the basis of this analysis, we attributed most of the hepatic microsomal activation of 3-NBA to NADPH:CYP reductase. None of the CYP enzymes examined (CYPs 1A1, 1A2, 3A4, 2C9, 2D6, 2E1) appeared to be involved in 3-NBA activation. The role of nine individual recombinant human CYPs (1A1, 1A2, 2A6, 3A4, 1B1, 2B6, 2C9, 2D6, 2E1) in the metabolic activation of 3-NBA catalysing DNA adduct formation was also examined. Therefore, we used microsomes of baculovirus transfected insect cells (Supersomes™) containing recombinantly expressed human CYPs (10 and 25 pmol) and human NADPH:CYP reductase. DNA adducts were observed in all Supersomes™ preparations, similar to those found with human hepatic microsomes. Of all recombinant human CYPs, CYP2B6 and CYP2D6 were the most active in 3-NBA activation, followed by CYP1A1 and CYP1A2. These results demonstrate the potential of human NADPH:CYP reductase and CYPs to contribute to the metabolic activation of 3-NBA by nitroreduction.
II.P.45. GENOTOXICITY of NITROSOHENOLS in the SOS CHROMOTEST: INHIBITORY EFFECT of ASCORBIC ACID

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In the examination of the causes of human cancer, a major role is ascribed to diet, based on epidemiological observations. Among the numerous chemical carcinogens are the nitrosocompounds. The relevance of these nitrosocompounds is that their precursors are commonly present in foodstuff.

Knowledge concerning the genotoxicity of nitrosophenols in the SOS chromotest is limited. $p$-Nitrosophenol and its methylated derivatives have been synthesized and tested for their genotoxicity towards Escherichia coli strain PQ37 in the SOS chromotest. Bacteriotoxic effects were analyzed in bacterial cultures. Effects of methylation, among the various positional isomers ortho or meta, were investigated. Different pathways of action were suggested for nitrosophenol or its methylated derivatives for their mutagenic activity.

On the other side, antimutagenic properties of ascorbic acid were determined. Ascorbic acid dramatically decreased the nitrosophenol synthesis reaction and its genotoxicity in the mutagenic test. Effective protection on bacterial cultures was also determined and quantified by ID$_{50}$ value.
II.P.46. The *pqi* GENE ROLE in STRESS RESPONSE of *Salmonella typhimurium* CELLS

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It has been recently established that under unfavorable environmental condition, Gram-negative nonsporulating bacteria of various species are able to transform into so-called «non-culturable state» (NS). Bacterial cells in non-culturable state (non-culturable forms) are characterized by a markedly reduced metabolic activity and temporary inability for growth and reproduction on solid nutrient media. Great epidemiological significance of non-culturable forms of microorganisms are confirmed by experimental data.

We developed a laboratory model for inducing the nonculturable state in *Salmonella typhimurium*, and identified several genes which controlled the process of reversible culturable form generation of *Salmonella*. The use of TnPhoA transposon as a mutagenic factor allows identification of genes encoding transmembrane or extracellular proteins.

In fact, one of mutants we isolated, TnPhoA-8, was capable of utilizing the exogenous substrate of alkaline phosphatase added to the medium; in this case, transposon insertion occurred into the gene encoding protein of this type. The cells of mutant clone PhoA-8 had more prolonged duration of cell survival (determined by the ability to form colonies) than the control strain. Results of nucleotide sequence analysis of the mutant gene showed that the sequence is homologous to that of the *pqi*-5 gene described only in *E.coli*.

The *pqi* gene is a component of the *soxRS* operon induced in cells of enteric bacillus under oxidative stress. The *pqi* gene product can be identified only in the membrane protein fraction; the function of the protein encoded by this gene remains unknown. By means of RT-PCR technique it was shown that gene *pqi* continues to be expressed not only during starvation and conversion to an NS, but also in NF. A low level of expression of some genes in NF of bacteria is likely to be necessary for fast recovery of growth and propagation when favorable environmental conditions supplant a negative situation. Resuscitation of NF bacteria depends on various inducers of biotic and abiotic nature. Consistent with this proposal, we demonstrated that the commercially available antioxidant Oxyrase® an oxygen radical-destroying enzyme, prepared from *E.coli*, is able to resuscitate a stressed population of *Salmonella* cells. The Oxyrase® is more effective in resuscitation of *pqi* mutant cells than in resuscitation of wild type cells.
II.P.47. The IMPACT of YELLOW LOCUS on DNA REPAIR and GERM CELL MUTABILITY in Drosophila

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Cell sensitivity to mutagenic factors can be due to activity of DNA repair. Ethyl methanesulphonate (EMS) was taken as a model mutagen. Experiments were carried out in D. melanogaster using wild type strain as compared with y or y ct v ones marked by recessive gene yellow. Germ cell mutability was estimated in excision repair deficient males from strains mei-9a and C(1)DX, y f/y mei-9a as compared to males of wild and yellow phenotypes, respectively. Basc flies were used to test for sex-linked recessive lethals (SLRLs), which are primarily point mutations.

In males, mutagen sensitivity was studied depending on spermatogenesis stages. For this purpose brooding technique was used to separate germ cells treated at different stages since DNA repair activity is known to be the highest in pre-meiotic cells. The SLRL frequency in spermatozoids of wild type males was shown to exceed the spermatogonia mutability 5.6 times. In yellow males these differences are significant but less pronounced (1.4). In y mei-9a males, the mutation frequency was practically similar in both spermatozoids and spermatogonia. It has been also shown that the SLRL frequency in pre-meiotic cells of yellow males (14.5%) exceeded substantially that in wild type males (3.7%) indicating the yellow mutation influence on repair of DNA adducts responsible for EMS-induced point mutations. In another series the effect of maternal repair was taken into account. EMS-treated Basc males were mated with intact wild type or yellow females. Embryonic and postembryonic lethality were analyzed in the F1. This value in yellow females was two – three times as high as in Berlin wild females. It is interesting to note that homozygous y mei-9 females laid unviable eggs.

These facts indicate inability of yellow females to repair primary lesions leading to chromosome breaks and death of individuals in ontogenesis. SLRL frequencies in F2 did not depend on female phenotype. However calculation of theoretically expected mutation frequencies taking into account embryonic and postembryonic lethality in F1 also allows us to assume the deficiency in maternal repair systems in yellow females as compared to the wild type. Hence, the impact of yellow locus on germ cell mutagen sensitivity seems to be due to deficient DNA repair.
Using HPLC or simple colorimetric MDA-TBA detection for the biomonitoring of oxidative stress in populations occupationally exposed to genotoxicants

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Free radicals are continuously formed in the body as a consequence of aerobic and anaerobic metabolism. Uptake of xenobiotics may lead to a higher production of free radicals and namely reactive oxygen species (ROS) in vivo which can react with cellular constituents such as DNA, lipids and proteins. Experimental and epidemiological evidence suggests that DNA oxidation is mutagenic and it is a major contributor to human cancer. Furthermore, oxygen radicals can oxidize lipid or protein molecules to generate intermediates that react with DNA to form adducts. It is well known that polyunsaturated fatty acids (PUFA) in biological membranes are among the main targets for free radical attack and malondialdehyde (MDA)—an end product of lipid peroxidation—has been reported to be mutagenic and carcinogenic.

One of the most frequently used tests for measuring the oxidative stress in biological systems is based on the reaction of MDA with thiobarbituric acid (TBA) but its specificity is questionable due to the presence of interfering chromogens. A comparative evaluation was made of the conventional spectrophotometric procedure and a High Performance Liquid Chromatography (HPLC) method for the determination of MDA in erythrocytes and urine from individuals of two populations occupationally exposed to acrylonitrile and pesticides. The statistical analysis only found a difference between non-smoker individuals that applied pesticides and the control group (P-value=0.03) when using the spectrophotometric method for the determination of urinary MDA-TBA levels. In the population exposed to acrylonitrile no significant differences in the values of MDA were found between the studied populations. A good correlation was found between the results obtained with the two methods (R=78.3%) although the HPLC yields lower values than the spectrophotometric method for the same sample. In a cost-benefit approach to population studies the HPLC approach is justified only if the results obtained with the spectrophotometric method suffer interference due to factors present in the sample and specifically related to the studied exposure.
II.PO.49. OXIDATIVE DNA DAMAGE ASSESSMENT in HUMAN SPERMATOZOA by MODIFIED COMET ASSAY

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The new techniques for \textit{in vitro} fertilization allow individuals with poor semen quality to procreate. On the other hand, the knowledge of the mechanisms regulating the efficiency, maintenance of information integrity and selection of spermatic cells is still rather deficient. In many instances, the decrease in fertility has been associated with the occurrence of lipid peroxidation of cell membrane. Furthermore, reactive oxygen species (ROS) can cause oxidative damage to other cell structures and particularly to DNA. It is also known that several antioxidant enzymes are not present in spermatic cells. However such deficiency can be compensated by the antioxidant properties of seminal plasma. Some techniques for \textit{in vitro} fertilization involve spermatic cells without seminal plasma. Under these conditions the sperm could be more vulnerable to the effects of oxidative stress and, therefore, present a higher DNA damage. Consequently, it appears particularly important to evaluate the oxidative DNA damage related to sperm manipulation before \textit{in vitro} fertilization. For this aim, we utilised a modified comet assay in order to evaluate the oxidative damage in such cells, by using two specific enzymes (FPG and Endo III), previously tested on somatic cells, able to recognise the oxidized bases. An endogenous oxidative damage has been detected on spermatic cells. In order to evaluate the sensitivity of the method and its capability to distinguish different kinds of DNA damage, genotoxic agents variously acting on DNA have been used (hydrogen peroxide, bleomycin, ethyl methanesulfonate). Preliminary data suggest a possible application of this modified comet assay to analyse DNA integrity in human spermatozoa to be used \textit{in vitro} fertilization.
Quantification of expression profiles of key genes in mammals is of great interest to assess fundamental processes of genetic toxicity. The array methodology is an invaluable procedure to generate genome-wide transcriptional profiles, but this method offers only an estimate of relative message levels and does not allow highly quantitative analysis on large scale of specimens. For investigations aimed at quantitative analysis, the sensitivity of PCR-based methods will hardly be reached by any other technique.

We have designed a reverse transcription multiplex PCR procedure for simultaneous detection and precise quantification of changes in the transcript levels of well-defined sets of genes (Pueyo et al Methods Enzymol, 2002). In this technique, up to 13 messages are exponentially amplified in a single MPCR reaction. Here, we used this method, in conjunction with real-time PCR assays, to quantify the exact number of molecules of different murine mRNAs. The genes studied allow to assess key cellular events such as DNA damage (OGG1, APE1), protein perturbation (HSP70), oxidative stress (HO1, A170, PRDX1) or transcriptional regulation (c-fos). Genes (GAPDH, ACTB) used as reference in most expression studies were also included.

Male BALB/c mice were divided into groups of 3 subjects. Oxidative stress was induced by paraquat (i.p. at 30 mg/kg). After killing, the liver, spleen, kidney, testis, heart, lung and brain were removed and immediately frozen in liquid nitrogen. Untreated animals were used for quantitation of basal expression levels. Basal levels showed outstandingly stable expression among mice (e.g. 3491±464 HO1 mRNA molecules/ng of total RNA from liver). Genetically diverse populations, such as the Algerian mouse (a rodent used as pollution bioindicator), had a variability in gene expression like that observed among inbred mice. In contrast, basal expression levels showed extensive variability across tissues: e.g. the spleen produced 44110 HO1 mRNA molecules/ng; this level being near 50-fold higher than that of brain. The housekeeping genes showed also considerable variability, highlighting the importance of absolute quantitation of mRNAs. Paraquat caused a large up-regulation of HO1 mRNA in liver, where a maximal induction of 28-fold was quantified at 120 min.

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II.P51. SIMPLE, ROBUST and COMPOUND-ECONOMICAL GENOTOXICITY and CYTOTOXICITY ASSESSMENT USING YEAST

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We have developed a simple endpoint assay ("GreenScreen") that detects the activation of DNA repair using a GFP reporter system in budding yeast. The test requires less than half a milliliter of compound at the highest test concentration. Genetically modified yeast cells, test compound and diluent are mixed in microplates, either manually or using commercially available liquid-handling robots, then stored overnight in a static incubator at 25°C. Cell density and fluorescence measurements are used to provide quantitative measures of cytotoxicity (reduction in cell proliferation) and genotoxicity (fluorescence normalized to cell yield). Automated data handling is available and simple interpretations are generated for cytotoxicity (positive, weak, negative) and genotoxicity (positive, weak, negative).

Previously, we reported a limited validation exercise of the assay [Afanssiev et al., Mut. Res. (2000) 464:297-308]. Here we report an extended validation using 110 compounds suggested by professional genetic toxicologists. Regulatory test data (Ames, ML, MN) for 87 of the compounds has been compiled from the literature. A comparison revealed that GreenScreen gives a very low incidence of 'False Positive' results.

GreenScreen has been assessed at two external pharmaceutical industry sites to test its robustness and reproducibility, and to establish the relevance of the data produced. Known genotoxic and non-genotoxic compounds with different toxicities, as well as coded proprietary compounds, were tested. The test was performed with ease by staff at the host organizations, known compounds gave the same results as those obtained in-house, and the results for proprietary compounds were in agreement with expectations.

The battery of regulatory genotoxicity tests is expensive and time consuming, and there has been a lot of interest in the development of cheaper, faster screens to give an early preview of the Ames test. GreenScreen is the first screening test designed to give an integrated preview of the whole battery of tests, and is presented in a robust format ready for automation.
II.P.52. DEVELOPMENT of PD1000: A NEW Escherichia coli STRAIN, HIGHLY SENSITIVE TOWARDS the DETECTION of ALKYLATING AGENTS

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We have reported previously on the E. coli mutagenicity tester strain BMX100 (Kranendonk et al., 1998). This strain detects efficiently chemical mutagenicity, monitored by the reversion of arginine auxotrophy. However, this strain failed to detect alkylating agents. Strain PD1000 was obtained by inactivation of both the ada and ogt genes in strain BMX100, to overcome this limitation. These genes encode for two types of DNA repair alkyltransferases.

The strain PD1000 was compared with the former strain BMX100, in the detection of mutagenicity using several alkylating compounds. The new strain was approximately 3 fold more sensitive towards N-Nitrosodipropylamine and N-Nitrosodiethylamine and 9 fold more sensitive towards MNNG (N-methyl-N'-nitro-N-nitrosoguanidine). The improvement in sensitivity for detecting alkylating agents by PD1000 is best demonstrated with NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), inducing 298 revertants per µmol, in comparison with strain BMX100 which did not demonstrated any mutagenicity when tested up to 200 µmol per plate.

The sensitivity for detection of mutagenicity for various types of non-alkylating mutagens, such as 4NQO (4-nitroquinoline-1-oxide), B(a)P (benzo[a]pyrene) and CHP (cumene hydroperoxide) was not impaired. Moreover, the level of spontaneous reversion in PD1000 was equal to the former strain.

We described previously the co-expression of human NADPH cytochrome P450 reductase together with several forms of human cytochrome P450 (Kranendonk et al., 1999). Results reported here suggest that PD1000 could be an appropriated strain for the study of the role of human cytochromes in the bioactivation of alkylating promutagens.


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II.P.53. A beta-LACTAMASE GENE REVERSION ASSAY
For SHORT-TERM DETECTION of MUTATIONS

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In order to overcome known disadvantages of current genotoxicity and mutagenicity assays a new bacterial short-term assay has been developed using base pair and frameshift mutations in ampicillinase genes located on a low copy plasmid. The approach of the new assay is to combine the conventional reverse mutation concept with the indicator test related detection of genotoxicity by reporter gene induction. The constructed plasmids contain all the necessary genetic elements to guarantee a sensitive, fast and specific detection of mutagens like mucAB genes, a tightly tetR regulated lacZ gene inducible by anhydrotetracyclin, a gene for the tet-repressor and the mutated ampicillinase. Frameshift mutations were integrated into repetitive GC-sequences and G-repeats known to be mutagenic hot-spots. Base pair substitutions were inserted in or around the active site of the ampicillinase gene thus generating reversible ampicilline sensitivity. The tester strains can grow in full medium during all parts of the assay. Thus the detection of revertants which only grow after reversion to ampicilline resistance is possible within few hours by induction of the lacZ gene and subsequent luminometric measurement by galacton® substrate. Nevertheless a conventional detection of revertants by pH shift using bromocresol purple or enzymatic β-lactamase assay using nitrocefin is possible even within overnight selection. With this new mutagenicity assay more than 20 representative chemicals have been tested with and without metabolic activation in a 384-well format. The first results show high specificity and the same, in some instances even better sensitivity compared to umu test and Ames microsuspension assay. The test is easy to perform and results on the mutagenicity of a sample are available within one working day or overnight. Furthermore, sterile working conditions are not necessary because contamination risks are minimized and the test is generally suitable for environmental samples.

A test kit has been produced which contains combinations of lyophilized strains, granulated growth medium and a granulated supplement for the selection medium making the assay applicable as high throughput screening test.
II.P.54. STATISTICAL EVALUATION and COMPARISON of COMET ASSAY RESULTS

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The aim of the present work is to find a mathematical distribution describing the comet assay data property and allowing for comparison of the results of various experiments using the parameters of the fitted distribution. Histograms of the comet assay data are generally unsymmetrical. That is why an asymmetrical theoretical distribution, the Weibull one, was selected.

The statement that a numerical property characterizing a given population of items follows the twi-parameter Weibull model implies that the probability density function has following functional dependence:

\[ f(x) = \frac{\alpha x^{\alpha-1}}{\beta^\alpha} \exp\left[-\frac{x}{\beta}\right] \]

where \( \alpha \) is known as the shape parameter and \( \beta \) the scale parameter or characteristic value of the property.

Presently Weibull distributions were fitted to the histograms of the tail moments and %DNA in the tail of the CRL 2088 cells (normal human skin fibroblasts) and CLV 102 (human embryo cells) after bleomycin (BLM) treatment.

![Fig. 1 portrays the fitting of Weibull density distribution to a histogram](image1.png)

![Fig. 2 shows Weibull plots and their parameters α and β for the tail moments of the CRL 2088 cells after BLM treatment. BLM conc. in µg/ml.](image2.png)

It has been shown that Weibull distribution can be easily characterized also by percentiles, e.g. 95th percentile, m95 or p95, for moments and %DNA, respectively.
II.P55. DEVELOPMENT of a $^{14}$C-POSTLABELLING PROCEDURE for the TRACE DETECTION of DNA ADDUCTS

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Accelerator mass spectrometry (AMS) is currently the most sensitive method for DNA trace adduct detection. AMS measures an isotope ratio, commonly $^{14}$C:$^{13}$C to determine the concentration of labelled compound in a sample. At present, analysis of adducts in humans requires administration of a $^{14}$C-labelled compound prior to sample collection. The objective of this investigation is to develop a $^{14}$C-postlabelling technique to incorporate $^{14}$C-labelled groups into DNA adducts followed by AMS detection, enabling the detection of DNA adducts in humans at very low levels without the need to administer a $^{14}$C-labelled compound.

A method has been developed to acetylate 2'-deoxyguanosine (dG) and O₆-methyl deoxyguanosine (O₆-MedG) with acetic anhydride resulting in a tri-acetylated product. The reaction products are purified on a water-methanol reversed phase HPLC system and have been structurally verified by mass spectrometry, with yields of greater than 90% consistently achieved. DNA was methylated with $^3$H-methyl nitrosourea to produce methylated adducts for use in method development. Studies have been carried out to ensure that O₆-MedG can be isolated pure from other methyl adducts by reversed phase HPLC. Isolated $^3$H-O₆-MedG has been used to ensure that the adduct does not degrade at any stage of the labelling procedure.

Different methods of detection were employed to ascertain that the acetylation reaction was viable at increasingly lower levels. Calibration lines have been produced for O₆-MedG separated by HPLC with UV detection. The limit of detection is 1 nmole. Concentrations of O₆-MedG ranging from 3 nmoles to 5 fmoles were acetylated with d₆-acetic anhydride to produce deuterated tri-acetyl O₆-MedG. This was analysed LC-MS using unlabelled tri-acetyl O₆-MedG internal standard. The ratio of peak areas was measured to produce a calibration line. The limit of detection is 10 fmoles. O₆-MedG will now be acetylated with $^{14}$C-acetic anhydride to detect adducts at increasing lower levels using liquid scintillation counting and ultimately AMS.
II.P.56. GENOTOXIC AGENTS in HUMAN MILK

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Genotoxic agents of dietary and/or environmental origin may play a role in the aetiology of breast cancer. Breast milk is known to contain components capable of causing DNA damage in *in vitro* assays and is potentially a means of investigating the exposure of breast epithelial cells to environmental agents. In the present study, extracts of human milk (8 - 48 g-equiv.) were examined for their ability to cause DNA single-strand breaks in MCL-5 cells ('Comet’ assay) in the presence of DNA repair inhibitors hydroxyurea and cytosine arabinoside, and to cause mutations in *Salmonella typhimurium* strain YG1019 (Ames assay) in the presence of Aroclor 1254-induced rat liver S9. Cows’ milk was a negative control. Extracts of milk were prepared by a solid-phase tandem extraction (64 g-equiv.) and fractionated (8 fractions, 10 mls) by reverse-phase HPLC. Fraction 3 was significantly positive for comet formation (16 g-equiv., median comet tail length = 121.5 µm), compared with control (15.0 µm), ($P<0.0001$, Mann-Whitney test) and also significantly mutagenic in YG1019, (880 ± 47 revertants/plate, 16g-equiv., compared with 21 ± 4 for controls). In all experiments, genotoxicity was observed in the absence of cytotoxicity. Furthermore, fractionated blue rayon extracts from pooled milk samples gave rise to additional positive fractions (3, 4 and 6) over a dose range of 2.5 – 25 g-equiv. milk/plate in the Ames assay. In further studies, partitioning of activity between lipid and skimmed milk was examined. Milk phases, separated by centrifugation, were extracted. After HPLC, approximately two-thirds of the total Ames activity (fraction 3) partitioned in the aqueous and one-third in the lipid phase when compared with whole milk, suggesting that the agent/s responsible for the observed genotoxicity are moderately polar. This type of approach may provide new clues as to the role of environmental agents in the initiation of breast cancer.
II.P.57. MECHANISM of NICKEL ASSAULT on the ZINC FINGER of DNA REPAIR PROTEIN XPA

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XPA is a member of the protein complex of the nucleotide excision repair (NER) pathway of DNA repair, participating in the recognition of damage. The 4S zinc finger domain of XPA is involved in the interactions with other NER proteins. As demonstrated previously, the activity of XPA is compromised by several metal ions implicated in DNA repair inhibition, including Ni(II), Cd(II) and Co(II) (1). In order to study the possible molecular mechanisms of XPA inhibition, we investigated Zn(II) and Ni(II) interactions with the synthetic 37-peptide (XPAzf), representing the XPA zinc finger sequence (2). The binding constants were determined using fluorescence and UV-Vis, structural insights were provided by circular dichroism and oxidative damage to XPAzf was studied with HPLC. The binding constants for Zn(II) and Ni(II) are $8.5 \pm 1.5 \times 10^8$ and $1.05 \pm 0.07 \times 10^6 \text{M}^{-1}$, respectively in 10 mM phosphate buffer, pH 7.4, and $6 \pm 4 \times 10^8$ and $2.9 \pm 0.5 \times 10^6 \text{M}^{-1}$ in 50 mM phosphate buffer, pH 7.4, yielding binding constant ratios Zn/Ni of ca. 800 and 2000, respectively. The Ni(II) ion forms a square-planar complex with the sulfurs of XPAzf, opposed to the tetrahedral structure of the native Zn(II) complex. Consequently, the overall zinc finger structure is lost in the Ni(II)-substituted peptide. Zn(II)-saturated XPAzf is remarkably resistant to air oxidation and is slowly oxidized by 0.01 mM, 0.1 mM and 1 mM H$_2$O$_2$ in the concentration-dependent fashion. However, the presence of just 10-fold molar excess of Ni(II) is sufficient to accelerate this process for all three H$_2$O$_2$ concentrations tested. Overall, our results indicate that XPAzf can undergo Ni(II) assault in specific conditions.

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II.P.58. PUVA-INDUCED LIPOAPOPTOSIS in the HUMAN LYMPHOCYTES: ARACHIDONIC ACID and its PHOTOPRODUCTS.

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The phototherapy PUVA, the combination of psoralen with UVA irradiation, is directed towards activated T lymphocytes in order to induce transient immune suppression and decrease of local inflammation via increased apoptosis.1

We have been studying the effect of exogeneous arachidonic acid (AA) and its psoralen photoadduct on the induction of apoptosis in the cultured human peripheral blood lymphocytes (Ly). The photoadduct, obtained in vitro and purified, has been characterized by NMR and MS spectrometry as cycloaduct of psoralen to vinylene bond of acid (AA<>PSO).

The physiological reaction towards additives, 20 – 240 µM per ml containing 10⁶ cells have been monitored after 24 h by flow cytometry, based on the reaction with annexin V (An⁺) and propidium iodide (PI⁺). The total number of Ly was not decreased within tested range of additives after 24 h. The low concentration up to 60 µM AA induced an early apoptosis (An⁺, single positive cells) that in a dose dependent manner shifted to late apoptosis (An⁺PI⁺, double positive cells) reaching plateau level at 160 µM AA (toxicity level). The adduct, AA<>PSO, presumably formed during PUVA treatment in vivo, induced apoptotic changes similar to those induced by free AA, but at concentration 2,5 times higher. Cells treated with pharmacological doses of UVA (2.5 – 7.5 J/cm²) revealed accelerated loss of viability and appearance of late apoptotis in the presence of psoralen (1µM) and arachidonic acid (20 – 160 µM).

Our results indicate that UVA irradiation induces synergistically apoptosis in the presence of psoralen and arachidonic acid additives. Thus it is possible that PUVA-induced apoptosis may proceed in part by a lipoapoptosis pathway born in lymphocyte’s membranes.

1. Z. Zarębska, E. Waszkowska, S. Caffieri, F. Dall'Acqua, Il Farmaco 55 (2000) 515-520
III.P.1. LECTINS as MODULATORS of Ni(II) INDUCED GENOTOXICITY and MUTAGENESIS in REC+ and REC- STRAINS of BACILLUS SUBTILIS

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Lectins (sugar-binding proteins) have a wide spectrum of biological activity in different cell types and are thought to be involved in the modulation of genome functions via transmembrane signal transduction. Study of lectins as pharmacologically prospective preparations with carcinostatic potential needs more detailed information about their mutagenic/antimutagenic effects. In B. subtilis there are known about twenty mutations deficient in recombination/reparation with increased sensitivities to DNA-damaging agents. Hence bacterial cells may provide a model for investigation of mechanisms of unknown genetic effects of lectins. In our work we use commercial lectins (LECTINOTEST, Lviv, Ukraine) and isolectins from Sambucus nigra inflorescenses [1] in modified rec assay [2] as possible protectors and Ni(II) ions (known human carcinogen) as a mutagen [2]. The toxic effect was tested in agar diffusion assay or in terms of % reduction in titer. Frequency of reversions to prototrophy was the measure of mutagenic effect. A comparative analysis showed that among mutations being tested (recA, recB, recF, recG, recL, adaA, recP), the recP, supposed to be involved in SOS reparation, was most sensitive to genotoxic effect of Ni(II) ions. Preincubation with legume lectins can significantly protect Rec+ cells in range ConA>PHA>LAA, Rec strains being almost insensitive. Isolectins of PHA and SNA demonstrated different patterns of such protection depending on their structure, molecular organization, dose and environmental conditions. Hybrid and homo-tetramers of PHA demonstrated different patterns of modulation of the frequency of reversions, supporting the idea of different affinity of subunits for some targets. Hence, in Rec strains lectins may act as modulators of genotoxic and (shown for PHA and SNA) mutagenic effects of Ni(II) ions. Low sensitivity of Rec strains to such effects may indicate an indirect mechanism which involves participation of reparation/recombination system.

III.P.2. UV-INDUCED UNSCHEDULED DNA SYNTHESIS as MEANS of the GENOTOXICOLOGY MONITORING in HUNGARY

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Since 1985, as part of a multiple end-point genotoxicology monitoring system we have scored UV-light induced unscheduled DNA synthesis (UDS) levels in controls and in occupational exposures. Here we present the data of 500 donors including 175 controls, 43 benzene-, 10 styrene-, 26 acrylonitrile- and dimethylformamide-, 21 polychlorinated biphenyl-, and 31 Adriamycin-exposed workers, 22 road-pavers, and altogether 172 hospital nurses exposed to either cytostatics, or anaesthetics, formaldehyde, or ethylene oxide. The scored biomarkers in peripheral lymphocytes were the chromosome aberration (CA), premature centromere division, sister-chromatid exchange (SCE) and \textit{hprt} mutation frequencies, labelling and proliferation rate indices, and UDS measured by liquid scintillometry in hydroxyurea-treated samples. UDS data were compared with the CA and SCE frequencies. The effect of confounders like age, gender, smoking and drinking habits was also considered.

The mean UDS levels in unexposed and industrial controls were 5.56±0.38, and 7.11±0.37 (relative units), respectively. Among workers exposed to acrylonitrile and dimethylformamide we observed increased mean UDS levels with increased exchange type CAs. In the hospital staff groups exposed to either cytostatic drugs, or anaesthetics, formaldehyde or ETO (in the latter case with extremely high exchange type CA frequency) the mean UDS was lower than in the industrial control.

The observed alterations in UDS levels followed the changes in working conditions during the follow-up investigations of Adriamycin-, benzene-, styrene, and PCB-exposed workers and among the road pavers. In these cases the UDS levels normalised parallel to the improvement in working conditions, while among Adriamycin producers UDS levels increased parallel to the deterioration of the working conditions.

In conclusion, the alterations in DNA repair capacity on either individual or group level reflected the changes in exposures, and UDS was a useful additional biomarker of the genotoxicology monitoring.

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III.P.3. GENOTOXICOLOGICAL and IMMUNOTOXICOLOGICAL MONITORING of FORMALDEHYDE EXPOSED WORKERS

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Authors present the results obtained by genotoxicological and immunotoxicological investigations on peripheral lymphocytes of four groups of formaldehyde exposed workers. We investigated 17 hospital employees with low dose exposure, 18 workers producing formaldehyde, 67 workers manufacturing school-furniture, and 11 workers impregnating wood with formaldehyde. This is the first publication of immunotoxic and apoptosis inducing effects in a formaldehyde exposed human group.

Genotoxicological biomarkers: HPRT variation frequency (VF), UV-induced unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE) and chromosomal aberration (CA) frequencies. Cytofluorometric S-phase fraction, lectin stimulation (LI), and proliferation rate (PRI) indices, apoptosis and immunotoxicological investigations were only performed in hospital staff. Immunotoxicological parameters: lymphocyte subpopulations (helper and cytotoxic T-cells, B-lymphocytes, NK-cells) and expression of activation markers CD25 and CD71. The means were compared with 87 controls.

SCE and CA showed no significant difference compared to the controls in any investigated group in accordance with the literature. LI was significantly decreased in all exposed groups. In the two groups of woodworkers but not in the hospital staff, VF and UDS were significantly increased. In the hospital staff we observed a rise in the percentage of helper T cells, a decrease in cytotoxic T cells, and no difference in the expression of activation markers. In these donors the lectin stimulated S phase fraction was decreased while the spontaneous apoptosis was increased.

These data demonstrate that formaldehyde is immunotoxic and, in accordance with the in vitro literature data, can decrease cell proliferation in exposed humans. However, formaldehyde can also increase spontaneous apoptotic activity, which may explain the observed low genotoxic effect in the groups exposed only to low doses of formaldehyde.

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III.P.4. FURTHER DATA on the POSSIBLE GENOTOXIC ACTIVITY of ARSENIC

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Arsenic is a well known carcinogen in humans, although its mode of action has not been yet well characterized. To obtain more information on the possible genotoxicity of arsenic, studies with Drosophila, with human cells treated in vitro, and with human populations environmentally exposed have been conducted.

In Drosophila, sodium arsenite treatment during larvae development was unable to induce any significant increase in the frequency of mutant spots in the wings of transheterozygous individuals. Nevertheless, pre-treatments and co-treatments with potassium dichromate produced a significant reduction of the genotoxic effects induced by potassium dichromate alone. When the study was repeated with ethyl methanesulphonate, sodium arsenite had no effects on the frequency of mutant clones induced by EMS. Thus, from these data it appears that arsenic acts as an agent which reduces oxidative stress damage.

In the human lymphoblastoid TK-6 cell line, different organic and inorganic arsenic compounds were tested by using the comet test. The list of compounds tested is as follows: sodium arsenite, sodium hexafluorarsenate, sodium arsenate, arsenobetaine, monomethylarsonic acid, dimethylarsonic acid, tetramethylarsonium iodide and tetraphenylarsonic chloride. Results indicate that only the inorganic form sodium arsenite induces a significant increase in the level of genetic damage evaluated by the comet test (DNA breaks as well as alkali labile sites). In addition, the compound tetraphenylarsonic chloride gives a slight increase around the statistical level. The other compounds tested give negative results. These data would support the view that inorganic agents show more genotoxic risk than the organic compounds.

Finally, a biomonitoring study has been carried out by evaluating the frequency of micronuclei (MN) in peripheral blood lymphocytes from 106 exposed people from the north of Chile and 111 controls from the area of Concepcion, also in Chile. The preliminary results indicate that the frequency of MN in the exposed group is higher than in the controls. Possible correlation between exposure, measured by the arsenic content in urine, and genetic damage is under investigation.
Inorganic compounds of arsenic are known to cause serious health problems, including cancer. The mechanism by which arsenic compounds cause human cancer has not yet been fully clarified. There are some suggestions that arsenic indirectly affect DNA, for example through inhibition of DNA repair processes, induction of oxidative stress, and modulation of cellular proliferation.

The present study was focused on estimating the chromosomal damages measured by frequency of micronuclei (MN) induced by occupational exposure to arsenic. Micronuclei are extranuclear bodies composed of chromosome fragments or whole chromosomes that failed to be incorporated into daughter nuclei at mitosis. Therefore, MN can be a consequence of chromosomal aberrations or spindle dysfunction, indicating clastogenic as well aneugenic effects, respectively.

The study was conducted among 70 workers employed in the nonferrous metal smelters where arsenic exposure occurs via inhalation route. The concentration of arsenic in the breathing zone of workers ranged from 3-80µg/m² of air. As the reference group eighty individuals with no exposure to genotoxic agents and of similar age, health status and smoking habit were selected. All subjects in exposed and control groups were male. The venous blood samples from each person were taken by venous puncture and used to perform 72 hours lymphocyte cultures. The incidence of micronuclei was determined in 1000 cytokinesis-blocked lymphocytes for every subject. The mean frequency of MN in exposed workers was about two times higher than in the control group. The differences were statistically significant (p<0.05). These findings indicate that exposure to arsenic in examined occupational environment creates genotoxic effects at the chromosomal level.

Study supported by 5th Framework Programme of UE, Contract No.QLK4-CT – 1999-01142.
III.P.6. CHROMOSOMAL RADIOSENSITIVITY in BREAST CANCER PATIENTS with a KNOWN or PUTATIVE GENETIC PREDISPOSITION

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Ionising radiation induces chromosomal damage. Several studies have shown that breast cancer patients are, on average, more sensitive to ionizing radiation than healthy individuals. In this study we investigated the chromosomal radiosensitivity of breast cancer patients with a known or putative genetic predisposition and compared it to a group of healthy women. The chromosomal radiosensitivity was assessed with the G2 and the G0-micronucleus (MN)-assay. For the G2-assay lymphocytes were irradiated in vitro with a dose of 0.4 Gy $^{60}$Co $\gamma$-rays after 71h incubation and chromatid breaks were scored in 50 metaphases. For the MN-assay lymphocytes were exposed in vitro to 3.5 Gy $^{60}$Co $\gamma$-rays at a high dose rate (HDR) or low dose rate (LDR). 70h post-irradiation cultures were arrested and micronuclei were scored in 1000 binucleate cells.

The results demonstrated that the group of breast cancer patients with a known or putative genetic predisposition was on the average more radiosensitive than a population of healthy women and this as well with the G2 as with the HDR and LDR-MN-assay. With the G2-assay 43% of the patients were found to be radiosensitive. A higher proportion of the patients were radiosensitive with the MN-assay (45% with HDR and 61% with LDR). No correlation was found between the G2 and the G0-MN-chromosomal radiosensitivity. Of the different subgroups considered, the group of the young breast cancer patients without family history showed the highest percentage of radiosensitive cases and this as well in the G2 (50%) as in the MN-assay (75-78%).
III.P.7. RADIOSENSITIVITY of AT and NBS HOMO- and HETERO-ZYGOTES as DETERMINED by THREE-COLOR FISH CHROMOSOME PAINTING

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A three-color chromosome painting technique was used to examine the spontaneous and the radiation-induced chromosomal damage in peripheral lymphocytes and lymphoblastoid cell lines from 11 patients with ataxia telangiectasia (AT) and from 14 individuals heterozygous for an AT-allele. In addition, two homozygous and six obligate heterozygous carriers of mutations in the Nijmegen breakage syndrome (NBS) were investigated. The data were compared with chromosomal damage in 10 unaffected control individuals and 48 cancer patients without any therapeutic treatment. Based on the well-documented radiation sensitivity of AT- and NBS-patients, it was of particular interest to determine whether the FISH-painting technique used allows the reliable detection of an increased sensitivity to in vitro irradiation of cells from heterozygous carriers. Peripheral blood lymphocytes and lymphoblastoid cells from both the homozygous AT and NBS patients presented the highest cytogenetic response, whereas the cells from control individuals showed only a low number of chromosomal aberrations. The response of cells from heterozygous carriers was intermediate, and in double coded studies clearly could be differentiated from both other groups. AT and NBS heterozygosity could be distinguished from other genotypes by the total number of breakpoints per cell, and also by the number of the long-lived stable aberrations in both AT and NBS, but by the fraction of unstable chromosome changes only in AT. Thus, the three-color painting technique presented here proved to be well-suited as a supplementing if not superior method compared with conventional cytogenetic techniques for the detection of heterozygous carriers of these diseases.
III.PO.8. A NOVEL DISORDER CHARACTERISED by RADIOSENSIVITY

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Sensitivity toward DNA damage-induced by ionising radiation may arise as a result of DNA repair, activation of cell cycle checkpoints or abnormal radiation response pathway.

Here we report a novel disorder characterised by chromosome instability, radiosensitivity, immunodeficiency, development defects and typical NBS bird-like face, with normal cellular levels of nibrin and no mutations in the NBS1 and LIG4 genes.

Spontaneous chromosome instability was assessed in human peripheral blood lymphocytes; we find that patient has an increased frequency of spontaneous chromosome aberrations when compared to healthy age-matched controls. Radiosensitivity was ascertained by irradiating lymphoblastoid cell lines; cells were collected three hours later in order to score chromatid breaks. The proband appeared to be sensitive to ionising radiation, although his sensitivity reached an intermediate level compared to that of normal and NBS cells. The analysis of treatment-induced cell cycle perturbations, revealed a proficient activation of G1 to S-phase transition checkpoint and the depletion of S-phase cells as well as the extent of G2-phase accumulation.

DNA was extracted from peripheral blood lymphocytes or from LCLs and NBS1, LIG4, XRCC4 and Artemis genes mutation analysis was carried out by dHPLC and direct sequencing: no mutation was found in any gene. Sequence analysis of the Artemis gene revealed the presence of three base changes at the homozygous state. Immunoblotting and protein detection indicated normal levels of NBS, hMre11, Rad50 and DNA-ligase IV.

Experiments aimed at evaluating the rejoining of DNA strand breaks are in progress by means of gel pulse electrophoresis technique, after X-rays treatment of control, NBS and proband lymphoblastoid cell line.

Our results point to the discovery of new human diseases characterised by radiosensitivity, which may represent valuable biological material to investigate the molecular pathways involved in DNA repair processes and cell cycle checkpoints alterations.
III.P.9. IDENTIFICATION of FIVE SINGLE NUCLEOTIDE POLYMORPHISMS in the DNA REPAIR GENES: MGMT, XPA, XPD and XRCC1 in SLOVAK POPULATION

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Sequence variations have been identified in a number of DNA repair genes in several studies. We report initial results from a biomonitoring study aimed at estimating the polymorphisms in coding regions of genes involved in repair of DNA damage in 350 persons from the Slovak Republic. Subjects consisted of men and women working in 3 factories, either exposed to asbestos (61 subjects), rockwool (98), glass fibres (80) or controls (altogether 138 subjects).

PCR-restriction fragment length polymorphism (RFLP) assays were used to determine five polymorphisms: X-ray repair cross complementing group 1 (XRCC1, exon 10, G/A, Arg399Gln), xeroderma pigmentosum complementation group D (XPD, exon 10, G/A, Asp312Asn and exon 23, A/C, Lys751Gln), xeroderma pigmentosum complementation group A (XPA, 5' non-coding region, 23A/G) and O'-methylguanine-DNA methyltransferase (MGMT, promotor-enhancer, 1099C/T).

The substitution located in exon 10 of XPD was found in 9.6%, in exon 23 of XPD in 13.6% and in region of enhancer of MGMT in 8.6%. In contrast, the substitutions located in 5' non-coding region of XPA and in exon 10 of XRCC1 were frequent (35.5% and 55.7%, respectively). A number of phenotypic biomarkers are being investigated within this study, including oxidative DNA damage and repair. We suppose that the polymorphism in repair genes may contribute to inter-individual variations in DNA repair capacity, but the data have yet to be analysed.

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Oxidative DNA damage is mediated by reactive oxygen species (ROS). In cells, ROS are formed either as by-products of aerobic metabolism or by exposure to environmental mutagens. 7,8-Dihydro-8-oxoguanine (8oxoG) is one of the most important oxidative DNA lesions produced by oxygen radical-forming agents. The presence of 8oxoG in DNA causes G:C to T:A transversions. In humans, it is assumed that 8oxoG is repaired by the protein product of the 8-Oxoguanine DNA glycosylase 1 gene (hOGG1). The presence of several polymorphic sites is described inside the hOGG1. Among them, the polymorphism Ser326Cys is suggested to play a role as a risk marker for diverse types of chronic diseases, including cancers. We examined genotype distributions of the Ser326Cys polymorphism in the healthy males (Caucasians) occupationally exposed to air pollution in the downtown of Prague as well as suburban employees. For that purpose, we developed a novel PCR-based RFLP approach for the genotype examination. The analyses based on 136 subjects showed that individuals carrying Ser/Ser homozygous genotype were more frequent (63%) in comparison with Ser/Cys heterozygotes (27%) and/or Cys/Cys homozygotes (10%). The frequency of Ser allele was determined to be 0.77. The significance of the Ser326Cys polymorphism to the frequency of chromosomal aberrations determined by conventional method and FISH (Fluorescence In Situ Hybridization) will be discussed.

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III.P.11. The EFFECTS of POLYMORPHIC XPD GENOTYPES on CHROMOSOMAL ABERRATIONS in the CZECH MALE POPULATION

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The xeroderma pigmentosum group D (XPD) gene encodes an ATP-dependent DNA helicase that is involved in both nucleotide excision repair (NER) pathway and basal transcription. The NER pathway enables cells to eliminate a variety of genome damage, including cross-links, UV light-induced DNA damage, and oxidative damage. Considering the number of sequence variations within the XPD gene, we were interested in impact of these polymorphisms on chromosomal aberrations (CAs) in the Czech male population. For that purpose we genotyped (PCR-based RFLP approach) 105 healthy males (53 exposed to air pollution in the downtown of Prague, 52 suburban employees) for “A to C” polymorphisms in exons 6 and 23 of the XPD gene. CAs were assessed by conventional method as well as by fluorescence in situ hybridization (FISH) assay. Our analyses showed significantly higher genomic translocation frequency (FG/100) in exposed C/C homozygotes (exon 23) as compared with control C/C individuals (2.36±1.93 vs. 1.22±1.19, P=0.033 by non-parametric Mann-Whitney test). These data suggest C/C exon 23 as a higher-risk XPD genotype for chromosomal aberrations.

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III.P.12. A SINGLE NUCLEOTIDE POLYMORPHISM in the RAD51 GENE in BREAST CANCER

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Breast cancer suppressor proteins BRCA1 and BRCA2 interact with RAD51, which plays a central role in homology-dependent recombinational repair of DNA double strand breaks, being essential for maintaining genomic stability. Therefore, genetic variability in the RAD51 gene may contribute to the appearance and/or progression of breast cancer. Preliminary reports suggest that a single nucleotide polymorphism in the 5’ untranslated region of RAD51 (a G to C substitution at position 135, the G/C polymorphism) may modulate breast cancer risk. We investigated the distribution of genotypes and frequency of alleles of the G/C polymorphism in breast cancer. Tumor tissues were obtained from postmenopausal women with node-negative and node-positive ductal breast carcinoma with uniform tumor size. Blood samples from age matched healthy women served as control. The G/C polymorphism was determined by PCR-based MvaI restriction fragment length polymorphism. The distribution of the genotypes of the G/C polymorphism did not differ significantly (P > 0.05) from those predicted by the Hardy-Weinberg distribution. There were no differences in the genotype distribution and allele frequencies between node-positive and node-negative patients. There were no significant differences between distributions of genotypes in subgroups assigned to histological grades according to Scarf-Bloom-Richardson criteria and the distribution predicted by Hardy-Weinberg equilibrium (P > 0.05). Our study implies that the G/C polymorphism of the RAD51 gene may not be directly involved in the development and/or progression of breast cancer.
III.P.13. A NEW POLYMORPHISM in $DDB1$ and $DDB2$ GENES – IDENTIFICATION and LUNG CANCER CASE-CONTROL PRELIMINARY STUDY

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Damaged DNA-binding protein, DDB, is a heterodimer of p127 (DDB1) and p48 (DDB2) subunits with a high specificity for binding to several types of DNA lesions and is a part of repair machinery in global genome nucleotide excision repair (GG-NER). Mutations in the p48 gene ($DDB2$), causing a loss of DDB activity, were found in patients with xeroderma pigmentosum group E (XP-E), characterized by a high predisposition to skin cancer. Recently, several polymorphisms in other NER genes (e.g. $XPD$, $XPA$, $XPF$, etc.) have been identified in human population and some of them were associated with increased risk of cancer. We searched for new polymorphisms in coding regions of $DDB1$ and $DDB2$ genes. We subsequently investigated the frequency of the found polymorphisms in 96 non-small cell lung cancer (NSCLC) patients and 96 healthy controls – all inhabitants of a highly industrialized and polluted region of Upper Silesia, Poland. New polymorphic variants were searched in 35 randomly selected individuals by RT-PCR and cDNA sequencing and afterwards PCR-RFLP analysis was used to genotype cases and controls. Three polymorphisms have been detected – (i) two silent substitutions in coding region of the $DDB1$ gene: 262C→T (Pro51→Pro), 1228G→A (Gly373→Gly), and (ii) 1536C→T substitution in 3’ non-coding region of the $DDB2$. The $DDB1$-373 and $DDB2$ polymorphisms were very rare in our group – 1228A allele frequency was 0.014 and 1536T allele frequency was 0.07. No variant homozygotes were found. Since the $DDB1$-51 polymorphism was found to be common (262T allele frequency = 0.13), we screened the NSCLC case-control group in order to assess a possible relationship between the polymorphism and cancer risk. There were no differences between cases and controls in frequency of the $DDB1$-51 genotypes when analysed according to diagnosis, cigarette smoking and occupational exposure. There was a statistically significant preponderance of CC homozygotes among younger cases, below 53 years of age, when compared with younger controls (96% versus 74%, $P = 0.04$). Our preliminary results possibly suggest the association between the $DDB1$ polymorphism and susceptibility to cancer disease but this needs to be verified by additional studies.
Gene polymorphism of DNA repair genes may be associated with modulation of cancer risk. Our investigation focused on MGMT gene encoding an alkyltransferase involved in direct DNA repair. The aim of our study was to determine the frequency of four polymorphic alleles of MGMT gene: 84Leu>Phe, associated substitutions 143Ile>Val-178Lys>Arg, enhancer polymorphism 1099C>T (Acc. X61657) and 79G>T (Acc. U95038) in 96 non-small cell lung cancer (NSCLC) cases and 96 cancer-free individuals from Upper Silesia. We also performed genotyping of three frequent MGMT alleles (84Phe, 143Val-178Arg, 1099C>T) in 164 NSCLC cases to examine the association of MGMT alleles with basic clinical and epidemiological characteristics of the patients. We studied “differential expression” of MGMT alleles and its association with polymorphisms in regulatory region of MGMT. Material for the expression study was RNA and DNA from white blood cells of 45 healthy inhabitants of Upper Silesia. We found that no MGMT allele showed statistically significant frequency difference between cancer cases and controls. The enhancer polymorphism was less frequent in never smokers (4%) than in smokers (18%), in females (5%) than in males (17%) and in never smoking cancer patients (6%) compared with never smoking controls (24%). We detected strong association between 84Phe allele and the clinical stage of cancer. MGMT alleles in heterozygotes revealed “differential expression” in white blood cells (14% of informative samples) and this phenomenon was associated with the enhancer polymorphism of MGMT. To further investigate the functional significance of the enhancer polymorphism, we cloned 275 bp wild-type and polymorphic promoter-enhancer element into pGL-3 vector in a way that the element controlled the expression of firefly luciferase gene. After transfection into three different cell lines, we found that the polymorphic variant was invariably associated with significantly increased activity of the luciferase enzyme. These observations are consistent with the hypothesis that the enhancer polymorphism is of functional significance since it is associated with increased expression of the MGMT gene and may decrease lung cancer risk at low carcinogen exposure.
III.P.15. DISTRIBUTION of GENETIC VARIANTS in DNA REPAIR GENES and DNA REPAIR CAPACITY in NON-SMALL CELL LUNG CANCER PATIENTS

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Reduced cellular DNA repair capacity (DRC) is associated with elevated lung cancer risk which, in part, may be modulated by polymorphisms in DNA repair genes. In an ongoing lung cancer case-control study, we are investigating the influence of DNA repair gene variants on cellular DRC (Int. J. Cancer 95:86, 2002). The individual DRC was determined in peripheral blood lymphocytes using the alkaline single-cell microgel electrophoresis (comet) assay and bleomycin as a DNA-damaging agent. In addition, four DNA polymorphisms were analysed for which a correlation with increased lung cancer risk has been reported: XRCC1 (Arg399Gln), XRCC3 (Thr241Met), and XPD (Lys751Gln and Asp312Asn). These variants are localised in three genes of different DNA repair pathways. PCR-based restriction fragment length polymorphism assays and melting point analysis of allele specific hybridisation probes (LightCycler) were applied. 155 lung cancer cases and 129 tumour-free control subjects were analysed by the comet assay. Impaired DRC was significantly associated with increased lung cancer risk (OR=2.1fold, p<0.001). The allele distribution between cases and controls was very similar for the XRCC1 and XRCC3 genotypes. The two polymorphisms determined on the XPD gene were in strong linkage disequilibrium. The variants Gln/Gln and Asn/Asn were detected with a higher frequency in cases; this difference did not reach significance, possibly due to the limited number of individuals screened so far. Furthermore, 53 individuals were homozygous for variant bases at 2 or 3 of the 4 sites investigated. These combinations were found to be more frequent among cancer cases than controls (12 vs 21%; p = 0.068) and these cases also showed a reduced DRC. These interim results indicate that lung cancer risk is increased with impaired DRC, which is not influenced by a single genetic variation alone. Our data rather suggest that a combination of sequence variations in DNA repair genes may affect both DRC and lung cancer risk. Analysis of additional DNA repair gene variants in a larger number of individuals is warranted.
Lung cancer is the most common carcinoma among European men. Among females, lung cancer incidence is still increasing in most countries, mainly because of smoking among women. Increasing incidences have also been confirmed in Poland. Apart from cigarette smoking, most other risk factors for lung cancer have been identified in occupational settings. The aim of this study was to assess the level of DNA damage in leucocytes of patients with lung cancer exposed to carcinogenic agents during the occupational work. DNA damage was assessed by the comet assay. The efficiency of leukocytes to repair damaged DNA was parallely assessed. The DNA damage was quantified by visual classification of cells into five categories according to the tail lengths of “comets”. The extent of DNA damage was expressed as the mean percentage of cells with damaged DNA. The cohort with lung cancer consisted of 208 patients from Lodz region. Their mean age was 58.8 ± 9.0 years. Most of them were men and smokers. The lung cancer in patients was assessed histologically and classified into the main types, such as: small cell carcinomas (SCC), adenocarcinoma (AC), non small cell carcinomas (NSCC), squamous cell carcinoma (SqCC) and others. Accordingly, patients were divided into the respective groups. The control groups included 180 persons from the same region. A statistically significant increase in percentage of cells with migrating fragments of DNA strands in patients with lung cancer compared to the control persons was found. The percentages of leucocytes with damaged DNA in each group of patients with diagnosed type of lung cancer were significantly higher (and amounted respectively to 20.8 ± 13.4, 15.3 ± 8.9, 16.7 ± 12.1, 23.8 ± 14.6, 21.0 ± 13.9) than in control group (12.7 ± 7.1%). Incubation of cells in the culture medium to assess the repair of damaged DNA increased the extent of DNA damage only in patients with lung cancer. Additionally, the high percentage of cells with damaged DNA was found among the painters, welders, metalworkers and operators of machine tools without lung cancer. Summing these results we can suggest that it is necessary to determine more precisely the exposure to establish the relationship between the frequency of lung cancer and the occupational exposure to carcinogenic agents.
III.PO.17. The EXPRESSION of a DNA REPAIR PROTEIN – XPA in NON–SMALL LUNG CANCERS CELL

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Lung cancer is the most common cause of cancer death in industrialized countries. The process of cancer development is connected with damage in DNA repair pathways and accumulation of somatic mutations in genes coding for proteins involved in regulation of cell proliferation, migration and apoptosis. Deficiencies in DNA repair correlate with an increased sensitivity to the cytotoxic effect of particular DNA damaging agents, as well as the tendency for chromosomal abnormalities and mutations in specific oncogenes and tumor suppressor genes. Studying the processes preventing carcinogenesis, e.g. DNA repair pathways, may lead to better ways of cancer prevention. There are several systems that repair damaged DNA in human cells. Nucleotide excision repair (NER) is considered to be a major DNA repair mechanism, involved in the removal of a wide spectrum of bulky DNA adducts modifying DNA double helix conformation. The XPA protein is a component of NER and plays a central role in DNA lesion recognition. Regulation of XPA gene expression has not been extensively studied. We analyzed the expression of XPA protein in non-small cell lung cancer (NSCLC) tissue using an immunohistochemical method. We detected high expression of XPA protein in cancer cells. This was surprising. We expected low XPA expression in cancer cells because null mutation in XPA gene causes cancer-prone human disorder xeroderma pigmentosum. We also compared the expression of XPA gene in primary human diploid fibroblasts (GMO7532) and lung cancer cell line A549. Using western-blot and immunochemical methods we found overexpression of XPA in the A549 line and low expression in GMO7532 cells. Additionally, we found that expression of XPA is reduced in human diploid fibroblasts after contact inhibition of their growth. It is possible that overexpression of XPA in lung cancer contributes somehow to the cancerous phenotype of cells. A study of XPA expression in an extensive set of lung cancer cell lines is currently under way.
III.P.18. DNA ADDUCTS and REPAIR CAPACITY in LUNG CANCER

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Oxidative stress and lipid peroxidation (LPO) generate promutagenic DNA lesions such as 1,N⁶-ethenoadenine (εA) and 3,N⁴-ethenocytosine (εC) that are likely to contribute to lung cancer development and progression.

We measured the levels of εA and εC by immunoaffinity/³²P postlabeling in the DNA of tumour and normal lung tissue of lung cancer patients (33 cases) as well as in leukocytes of the same group. Activities of DNA-glycosylases repairing εA and εC were also measured in the same tissues (57 cases) and in leukocytes of healthy volunteers (26 individuals).

High individual differences (up to 10-fold) were observed both in adduct level and glycosylase activities. No difference in εA and εC level between tumour and non-affected lung tissue was recorded. However, leukocytes accumulated statistically significant higher number of DNA adducts than lung tissues. Repair activities for both εA and εC were significantly higher in tumour than in normal lung tissue. There was an inverse correlation between the level of εC and the activity of εC-glycosylase in normal and tumour lung tissue; however for εA such a correlation was found only in tumours. This suggests that εC-DNA-glycosylase is the “first choice” enzyme for removing this lesion from DNA in humans.

There was no difference in εA and εC-glycosylases activities between men and women as well as smokers and ex-smokers.

εC-glycosylase activity was decreasing gradually with age in normal lung (3.22-fold difference between groups of 40-50-years old and over 70-years old patients).

We observed differences between two histological types of lung cancer: squamous cell carcinoma (SQ) and adenocarcinoma (AD). In patients with SQ the ratio of εA/εC was higher in non-affected lung tissue than in AD patients. In AD individuals εA and εC DNA-glycosylase activities were significantly lower than in SQ; however, normal lung of AD patients revealed higher deficiency in εA-glycosylase (2.72-fold decrease) than in εC-glycosylase (1.8-fold) in comparison with SQ type of tumour. This may suggest that people developing inflammation-related adenocarcinoma might have defective “first choice” defense mechanisms against LPO-induced DNA damage.

Plasma of healthy volunteers contained higher level of vitamin E and A in comparison with that of cancer patients.
III.P.19. ASSESSMENT of DNA DAMAGE in LEUCOCYTES of PHARMACY PERSONNEL OCCUPATIONALLY EXPOSED to CYTOSTATIC DRUGS

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Data from the medical and laboratory examinations, performed among the medical personnel in the biggest hospital in our city, were not sufficient for evaluation of health hazard of exposure to cytostatic drugs. The aim of this study was to assess the DNA damage in leukocytes of hospital personnel regularly exposed to cytostatic agents. The study was performed in 6 units of the hospital, where workplaces contain cabinets with vertical laminar flow and personnel wear adequate personal protective equipment. The comet assay was used to evaluate DNA damage in the leukocytes. The DNA damage was quantified by visual classification of cells into five types of comets and expressed as the mean percentage. DNA repair was assessed parallely. Before DNA repair the percentage of cells with comet in the exposed subjects (14.8±6.5%) was significantly higher than in controls (11.8±4.0%). Among nonsmokers the difference in DNA damage between exposed and control groups was more clear than among smokers. After repair the DNA damage was lower both in the medical personnel and controls. The highest percentages of cells with comets were found for the subpopulations in the chemotherapy and diagnostic units (18.1±5.1% and 16.8±4.2%, respectively). This study may suggest that occupational exposure of medical personnel to cytostatic drugs in this hospital is at the low level.
III.P.20. BULKY DNA ADDUCTS in HUMAN SPERM; RELATION to SMOKING, ENVIRONMENTAL FACTORS and MEASURES of SEMEN QUALITY

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The integrity of DNA of spermatogenic cells can be affected by endo- and exogenous genotoxic factors. Resulting DNA damage in spermatozoa may significantly contribute to impaired fertility. Here, the 32P-postlabeling method was used to analyze the levels of bulky DNA adducts in sperm cells in a group of 179 males, either healthy donors or patients with an impaired fertility. Only a modest difference in the levels of bulky DNA adducts (1.2-fold increase, p=0.054) was found between smokers and nonsmokers when all donors were analyzed. However, statistically significant difference has been found between smokers and nonsmokers in the groups of healthy individuals (1.5-fold increase, p=0.026). No correlation between alcohol or coffee drinking and sperm DNA adducts has been found. The levels of DNA adducts in sperm seemed to be unaffected by environmental and occupational factors. On the other hand, a significant negative correlation between DNA adducts and sperm concentration or sperm motility has been observed (p<0.001; rS=−0.249 and −0.3048, respectively). The data suggest that DNA adducts in sperm cells can be applied as a potential biomarker in studies of human infertility.

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III.P.21. REPAIR of ALKYLATION DAMAGE by DIFFERENTIATING MOUSE TERATOCARCINOMA CELLS

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O⁶-alkylguanine is the major mutagenic and carcinogenic lesion in DNA induced by monofunctional alkylating agents that are widely distributed in the environment. The consequences of alkylation are cell- and tissue specific and depend on different repair capabilities in the targets. O⁶-alkylguanine-DNA alkyltransferase (AGT; EC 2.1.1.63) is a ubiquitous protein that repairs O⁶-alkylguanine via a unique mechanism when it accepts alkyl group on a Cys residue in a single step stoichometric reaction, thereby itself being inactivated. Consistent with former studies we revealed gradually decreasing AGT activity in developing mouse brain. To analyze the early commitment and differentiation events we used mouse teratocarcinoma cell lines, which were induced to differentiate into neuronal direction as a model for early embryonal development. In the present study we examined the AGT activity in mouse teratocarcinoma cell lines (P19, PCC7), and transfected derivatives of P19, harbouring a human AGT cDNA driven by a housekeeping promoter in order to increase the very low endogenous AGT activity of parental cells. In teratocarcinoma cell lines and in the transfectants as well, the AGT activity decreased significantly (by 20-40%) during differentiation. The transfectants were used to examine the possible reasons for reduction of AGT activity in detail and we detected an alteration in substrate specificity. Using in vivo labeling of phosphorylated proteins in undifferentiated and differentiated transfectants we quantified the phospho-AGT. Our findings that decreasing of AGT activity is accompanied by increasing phosphorylation of AGT during differentiation is in concordance with Srivenogupal’s observation, that phosphorylation functions to down-regulate or inactivate AGT activity (Cancer Research 60, 282-287, 2000).
III.P.22. MICROSATellite INSTABILITY and DNA REPAIR in COLON CANCER


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The main causes of colorectal carcinoma (CRC) include: (i) inflammatory processes and high fat diet resulting in the increase of DNA damage, among others – lipid peroxidation (LPO) derived etheno-DNA adducts formation; (ii) deficiency in mismatch repair (MMR) resulting in increased genomic instability.

We have compared microsatellite instability (in 5 microsatellites: Bat26, Bat40, D2, D5 and D17), expression of MMR proteins (MLH1, MSH2, MSH6) as well as the activity of DNA glycosylases specific for 1,N\(^2\)-ethenoadenine (εA) and 3,N\(^4\)-ethenocytosine (εC) in tumour and normal colon epithelium obtained during surgical intervention on 50 sporadic CRC patients in Norway.

Out of 50 analyzed CRC cases, in 6 patients disturbances of genomic stability and/or MMR deficiency was observed. Four of them revealed MSI in tumour tissue: two in all five microsatellites, one in BAT26, BAT40 and D5, and one in BAT26, BAT40 and D2. Disturbances in MMR proteins expression were noted in tumour tissue of only 3 out of 50 patients, and they involved exclusively deficiency of MLH1 protein expression as measured by antibodies staining. Interestingly, only in one case (patient no 4) microsatellite instability coincided with the dysfunction of MLH1 protein. In one case with MSI, MMR deficiency has not been measured yet. In the other four cases there was no correlation between microsatellite instability and expression of MMR proteins, suggesting that other factors than MMR deficiency can also contribute to genomic instability in colon cancer.

DNA glycosylases were measured only in 2 out of 6 patients, who revealed either MSI or MMR disturbances. For one of them (patients No 4), neither εA- nor εC-glycosylase activity was detectable in normal and tumour colon epithelium. This patient had also defective MMR and thus high level of DNA damage could be expected. For the second one, both εA- and εC-glycosylases activities had significantly higher values in normal colon and in tumour than in other patients, and moreover the εA-glycosylase activity increased tremendously in the tumour to the value 41.8 εA pmoles/h/mg protein, which was at least 3-fold higher than in any other tissue measured in this study. Further investigations are now in progress to evaluate the role of etheno-DNA adducts and DNA-glycosylases activities for the genomic stability in colon cancer.

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III.P.23. MINISATELLITE INSTABILITY is LINKED to the P53 GENOTYPE in MICE

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Minisatellite instability involves distinct mutation processes in somatic and germline cells. In germ cells, tandem repeat instability most likely arises at meiosis as a by-product of high-frequency meiotic recombination events occurring in and near minisatellites. Minisatellites not only show high-frequency spontaneous mutation in the germline, but also appear to be very sensitive to mutation induction by ionising radiation, both in experimentally irradiated mice and in human populations exposed following the Chernobyl accident: the mechanisms of mutation induction by radiation remain enigmatic.

The p53 gene has recently been shown to play an important role in the development of malformations in mouse embryos irradiated at the zygote, gastrula stage and during organogenesis (Baatout et al, In Vivo, in press). P53 heterozygotes mice were mated and embryos were X-irradiated with 0.5 Gy 1, 8 or 11 day after fertilisation. Both in control and irradiated pregnancies, developmental abnormalities (mainly exencephaly) were recorded, affecting mainly the homozygous null embryos and to a lesser extend the p53 heterozygotes and with a sex bias towards females. Over all foetuses, irradiation at day 8 had about doubled the malformation rate from 12.9 to 23.4% with the presence of different kinds of external abnormalities (exencephaly, gastrochisis, cleft palate, polydactyly). We analysed genetic instability by minisatellite study in embryos irradiated at different times during development (1, 8 and 11 days) and harvested at day 19 of pregnancy in order to see whether the development of external abnormalities, the genotype or the sex was linked to genetic instability. Germline mutation rates was analysed at one minisatellite locus (probe M) because previous work has shown that germline mutation at that particular locus was remarkably sensitive to ionising radiation in mice (Paquette and Little, Cancer Res, 1992).

Preliminary results showed that amongst control and irradiated groups, two populations exhibited around 50% of genetic instability among p53 +/+ females and p53 −/− males. The other groups showed less (17% amongst the p53 +/− males) or even no genetic instability so far (in p53 +/- females and males and p53 −/− females). In 75% of the cases, the mutation was of maternal origin. In 18% of the cases, a genetic instability was linked to the presence of one particular type of malformation: polydactyly. These preliminary results that need to be extended to 350 foetuses are not going in the same direction as the results published by Ohashi et al (Jpn J Cancer Res, 1986), who showed that p53 gene deficiency did not enhance instability of mouse minisatellites in somatic cells of normal tissues (using probes Pc1 and Pc2).
III.P.24. MUTAGEN SENSITIVITY of LYMPHOCYTES of WOMEN CARRYING a BRCA1 MUTATION

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Various inherited cancer-prone conditions show an elevated sensitivity to the induction of chromosome damage in cells exposed to ionizing radiation, indicative of defects in the processing of DNA damage. We have previously shown that lymphocytes of women with a mutation in the breast cancer susceptibility genes BRCA1 or BRCA2 reveal clearly enhanced micronucleus frequencies after gamma-irradiation (1, 2). To further characterize the mutagen sensitivity and to better understand the underlying mechanisms, we now tested the effect of various mutagens on the micronucleus frequencies in lymphocytes of women with various BRCA1 mutations in comparison to controls. Our results indicate increased sensitivity towards bleomycin, cisplatin, cyclophosphamide and BCNU. However, mutagen sensitivity towards cisplatin and BCNU did not lead to enhanced induction of sister chromatid exchanges (SCE), suggesting that recombination is not affected. In contrast to the findings for various DNA-damaging agents, there was no clear difference in the response to vincristine and taxol. FISH analysis revealed that the two aneugens mainly induced centromer-positive micronuclei and the relative amount was similar in lymphocytes with and without BRCA1 mutation. We conclude that mutagen sensitivity of cells with a BRCA1 mutation can result from different kinds of DNA damage, in accordance with the proposed central role of BRCA1 in maintaining genomic stability. Although BRCA1 has been shown to interact with antimicrotubule agents, this interaction obviously does not result in enhanced induction of micronuclei. Since some of the DNA-damaging mutagens tested here are used in breast cancer chemotherapy, it might be that women with a BRCA1 mutation are at higher risk for the induction of mutations and secondary cancers by standard therapies.

III.P.25. CAN LYMPHOBLASTOID CELL LINES be USED as a MODEL for BRCA1–INDUCED MUTAGEN SENSITIVITY?

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The breast cancer susceptibility gene BRCA1 is involved in maintaining genomic stability but the precise nature of its contribution is not yet clearly determined. Evidence suggests that BRCA1 may play a role in a multitude of different processes including DNA repair, recombination, cell cycle control and transcription. We have previously shown that lymphocytes of women with a BRCA1 mutation show elevated mutagen sensitivity to the chromosome-damaging effects of gamma irradiation or hydrogen peroxide as revealed by the micronucleus test (Rothfuss et al., 2000; Trenz et al., 2002). We now wanted to see whether similar results could be obtained with lymphoblastoid cell lines (LCL) and whether such permanent cells are suitable as a model for the investigation of mechanisms involved in mutagen sensitivity. Six LCL (three with and three without a BRCA1 mutation) were comparatively tested. Our results show that there is no systematic difference in radiation sensitivity between LCL with and without a BRCA1 mutation. Spontaneous and gamma radiation-induced micronucleus frequencies were in same range. Furthermore, cytotoxic effects (reduced cell proliferation, reduced viability) induced by gamma radiation were not different. The only difference found was an induction of micronuclei by 10µM hydrogen peroxide in BRCA1 cell lines while a concentration of 20µM hydrogen peroxide was necessary to induce micronuclei in control cells. comet assay experiments did not reveal differences with regard to the induction and removal of primary DNA damage. Furthermore, expression of BRCA1 mRNA after gamma irradiation showed considerable variability and there was no clear difference between cell lines with and without BRCA mutation. These results indicate that LCL with a BRCA1 mutation do not generally show the same mutagen sensitivity as lymphocytes with the same BRCA1 mutation. Therefore, the use of LCL to study the mechanisms underlying mutagen sensitivity due to a heterozygous BRCA1 mutation seems to be limited.

III.P.26. MUTAGENICITY of MAMMOGRAPHY X-RAYS: IMPLICATIONS for BRCA1/2 FAMILIAL PREDISPOSED WOMEN

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The aim of our study was to measure the mutagenic potential of soft diagnostic mammography x-rays (29 kVp) relative to conventional X-rays (200 kVp) in human cells. We studied the induction of HPRT-mutants in SV40-transformed human lung fibroblast. The mutagenic potential of mammography X-rays at low doses (< 0.5 Gy) was 4.2-fold higher compared with conventional X-rays. Familial predisposed women heterozygous for an inherited mutation in a tumour suppressor gene (BRCA1 and BRCA2) are at a higher risk for loss of tumour suppressor gene function than normal women because only one instead of two intact alleles needs to be inactivated by a radiation-induced mutation. Assuming that the mutant yield measured for the human HPRT gene also holds for the human BRCA1 and BRCA2 genes the risk of induction of BRCA mutants can be roughly estimated. Based on the induction of 4.1 mutants per 10^5 survivors per Gy measured for the human HPRT gene (42.83 kb) at doses < 0.5 Gy, an average absorbed dose of 4 mGy per mammography and an average breast of 500g with about 10^11 epithelial target cells, it can be calculated that more than 16 000 heterozygous BRCA (+/−) epithelial breast cells will become homozygous (−/−) by a single mammogram. This number increases to 32 000 BRCA (−/−) deficient cells when taking into account that BRCA genes are on the average twice as large as the HPRT gene. In contrast, for non-predisposed women an average of 2.7 BRCA (−/−) deficient breast epithelial cells per mammography exposure were calculated. However, these numbers may vary considerably depending on the presence or absence of essential genes adjacent to BRCA1/2 genes. It is not known if and how mammography-induced BRCA (−/−) deficient breast epithelial cells promote breast cancer risk. These findings should, however, stimulate a re-evaluation of the risk assessment of mammography for familial predisposed women.
Hepatocellular carcinoma (HCC) is frequent in areas of high aflatoxin exposure and HBV prevalence, as Western Africa and South-East China. A specific mutation in TP53 (Ser-249) is a hotspot in HCCs from these areas, reflecting DNA damage by aflatoxin. We have analysed Ser-249 in HCCs from the Gambia, West Africa (29 cases, all biopsies) and from Qidong, Eastern China (20 cases, including 12 biopsies and 8 surgical specimens). We have also analysed free DNA isolated from 24 plasma samples from Gambian HCC patients, including 17 matched with biopsies. These specimens were collected at the time of clinical diagnosis. Next, we have investigated Ser-249 in 257 plasma samples from Qidong, collected prospectively during the follow-up of a cohort of HBV chronic carriers. DNA extracted from plasma or tissues was amplified and analysed by restriction digestion with HaeIII, that cuts within codon 249 (1). Mutation was confirmed by sequencing. Ser-249 mutation was found in 35% (10/29) of Gambian HCCs. The prevalence in plasma DNA was 38% (9/24). Concordance between matched tumour and plasma was 71%. In Qidong HCCs, Ser-249 was found in 15/20 cases (65%), and also in 3 of 8 non-tumoral, adjacent tissue available. In plasma samples collected prospectively, only 0.9% (2/257) contained Ser-249. In addition, the prevalence of aflatoxin exposure was low based on serum aflatoxin-albumin adduct levels. In conclusion, the prevalence of Ser-249 shows large variations between areas of high incidence of HCC. The prevalence in The Gambia is lower than reported in neighbouring Senegal (2). Plasma of cancer patients is a good source of material to evaluate the presence of Ser-249. However, a prospective follow-up of chronic carriers from Qidong does not show that plasma Ser-249 is a marker of early tumorigenesis. Further studies are needed to investigate correlations between plasma levels of Ser-249 mutants and levels of aflatoxin exposure.

Barrett’s oesophagus is a pre-malignant metaplastic condition affecting up to 1% of the Western population and predisposing sufferers to oesophageal adenocarcinoma. p53 abnormalities have been implicated in the early stages of Barrett's progression. In this study, biopsies obtained from Barrett’s patients were analysed for p53 allelic deletions using FISH. In addition the same biopsy samples were concurrently analysed for rare p53 mutations using a genotypic method called the Restriction Site Mutation (RSM) assay. Finally, tumours arising in Barrett’s patients were analysed for p53 mutations by sequencing. Loss of the p53 allele was a rare event in Barrett’s progression, being present in approximately 12% of metaplastic biopsies and 15% of dysplastic biopsies. P53 mutations were more widespread, being detectable in approximately 20% of metaplastic biopsies and approximately 30% of dysplastic biopsies. In contrast, sequencing of Barrett’s related tumours revealed a low (10%) level of p53 mutations. However, the method used to identify the precancerous p53 mutations (the RSM assay) is far more sensitive than direct sequencing which was employed to detect the tumour mutations. Hence, it is possible that the tumours contain p53 mutations in a subset of the cells. As only 10% of Barrett's patients progress to cancer, does the presence of a p53 mutation or an allelic deletion of p53 identify high risk cancer patients? We are currently following up these patients to assess the cancer progression of patients with and without p53 abnormalities in an attempt to ascertain if early p53 abnormalities are predictive markers of cancer development in Barrett's oesophagus.
The heterozygous p53 knockout mouse is being used as a short-term alternative model for carcinogenicity screening of chemicals. In most cases, these mice develop tumours within 6 months of exposure to genotoxic carcinogens. Few data are available for these mice on chemical mutagenicity relative to their parental wild type strain. Unexpectedly, a significantly lower level of micronucleated erythrocytes was detected in p53 +/- than in p53 +/- mice at certain time points after chronic inhalation of benzene (Healy et al, Mutagenesis 16, 163, 2001).

We have studied the induction of micronuclei in bone marrow polychromatic erythrocytes (PCEs) of p53 +/- and p53 +/- mice (over a C57Bl genetic background) after acute or subchronic exposure to melphalan (MLP). Heterozygous knockout mice were given a single oral dose of 0, 0.5, 1 or 2 mg/kg MLP and sacrificed 24 h later, or 4 weekly administrations of the same dose levels and sacrificed 24 h after the last treatment. Wild type mice were treated either acutely or subchronically with 2 mg/kg MLP or solvent and similarly sacrificed 24 h after the end of treatment. After acute treatment the frequency of micronucleated PCEs was significantly higher in p53 +/- than in p53 +/- mice; in addition, only wild type animals showed a toxic effect by a reduction of the PCE frequency over total erythrocytes. After subchronic treatment, the opposite situation was observed, with p53 +/- mice showing a statistically higher frequency of micronuclei than p53 +/- mice. The frequency in subchronically treated wild type mice was also significantly higher than the frequency measured in the same mice after acute treatment. These data could be explained by a higher acute cytotoxicity induced by melphalan in wild type animals which triggered an error-prone increase in cell proliferation rate.

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III.P.30. The EXPRESSION of HEAT SHOCK PROTEINS in HUMAN DIPLOID LUNG FIBROBLASTS INCUBATED with ACN, PAHs and EOM in vitro

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Heat shock proteins (hsp) fall into the group of stress proteins, i.e. proteins, the expression of which is induced by stress. They are supposed to be an early sensitive biomarkers of environmental pollution. These proteins are divided into several groups according to their sequence homology and molecular weight. They are specifically induced by proteotoxic factors.

We studied the influence of environmentally relevant chemicals, polycyclic aromatic hydrocarbons (PAHs) and acrylonitrile (ACN), and extractable organic matter (EOM) adsorbed on air particles on the expression of hsp32 and hsp70 proteins in human diploid lung fibroblasts (HEL) in vitro. The main role of hsp32 is to regulate the biotransformation processes depending on cytochrome P450. hsp32 is important in the protection of cells against oxidative damage. Protein hsp70 takes part in folding of newly synthetized proteins, as well as in folding of proteins damaged by stress factors.

The incubation of HEL cells with different concentrations of ACN resulted in dose-dependent expression of hsp32. The time-dependent expression of hsp32 after incubation with ACN was also observed. The expression of hsp70 was induced in cytotoxic range of ACN concentrations, but not in a dose-dependent manner. The incubation of cells with ACN for at least 1 hour induced the expression of hsp70, but not in time-dependent manner. The effect of dibenzo[a,l]pyrene (DB[a,l]P), the most carcinogenic PAH ever tested, on the expression of both heat shock proteins was assessed. We observed neither time-, nor dose-dependent effect of DB[a,l]P on the expression of hsp32 and hsp70 protein. We also analyzed the effect of EOM on the expression of both proteins. The increased expression of either protein was not found in either time- or dose-dependent manner.

We conclude, that hsp32 and hsp70 are not the suitable markers of exposure to DB[a,l]P, as well as EOM in vitro. The effect of ACN on the expression of either protein was observed, however, only under the cytotoxic conditions.

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Aristolochic acid nephropathy (AAN) is a unique type of rapidly progressive interstitial fibrosis, associated with urothelial cancer and related to the prolonged intake of Chinese herbal remedies (*Aristolochia* species) containing nephrotoxic and carcinogenic aristolochic acid (AA). On both clinical and morphological grounds, AAN is very similar to another fibrosing nephropathy, the Balkan endemic nephropathy (BEN), including the association with urothelial tumours. It has been suggested that the mycotoxin ochratoxin A is associated with BEN, but also AA was considered as a possible causal factor in BEN. In this pilot study we assessed AA exposure in endemic areas for BEN. Using the $^{32}$P-postlabelling method we analysed renal tissue from three female farmers who lived in endemic areas and in two of whom an upper urinary tract cancer had developed, for AA-DNA adducts. In two of these patients, one of whom had a urothelial tumour, the major adenosine adduct of aristolochic acid I, 7-(deoxyadenosin-$N^6$)-aristolactam I (dA-AAI), the most abundant AA-DNA adduct found in AAN patients, was observed and identified by cochromatographic analyses on TLC and HPLC. Adduct levels ranged from 5.6 to 17.1 adducts per $10^9$ nucleotides. Our data clearly demonstrate that people living in endemic areas for BEN have been exposed to AA. Thus, AA should be considered as a potential risk factor in the development of urothelial cancer in endemic areas. The epidemiology of AAN and AAN-associated urothelial malignancies might provide a clue to urothelial malignancies in endemic areas. Whether AA plays a role also in BEN remains to be further assessed in patients with unequivocal diagnosis of BEN and in patients with other nephropathies but living in endemic areas for BEN.
During a 16-year-period (from 1986 till 2001) we analyzed the frequency of chromosomal aberrations of 1414 healthy Hungarians. The purpose of the study was to determine how the genetic instability measured by spontaneous rate of chromosomal mutations under the same methodological circumstances changes with regard to the time, age, sex and smoking status of the individuals.

People from three different residential areas of Hungary were examined: the capital city Budapest, an industrial area and a greenbelt. Persons exposed previously to known mutagens were excluded from the study. The average age of the entire subpopulation was 36.2±10.9 years (17-77).

Chromosomal aberrations were evaluated in 100 cells of each person. Aneuploidy, chromatid breaks, chromosomal fragments, dicentric and ring chromosomes were counted. Regression analysis and Chi-square tests were used to compare the data within and between the groups.

During the 16-year period we found that the number of aberrant cells of persons increased between 35-300% in all the three types of residential areas. The growth rates of aberrations were more significant in Budapest and in the greenbelt area than in the industrial region.

There was no difference between sexes similarly to our earlier findings between 1978 and 1983. Smokers revealed higher aberrant cell frequency than non smokers in all the three places of residence (1.85% vs. 1.46% of aberrant cells). Formerly these differences had not been found. When the average number of aberrant cells of inhabitants was compared regarding the type of residence, there was no significant difference among them; however, chromosome-type aberrations characterized the Budapest- and greenbelt area individuals rather than those from the industrial towns. The increasing tendency of the chromosomal aberrations coincides well with the morbidity and mortality rates, particularly of environmental-related cancers in Hungary.
Pesticides are extensively used all over the world. Large amounts of these chemicals are released into the environment and many of them affect non-target organisms, being a potential hazard to human health. Individuals occupationally exposed to pesticides (as field workers, mixers, loaders, applicators, etc.) who are in direct contact with these chemicals may offer us a good opportunity to study their adverse health consequences. The results obtained in the framework of an EU-research project, aimed at investigating the relationship between occupational exposure to pesticides and the induction of cytogenetic damage are presented. Populations from Greece, Spain, Poland and Hungary, all of them characterised by intensive agriculture activity, were the subject of the study. A total of 239 agricultural workers and 231 unexposed controls were examined for cytogenetic effects in lymphocytes of peripheral blood and exfoliated cells of the buccal mucous. The frequency of micronuclei (MN) was evaluated in both cell types, and their relationship with different confounding factors (e.g. sex, country, smoking habit, etc.) was determined. The results obtained indicate no increases in the MN frequencies in the agricultural workers when compared to the controls for either lymphocytes or buccal cells. However, exposed individuals showed a significant decrease in the cytokinesis-block proliferation index (CBPI) when compared to the controls. When the effect of the different confounding factors was evaluated, age was positively related with MN in lymphocytes, and the Polish population showed a MN frequency significantly higher than those observed in the other populations. For the buccal cells, the Spanish population showed the highest MN frequency, attaining significant differences with regard to the other populations. Finally, the CBPI was found to be inversely influenced by age, and Hungarian exposed men were the group that showed lower values.
Single cell gel electrophoresis (SCGE) has been widely used to detect DNA damage of cells exposed in vitro and in vivo to various physical or chemical agents. In molecular epidemiology studies DNA damage evaluated by the comet assay is considered as a non-specific biomarker of exposure effects [1]. However, attention has been paid to the experimental variability of this assay [2]. When large numbers of samples need to be analyzed, an internal standard is indispensable.

We have applied this method for studies of an influence of occupational exposure to polycyclic aromatic hydrocarbons (PAH) on the DNA damage detected in lymphocytes of exposed people, and cellular susceptibility to the induction of the oxidative damage. For the latter purpose the SCGE assay was applied to evaluate the DNA damage induced by 2 Gy of the challenging dose of X-rays (as an oxygen radicals and oxidative damage inducing agent), and again after a certain time of incubation during which a "completed" repair of the induced damage should significantly diminish the damage detected in the cells. For the purpose of proper adjustment of the residual (unrepaired) damage, studies of the DNA repair kinetics were performed. The half-life time of the repair process for a young male donor estimated from the kinetics was ~5 min, so, incubation of irradiated lymphocytes for the period longer than one hour did not decrease anymore the amount of residual damage.

Lymphocytes of the unexposed and exposed donors were divided into three parts for the estimate of the damage: a/ induced in vivo, b/ by challenging dose of X-rays, and c/ a residual damage after repair during the incubation. To control a stability of the assay experimental conditions, a group of cells from the same sampling probe of Mr. Standard's was divided into two parts for an analysis after challenging X-rays exposure and after repair of X-rays induced damage. To evaluate the DNA damage three parameters have been chosen (TL – length of the comet tail, tDNA – fraction of the DNA in the comet tail, TM – comet tail moment equal to percentage of DNA in the tail multiplied by the tail length). Experiments were run for the group of 100 donors from a reference group and persons exposed to PAHs (town policemen) from Czech Republic.

Comparison of preliminary results showed a high reproducibility between an independent electrophoresis for the Mr. Standard samples, and no significant difference between exposed and unexposed subgroups for various measures of the DNA damage induced in vivo. Preliminary results also suggest a difference between unexposed and exposed donors' responses to radiation and the efficiency of the X-rays induced damage repair.

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The aberration of functional activity of genotype and state of endogenic intoxication of an organism were investigated in 158 patients with bronchial asthma. The dependence of indexes chromatisation, nucleolar and pathologically altered nuclei of somatic cells and parameters of blood serum average molecules on the degree of disease severity was established.

The object of the cytogenetic research were epitheliocytes of mucous membrane of the oral walls and peripheral blood lymphocytes. Somatic cells kariogram indices changes were revealed in patients with bronchial asthma testifying to aberration of genome functional status. Decrease of chromatin transcription activity by 18-23%, increase of pathological nuclei number by 1.5-2.1 times, presence of heteropicnotic X-chromosome in males in 14.8-18.3% of cells have been the determined variations of indexes depending on the severity of disease.

Patients with bronchial asthma of average severity showed the total parameters of peptide remains: 0.246±0.008 and nucleotide ones: 0.294±0.019, whereas those with severe course showed 0.315±0.012 and 0.359±0.014, respectively.

Complex treatment of bronchial asthma decreased the endogenic intoxication of the organism and increased the function of the transcriptional and transmitting complex of somatic cells.
In the cell, oxidative or nitrosative stress can be reflected not only on the DNA level but also on the protein level. Redox modifications of cysteine thiols in regulatory proteins are known consequences of ROS/RNS activity. Such modifications are usually interpreted as the formation of intra- or intermolecular disulfide bonds and their impact on the structure and function of the modified proteins. Recently, it has been suggested that the mechanism of cysteine modification is more complicated than simply disulfide bond formation. Cysteine thiols may be, among other modifications, hydroxylated, nitrosylated or glutathionylated in vivo depending on the redox status of the cell.

S100 is a multigenic family of Ca\(^{2+}\)-modulated proteins of the EF-hand type, expressed exclusively in vertebrates. S100 proteins are closely associated with several human diseases including cardiomyopathy, neuro-degenerative disorders and cancer. Within cells S100 proteins have been implicated in the regulation of a variety of processes such as protein phosphorylation, enzyme activation, dynamics of cytoskeleton components, activity of transcription factors, calcium homeostasis and cell proliferation and differentiation. Certain S100 family members, i.e. S100A1 and S100B, are released into the extracellular space by a yet unknown mechanism. Extracellular S100 proteins stimulate neuronal survival and/or differentiation, astrocyte proliferation, cause neuronal death via apoptosis and stimulate or inhibit the activity of inflammatory cells. Extracellular activities of S100A1 and S100B strictly depend on the redox status of the cell. Intermolecular disulphide dimer was proposed to be the bioactive form for these proteins. This hypothesis is incompatible with available structural data. We propose that other oxidative modifications of cysteines might be responsible for regulation of their extracellular activity. We show that thiols in human S100 calcium-binding proteins S100A1 and S100B readily react with low molecular weight oxidants of physiological relevance. The different cysteine modifications impose significantly different effects on protein stability and conformation as seen by CD, NMR and fluorescence.

The interaction of Cys modified recombinant S100 proteins with their intra- and extracellular target proteins will be presented.
III.P.37. VALIDATION of the NEUTRAL COMET ASSAY

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Comet assay under neutral conditions allows detection of DNA double-strand breaks, considered to be the biologically relevant radiation-induced lesion. We describe a modification of the neutral comet assay, which simplifies and facilitates its use for estimation of DNA double strand breaks in X-irradiated mammalian cells. The results point to a satisfactory sensitivity of the modified comet assay and its specificity for DSB. The minimum detection level of the modified comet assay is about 5 Gy.

The specificity of the assay for DSB was confirmed by measuring the repair rate of X-ray-induced lesions in DSB repair-competent CHO-K1 cells and in the DSB repair-defective xrs-6 mutant. For CHO-K1, the repair of DNA damage was rapid during the first hour. In contrast, DSB repair-deficient xrs-6 cells did not repair the DNA damage so efficiently. The residual (24 h) DNA damage was indistinguishable from the background in CHO-K1 cells, but was 23% of the initial damage in xrs-6 cells.

The specificity of the method was further confirmed by measuring the DNA lesions that should be “invisible” under neutral, but not under alkaline conditions. Such criteria are fulfilled by DNA damage induced by H₂O₂ or during repair of UV-C-induced DNA damage. The differences between mean tail moment values of the control and treated cells were highly statistically significant when measured by the alkaline comet assay (Student’s test p<0.0001) but not significant (UV-C irradiated cells) or only slightly elevated (H₂O₂ treated cells) when measured by the neutral assay.

Under the neutral pH conditions used in this study, the DNA should be double stranded. The presence of single stranded DNA (ssDNA) in comets formed under neutral conditions was estimated by means of an anti-ssDNA antibody. In the alkaline version of the assay, the DAPI-stained comet DNA perfectly overlapped the antibody (FITC) -stained DNA. On the contrary, no comets were recorded with FITC staining when photographs of neutral comets were taken, although the DAPI-stained DNA was readily visible. The results clearly indicate that under conditions used in this study no ssDNA was present in the comet tail.
We examined the usefulness of bleomycin sensitivity assay, whether it serves as a biomarker of individual sensitivity and risk for head and neck cancer under our environmental conditions. The test postulates that the mutagen-sensitive phenotypes have a genetic basis, and an elevated number of in vitro induced chromatid breaks per cell (b/c) indicates a cancer susceptibility phenotype. The assay has up to now been used for distinction between cancer patients and healthy individuals. We wished to clarify if bleomycin assay is specific for head and neck cancer phenotypes as compared to alcoholics whose lifestyle and exposure-situations are similar to our head and neck cancer study-population.

Conventional chromosome analysis and bleomycin assay were done in 255 head and neck cancer patients, 156 healthy non-smokers and non-drinkers, 169 smokers and non-drinkers, and in 52 cancer-free alcoholic and smoking patients with liver diseases. The aberrant cell frequency in cancer patients, alcoholics and healthy smokers was completely identical (2.8%), and it differed significantly (P<0.04) from the non-smoking controls (2.25%) only. Chromosomal aberrations were clearly associated with the tobacco-smoking exposure, but not with the health status. Mutagen sensitivity measured by the bleomycin test showed weakly, but significantly elevated values not only in cancer patients (1.10 b/c) but also in alcoholics (1.29 b/c) as compared to the controls (0.98 b/c). We also found that considerable proportion of Hungarian controls (44%) showed twice as high mutagen sensitivity than that reported on US and Western-European study populations (10-22%).

We concluded that this biomarker does not characterise susceptibility to head and neck cancer in the Hungarian population due to insignificant differences between cancer patients and alcoholics, and the high proportion of sensitive persons among controls, respectively. The method indicated a slight cancer susceptibility in cases when aberrant cell frequency was higher than 2%, and b/c was greater than 1. Bleomycin assay, therefore, can be used for measuring cancer susceptibility only under well-defined circumstances.
III.PO.39. FORMATION of OXIDATIVE DNA DAMAGE in MAMMALIAN CELLS DEPENDS on LABILE IRON POOL LEVEL

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Labile iron pool (LIP) constitutes a crossroad of metabolic pathways of iron-containing compounds and is a midway between cellular need of iron, its uptake and storage. In the course of this study we investigated the oxidative DNA damage in two pairs of mouse cell lines differing in LIP level. The pair of mouse lymphoma L5178Y (LY) sublines (LY-R and LY-S) naturally differ in the level of LIP (0.57 and 0.18 µM Fe, respectively) due to the differences in iron storage and uptake regulation (Lipiński et al., Blood 95, 2960-66, 2000). The second experimental model consists of a pair of the mouse erythroleukaemia cell lines (MEL), the intact MEL cells (MEL(−)) and MEL cells overexpressing the heavy subunit of ferritin (H-Fr) (MEL(+)). Due to the overexpression of H-Fr the LIP level decreased from 1.2 µM Fe in intact MEL(−) cells to 0.58 µM Fe in MEL(+) cells (Picard et al., JBC, 273, 15382-86, 1998).

Using modified comet assay we compared the total DNA breakage in the studied cell lines treated with hydrogen peroxide. More DNA damage was found in LY-R and MEL(−) cell lines as compared to LY-S and MEL(+) cell lines. We compared also the yields of DNA lesions sensitive to specific DNA repair enzymes in both cell lines treated with H2O2. The yield of ENDOIII sensitive sites (oxidized pyrimidines) and FPG sensitive sites (oxidized purines, mainly FAPY and 8-oxo-dG) was found to be higher in LY-R cells than in LY-S cells. However, no spectacular differences were found in the abundance of ENDOIII or FPG sensitive sites in DNA of MEL(−)/MEL(+) cells.

Our data confirm the previous findings obtained with GS/MS (Zastawny et al., FRBM, 24, 1250-55, 1999) that sensitivity of LY-R cells to H2O2 is partially caused by the higher yields of the oxidative DNA damage, as compared to those in LY-S cells. The critical factor in this case appears to be the availability and abundance of transition metal ions that take part in the OH• radical-generating Fenton reaction (very likely in the form of LIP). The crucial role of the LIP iron was further confirmed by the use of MEL(−)/MEL(+) cells. Depletion of the LIP level in MEL(+) cells caused a decrease in the DNA breakage induced by H2O2.
A CYTOGENETIC ANALYSIS of PRIMARY and “TELOMERIZED” PROGERIA FIBROBLASTS

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The Hutchinson-Gilford Progeria Syndrome (Progeria) presents clinical features that resemble premature aging in childhood. The condition is rare with a reported incidence of 1 in 8 million. Because of a distinct paternal age effect and the low frequency of parental consanguinity, sporadic autosomal dominant inheritance with most cases resulting from new mutations appears to be the most likely mode of inheritance. The underlying genetic defect is unknown and currently, there is no cure.

In this study we report the analysis of three clonal fibroblast cell lines derived from progeria patients. These clones were immortalised by infection with a retroviral vector carrying the catalytic subunit of telomerase. However, other clones transduced with telomerase frequently failed to immortalise despite the stabalisation of telomere length and the presence of active telomerase holoenzyme. On the basis of this result and the likely mode of inheritance of progeria we hypothesise that the progeria mutation confers resistance to “telomerization”. This model predicts that the immortal clones we have isolated have arisen from founder clones that have lost the dominant allele as a result of somatic mutation.

To determine if such mutations could be identified karyotypic analysis of these three clones and their corresponding primary cell cultures was performed using CGH, dual colour FISH, M-FISH and Telo-FISH. Rearrangements of chromosomes 11 (deletions, amplification of 11q and translocations t(11,22)) were seen in the cell lines and in a small percentage of the primary cells. These findings are consistent with our model and suggest that molecular cytogenetic techniques may prove valuable in the identification of candidate progeria loci.
III.PO.41. A NEW ADULT-ONSET SYNDROME of DEMENTIA, SEVERE NEUROLOGICAL ABNORMALITIES and PREMATURE AGING ASSOCIATED with IMPAIRED DNA REPAIR CAPACITY DUE to a MUTATION in the XP-F GENE

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Two siblings, a brother and a sister, were diagnosed with an adult onset dementia, premature aging, and severe neurological degeneration. Up to the age of early forties, both were normal, with no apparent symptoms except for a mild sensitivity to sunlight. Within a few years from the initiation of the clinical symptoms, they developed some facial features resembling Cockayne syndrome as well as some skin photosensitivity resembling patients with Xeroderma pigmentosum (XP), but without any skin malignancies. Their mental and physical capacity deteriorated rapidly. Fibroblast cell cultures established from these patients exhibited reduced colony forming ability after UV-irradiation. Molecular studies of these cells revealed the presence of defective DNA repair capacity, using transcription-coupled DNA repair assay (TCR) and host cell reactivation assay (HCR). Transfection experiments in which the various normal XP genes were introduced into the patients’ cells assigned them to the XP-F complementation group. The 11 exons of the XP-F gene were amplified by PCR using proper primers and the mutation was identified at the beginning of exon 6. An insertion of C at nucleotide 14,094 resulted in a frameshift mutation. Most XP patients belonging to the XP-F complementation group express mild sun sensitivity and low rate of skin tumors. None of the XP-F mutations described so far resulted in clinical symptoms seen in these affected siblings. Our results indicate either the presence of a unique late onset combined CS/XP syndrome, the first to be reported for the XP-F complementation group, or a new late onset syndrome with some similarities to XP and CS. The role of the mutated XP-F gene in the late onset clinical manifestations is not yet understood.
IV.P.1. APOPTOSIS PROTECTS KERATINOCYTES AGAINST UVB-INDUCED DNA DAMAGE

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Epidermal keratinocytes are the primary cellular target for radiation-induced skin carcinogenesis. It is well known that keratinocytes are more resistant to the lethal effects of UV light than fibroblasts. However, the mechanism behind this phenomenon is still unclear.

In this study we investigated the biological response to UVB radiation in primary human keratinocytes and fibroblasts isolated from the same skin biopsy. Our results show that UVB exposure results in a different response in keratinocytes as compared to fibroblasts. In particular, keratinocytes i) are more resistant to the lethal effects as assessed by clonal cell survival (D37=1000 vs 500 J/m²); ii) are susceptible to apoptosis at doses (1000 J/m²) where fibroblasts are completely refractory; iii) present a lower level of DNA photoproducts; iv) repair CPDs more efficiently; v) present an attenuated G1/S arrest; vi) display a faster recovery of RNA and DNA synthesis; vii) show a lower level of P53 expression with a rapid decrease at 12 hrs post-irradiation while in fibroblasts P53 continued to accumulate up to 24 hrs.

The analysis of P53 induction in primary fibroblasts derived from XPC and CSA patients revealed that the defect in GGR (XPC) did not affect p53 response while the expression of P53 persisted in cells defective only in TCR (CSA). These findings support the hypothesis that the signal for p53 induction originates from actively transcribed genes.

Our results are consistent with a model in which apoptosis removes heavily damaged keratinocytes. The lack of this mechanism is responsible for the deleterious effects of UVB in fibroblasts. The existence of a “cellular proofreading” might prevent the replication of aberrant keratinocytes reducing the frequency of mutated cells and consequently the risk of skin cancer development.
IV.PO.2. MODULATION of CELL CYCLE CONTROL and GENE EXPRESSION by ARSENIC(III), CADMIUM(II), COBALT(II) and NICKEL(II)

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Different compounds of various metals, even though only weakly mutagenic, are known to be carcinogenic to humans as well as to experimental animals. Several pathways of indirect genotoxicity are known. Thus, As(III), Cd(II), Co(II) and Ni(II) inhibit different DNA repair pathways at non-cytotoxic concentrations; potential molecular targets are zinc finger proteins like PARP or XPA. Within the present study, we investigated whether these metals also interfere with cell cycle progression and control, the latter determined after UVC-radiation. When human lung cancer cells (A549) were exposed to non-cytotoxic concentrations of the above metals, Co(II) revealed a slight and Ni(II) a pronounced G1-arrest, As(III) arrested the cells mainly in G2/M-phase whereas Cd(II) did not provoke any arrest. UVC-radiation alone induced a S-phase arrest. However, this effect was impaired at increasing concentrations of As(III), Co(II) or Ni(II) and a normal cell cycle profile was observed. With increasing concentrations of Cd(II) a slight reduction in UVC-induced cell cycle arrest was seen. To elucidate which proteins are affected in altered cell cycle control, the expression patterns of different genes were studied. Our results indicate that As(III) induces p21 expression whereas GADD45 mRNA levels were increased upon Cd treatment. Incubation with Ni(II) enhanced the expression of both genes. These findings suggest that toxic metal compounds can modulate the regulation of expression of certain genes which could account for the observed interference with cell cycle control. The carcinogenic effects of these metals, especially in combination with other DNA damaging agents, may therefore not only be due to inhibition of DNA repair mechanisms but also to altered cell cycle progression.
IV.P3. EVALUATION of the EFFECTS of ANATOXIN-A in HUMAN LYMPHOCYTES in vitro USING the ALKALINE SINGLE CELL GEL ELECTROPHORESIS (COMET ASSAY)

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Cyanobacteria (blue-green algae) are well-known for their ability to produce highly toxic secondary metabolites. Anatoxin-a is a bicyclic aliphatic secondary amine with the structure 2-acetyl-9-azabicyclo [4:2:1] non-2-ene, a structural analogue of cocaine [Fawell J.K., 1999]. It is produced mainly by different species and strains of Anabaena, Aphanizomenon and Oscillatoria [Carmichael, 1992]. Anatoxin-a is a potent nicotinic agonist which can produce neuromuscular blockade and death by respiratory arrest [Pelander, 1996]. There is no information on chronic toxicity, mutagenicity or carcinogenicity of this alkaloid. The comet assay is a sensitive and rapid method for DNA strand break detection in individual cells. Three comet parameters were analysed: tail length (measured from the center of the comet head), percentage of DNA in the tail and tail moment (tail length x percentage of DNA in the tail). Lymphocytes were exposed for 1 h at 37°C to anatoxin-a at concentration: 1-2-5-10 µg/ml.
IV.PO.4. FURTHER INVESTIGATIONS into the MOLECULAR MECHANISM of BETA CAROTENE-ATTRIBUTED CO-CARCINOGENSE

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Several intervention studies assessing the anticarcinogenic properties of β-carotene in humans, unexpectedly found a higher risk for lung cancer in β-carotene supplemented smokers than in non-supplemented controls. Various hypotheses for the underlying mechanisms by which β-carotene may promote, rather than inhibit chemical carcinogenesis, have been suggested:

1. β-carotene may activate and/or induce phase I carcinogen-bioactivating enzymes, including activators of such cigarette smoke carcinogens as polycyclic aromatic hydrocarbons, and this may furthermore be associated with the generation of oxidative stress.

2. At high serum levels of β-carotene as obtained during these clinical trials applying β-carotene as a single agent, prooxidant characteristics of β-carotene may become manifest, yielding reactive oxygen species (ROS) and inducing oxidative DNA damage.

3. Linking the previous hypotheses, it has been suggested that cigarette smoke carcinogens such as benzo(a)pyrene (B[a]P) and/or B[a]P metabolites may directly react with β-carotene; furthermore oxidized β-carotene may have a role in the bioactivation of B[a]P analogous to the peroxide-shunt pathway of cytochrome P-450 supported by cumene hydroperoxide (CuOOH).

The aim of this study was to assess the effect of β-carotene, at concentrations as achieved during the clinical trials, on the formation of B[a]P-DNA adducts and 8-oxo-dG in vitro in isolated DNA, applying as metabolizing systems phenobarbital/5,6-benzoflavone-induced liver and lung microsomes from rats, and lung microsomes from human smokers. B[a]P-DNA adducts were analyzed by means of the 32P-postlabeling method and the formation of 8-oxo-dG by means of HPLC-ECD. In order to obtain more information on the generation of ROS and carbon-centered radicals during incubations of β-carotene with and without B[a]P, we applied electron spin resonance (ESR) spectroscopy with rat liver and lung, as well as human lung activating systems using both a NADPH-supported and a CuOOH-supported peroxidative system. We could provide evidence of the occurrence of a carbon-centered β-carotene radical which was found to be able to interact with B[a]P. Moreover, this carbon-centered β-carotene radical probably interacts with DNA through intercalation; the relevance with respect to the postulated co-carcinogenic effect of β-carotene remains to be proven.
IV.P.5. CHROMOSOMAL ABERRATIONS INDUCED by ETHOXYQUIN (EQ) in HUMAN LYMPHOCYTES

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Ethoxyquin (1,2-dihydro-2,2,4-trimethyl-6-ethoxyquinoline) is an antioxidant which is used as a food preservative, an antidegradation agent for rubber, a pesticide and plant growth regulator. It has been shown that EQ has an anticlastogenic effect in the micronucleus and chromosome aberration tests in vivo. It is also known that EQ inhibits tumor formation in a number of tissues, but predominantly in the liver. However, there are also reports that EQ may exert a carcinogenic effect in kidney.

The present study was undertaken to determine whether EQ has clastogenic properties in the chromosome aberration test in vitro. Chromosome aberrations were analyzed in human lymphocytes after their incubation with EQ (0.5 mM – 0.01 mM) in the presence and absence of liver supernatant (S9) from rats treated with Aroclor 1254.

It has been shown that EQ can induce dicentric chromosomes and translocations in human lymphocytes.
The clinical consequences upon nucleotide excision repair (NER) defects in humans are autosomal recessive diseases such as Xeroderma pigmentosum (XP). This syndrome is the most sun-sensitive disorder, and XP patients present high frequency of skin cancer. The majority of XP patients carry mutations in XPA or XPC genes, coding for proteins that act during initial steps of the NER process, playing a role in the recognition of DNA damage. Culture cells from XPA and XPC patients are hypersensitive to ultraviolet (UV) as a result of malfunctioning DNA repair. So far, there is no effective long-term treatment for these patients. Skin cancer prevention can only be achieved by strict avoidance of sunlight exposure or by the use of sun-screening agents. We have constructed recombinant adenoviruses carrying the XPA or XPC genes, which were used to infect XP-A and XP-C immortalized and primary fibroblast cell lines. Cells were infected with these viruses, which are unable to replicate and transiently express the proteins for the transgenes they carry. The data demonstrate that the complete population of cells is infected with the viruses, express the XPA or XPC proteins and there is full correction of DNA repair defect: the cells recover normal levels of UV resistance and DNA repair synthesis (Unscheduled DNA Synthesis- UDS) is completely restored. Thus, infection with XPA or XPC- adenovirus results in complete phenotypic reversion in XPA or XPC deficient cells, respectively, with no trace of cytotoxicity. Moreover, employing confluent cells, we were able to demonstrate that the transgene expression is maintained in the cells for at least 60 days after infection. The efficient adenovirus gene delivery can be an important tool for a better understanding of XP deficiency and the causes of DNA damage induced skin cancer.

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IV.PO.7. The MUTAGENIC POTENTIAL of $^{131}$I-LIPIODOL THERAPY and COMBINED $^{131}$I-LIPIODOL-CISPLATINUM THERAPY in HCC PATIENTS

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Primary hepatocellular carcinoma (HCC) is one the most common malignant tumors in the world. Of the approaches available for treating irresectable HCC, radionuclide therapy with $^{131}$I-lipiodol has shown promising results. The therapy can be combined with chemotherapy.

The aim of the study was to quantify the mutagenic potential of the therapy in patients treated with $^{131}$I-lipiodol or with combined $^{131}$I-lipiodol-cisplatinum. For this study dicentric chromosomes were scored in metaphase spreads of peripheral blood lymphocytes obtained with the classical technique and with the premature chromosome condensation technique (PCC) using calyculin A. The latter technique was necessary in view of the low proliferation of lymphocytes of these patients after treatment. The in vitro dose response curve for dicentrics obtained with both techniques was compared.

The population consists of a group of 12 HCC patients treated with $^{131}$I-lipiodol. Patients received a mean activity of 1.94 GBq of the radiofarmacon administered into the liver artery by catheterization. Half of the patients were also treated intravenously with cisplatinum. For each patient a blood sample was taken before therapy and one and two weeks after therapy. A partial analysis of the data show that the mean number of dicentrics for the patients, treated exclusively with $^{131}$I-lipiodol, was respectively 0.017, 0.078 and 0.104 per cell before therapy, one week after and two weeks after therapy. The patient dose for this subpopulation, after background correction, was found to be 0.92 Gy one week after therapy and 1.12 Gy two weeks after therapy. For the patients who received both radio- and chemotherapy the mean number of dicentrics scored in the lymphocytes resulted in a number of 0 per cell before therapy and 0.280 per cell two weeks after therapy. The latter corresponds with an absorbed dose of 2.34 Gy.

This indicates that the mutagenic potential of combined $^{131}$I-lipiodol-cisplatinum therapy is two to three times higher than in the case of $^{131}$I-lipiodol therapy alone.
IV.PO.8. HYPERTHERMIA DIFFERENTIALLY MODULATE the REPAIR of DOXORUBICIN-DAMAGED DNA in NORMAL and CANCER CELLS

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Hyperthermia can modulate the action of many anticancer drugs and DNA repair processes are temperature-dependent, but the character of this dependence in cancer and normal cell is largely unknown. This subject seems to be worth studying, because hyperthermia can assist cancer therapy. An 1-hr incubation at 37°C of normal human peripheral blood lymphocytes and human myelogenous leukemia cell line K562 with 0.5 or 2.0 µM doxorubicin gave significant level of DNA damage as assessed by the alkaline comet assay. The cells were then incubated in doxorubicin-free repair medium at 37 or 41°C. Lymphocytes incubated at 37°C needed about 60 min to remove completely damage to their DNA, whereas at 41°C time-period required for complete repair was shortened to 30 min. There was no such large difference between repair kinetics at 37 and 41°C in cancer cells. Therefore, hyperthermia may significantly affect the kinetics of DNA repair in drug-treated cells, but the magnitude of the effect may be different in normal and cancer cells. These features may be exploited in cancer chemotherapy to increase the effectiveness of the treatment reduce unwanted effects of anticancer drugs in normal cells and fight DNA repair-based drug resistance of cancer cells.

This work was supported by the grant 505/652 from the University of Lodz.
A search for new agents that can sensitize cancer cells to ionizing radiation is of continual interest and mainly due to the use of radiation in cancer therapy. Resveratrol, a powerful antioxidant has been shown to inhibit carcinogenesis in animal models. The purpose of this work was to examine whether resveratrol can sensitize cancer cells to X-irradiation. The human cancer cell lines examined were HeLa (cervix carcinoma), K562 (chronic myeloid leukaemia) and IM-9 (multiple myeloma). The assays that were performed following X-irradiation (doses from 0 to 8 Gy) and/or incubation in the presence of resveratrol (concentrations ranging from 0 to 200 µM), were the following: trypan blue exclusion test to determine cell viability, cell morphology after May-Grünwald Giemsa staining, DNA profile analysis by flow cytometry to assess cell cycle distribution and the presence of the sub-G1 peak. The cell lines showed different radiation sensitivity (IM-9, high radiation sensitivity, K562, intermediate radiation sensitivity and HeLa, low radiation sensitivity) as seen by the X-irradiation dose related inhibition of cell growth and induction of apoptosis. The addition of resveratrol alone to the cell cultures induced apoptosis and inhibited cell growth from 100 µM (IM-9) or 200 µM (EOL-1 and HeLa) concentrations. Concomitant treatment of the cells with either resveratrol and X-irradiation induced a synergical effect only at the highest dose of 200 µM. These results show that resveratrol can act as a potential radiation sensitiser only at high concentrations. Further studies need to address the toxicity of resveratrol on normal cells.
IV.P.10. EVALUATION of GENOTOXICITY in a POPULATION of PORTUGUESE WORKERS EXPOSED to STYRENE

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Styrene remains one of the most important organic chemicals used world-wide in several applications, including the production of synthetic rubbers, resins and plastics. In humans, exposure to styrene takes place by inhalation, and is biotransformed by cytochrome P450 monooxygenases to its main reactive metabolite styrene-7,8-oxide.

The objective of the present work was to investigate the possible genotoxicity induced in a group of 15 workers occupationally exposed to styrene in Portugal, as compared with 15 control individuals. Cytogenetic effects were evaluated by means of sister-chromatid exchanges (SCE) and micronucleus (MN) tests, and the DNA damage by means of comet assay. Levels of exposure were measured by determining the styrene metabolites mandelic and phenylglyoxylic acids in urine.

Data obtained show significant increases in SCE frequencies and in DNA damage (evaluated as comet tail length) in the group of workers exposed to styrene, according to the results presented by Brenner et al., (1991), Vodicka et al. (1995), Somorovská et al. (1999), and Laffon et al. (2002) for different styrene-exposed populations. We are currently evaluating the role of the individual genetic polymorphisms in genes associated with the metabolic fate of styrene (e.g. CYP2E1, mEH, GSTP1, GSTM1 and GSTT1) on the levels of DNA lesion induced by this compound in exposed individuals and extending the number of individuals analysed in both groups to enhance the statistical power of the study, and thus to check if the statistical difference with respect to the control population is maintained.

This work is supported by the Fundação da Ciência e Tecnologia (FCT) Project POCTI/33213/ESP/2000.

Cigarette smoke contains a relevant number of compounds arising from the process of tobacco combustion. Some of these, such as nitrosamines, are mutagens and/or carcinogens. The presence of such compounds is one of the major health risks for smokers. There are several strategies under study aiming at reducing the risk arising from the exposure to mutagens/carcinogens in cigarette smoke by reducing the presence of hazardous chemicals that gather in smoke condensate. One of the methods used to obtain this goal is to treat the “cut tobacco” with supercritical CO$_2$ in order to reduce the presence of several substances that can give rise to mutagen compounds. The technique of tobacco extraction by CO$_2$ supercritical is already effective in reducing nitrogenous compounds, unsaturated aliphatic hydrocarbons and several aromatic molecules. The effect of this “cut tobacco” treatment on the DNA damage induced by smoke condensate has been detected by treating an embryo rat fibroblast cell line (Rat1) with several samples of smoke condensate from “cut tobaccos” treated with CO$_2$ supercritical. The DNA damage induction, such as single and double strand breaks, alkali labile sites and oxidative damage, is measured by alkaline single cell gel electrophoresis (SCGE) assay. Preliminary results show that the treatment of cells with the smoke condensate induced a statistical significant increase of single and double strand breaks on DNA ($p=0.03$ Mann-Whitney U-test), however, the smoke condensate from “cut tobacco” treated with CO$_2$ supercritical induced a highly significant reduction ($p<0.001$ Mann-Whitney U-test) of DNA damage compared to the smoke condensate from untreated “cut tobacco”. 

Prof. Achille Cittadini and colleagues, Università Cattolica del Sacro Cuore, for the contribution to the study on DNA damage induction.
IV.P.12. GENOTOXICITY of a cAMP ANALOGUE, 8-Chloro Cyclic Adenosine 3’5’Monophosphate by MICRONUCLEUS TEST in vivo and in vitro

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Among many cAMP analogues characterized, 8-Cl-cAMP has been reported to have unique growth-inhibitory effects in different tumor cell lines. 8-Cl-cAMP acts via a site-selective activation of cAMP-depedent protein kinases and down regulation of its regulatory subunits, reverts the neoplastic-related predominance of PK-I type back to the ratio more typical to the normal phenotype. Genotoxic potential was assessed by the in vivo cytogenetic test using adult BALB/c mice and by cytohalasin-blocked (CBMN) micronucleus assay in vitro using human peripheral blood lymphocytes.

Micronucleus test in vivo results show a consistently increasing dose-dependent pattern, an increase of the dose regime (10 mg/kg b.w. 90 mg/kg b.w. and 160 mg/kg b.w.) there is an increase in the frequency of micronuclei in polychromatic erythrocytes (4,88 ± 0,35; 8,32 ± 0,57; 11,75 ± 0,37) compared to the negative control (2,04 ± 0,28).

Using three doses (1µM/ml; 5 µM/ml and 15 µM/ml) in the CB-micronucleus test in vitro test, 8-Cl-cAMP has shown threshold effect, i.e. 8-Cl-cAMP has no potential to increase the frequency of micronuclei in bi-nuclear lymphocytes (4,9± 2,13 and 5,4 ±2,9) in doses of 1µM/ml and 5µM/ml compared to the negative control group (5± 3). Statistical difference (p<0,05) has been estimated only for the highest dose of 15 µM/ml. The occurrence of the threshold effect and differential expression in vivo and in vitro of phosphodiesterases that metabolise 8-Cl-cAMP to its toxic metabolite 8-Cl-adenosine shows that the genotoxic potential seen in vivo is the consequence of the toxic metabolite 8-Cl-adenosine. These effects distinguish 8-Cl-cAMP as a non-cytotoxic and non-genotoxic antitumour drug, in contrast to classical antimetabolic cytotoxic, cytostatic and genotoxic drugs.
IV.P.13. TRIGGERING TYPE of PROTECTIVE ACTIVITY of ANTIMUTAGENS of DIHYDROPYRIDINE SERIES

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Effective antimutagens (AM) inhibiting spontaneous and induced mutagenesis in germ and somatic cells of animals were revealed among 1,4-dihydropyridine derivatives. Effects of these compounds were studied in detail in different test-systems that made it possible to establish peculiarities and some mechanisms of their action. It is typical that AMs display long lasting protective effects that were registered not only immediately after AM exposure but also after AM excretion from organism of experimental animals (Drosophila, laboratory mice, pond carp). Data obtained allow supposition that protective action of AMs in mouse somatic cells are likely to be due to activation of glutathion-S-transferase system. Experiments with Drosophila larvae and adults show that AMs do not interact with ethyl methanesulfonate (EMS) and most likely induce protective mechanisms counteracting mutation process at its different stages. Suppression of EMS-mutagenesis and clastogenesis proved to be owing to AM influence on DNA repair. In Drosophila females of different genotypes, AMs increased maternal repair efficiency of primary lesions induced by EMS in male spermatozoids. They were capable to modulate maternal repair systems for 5–14 days following treatment of females with AMs indicating long term and stable expression of appropriate genes. There are data confirming their capacity to induce heat shock gene expression. Thus, the protective action of these AMs and phenomenon of its long term manifestation are due to triggering the host defence mechanisms and prolonged expression of responsible genes. Here we put forward a hypothesis that AMs control gene expression at the transcription level.
IV.P.14. CHANGES of \textit{P53} and \textit{BAX} GENES EXPRESSION in A549 CELLS INDUCED by Pt (II) COMPLEX of 3-AMINOFLAVONE in COMPARISON WITH \textit{cis-DDP}

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\textit{Cis}-diamminodichloroplatinum (II) (\textit{cis}-DDP) is one of the most widely administrated antitumor drug. The use of \textit{cis}-DDP indicates severe limitations because of its toxic side effects. Therefore efforts are concentrated on the development of improved platinum compounds with a broader activity spectrum and lower toxicity. Approach to design more effective complex anticancer drugs is based on the biological activity of flavonoids, because these ligands have own antitumor properties. Complex of Pt (II) 3-aminoflavone is a structurally related to \textit{cis}-DDP, containing a flavone molecule instead of amine with two \textit{cis} bound labile chloride ligands. To minimize toxicity, 3-aminoflavone which possesses the desired NH$_2$ groups has been used as non leaving ligand [Zyner et. all 1999].

The \textit{P53} tumor suppressor gene plays a main role in induction of apoptosis. The \textit{P53} protein is an integral component of the cell cycle check point machinery and is rapidly up-regulated after DNA damage. In response to it \textit{P53} activates other genes eg. \textit{BAX}, which is a member of the \textit{BCL}-2 family of apoptosis regulating genes. There is evidence that \textit{P53} can upregulate an expression of the pro-apoptotic gene \textit{BAX}. We used a reverse transcription-PCR method (RT-PCR) to examine the effect of complex of Pt (II) 3-aminoflavone and \textit{cis}-DDP on the transcriptional level of mRNA for \textit{P53} and \textit{BAX} in A549 cell line (human lung carcinoma cell line). We indicated that the analogue of \textit{cis}-DDP cause greater increase of an expression of the both genes in comparison with \textit{cis}-DDP.

On the base of our results we can suppose that complex of Pt (II) 3-aminoflavone is a stronger inducer of apoptosis than \textit{cis}-DDP. Further studies on this compound could confirm its advances as a potential anticancer drug.

IV.P.15. COMPARATIVE STUDY of the MICRONUCLEUS TEST and the COMET ASSAY after COMBINED EXPOSURE of MICE to LOW DOSES of X-RAYS and ANTICANCER DRUGS

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Combination of the comet and the micronucleus (MN) assays are recently recommended for the study of genotoxicity. MN test detect both acentric fragments (due to DNA breakage) and chromosome loss. The direct DNA-breaking capacity can be estimated by alkaline single cell gel electrophoresis (SCGE).

On the basis of preliminary studies, the effects of combined exposure of male mice to low doses of X-rays and anticancer drugs (cyclophosphamide – CP, mitomycin C – MMC) on the induction of micronuclei and DNA damage (comet) have been investigated in bone marrow of mice. Males were whole body irradiated and/or injected i.p. with CP or MMC. The doses of 0.05 Gy + 3.15 mg/kg bw CP and 0.05 Gy + 0.25 mg/kg bw MMC for MN and 0.25 Gy + 3.15 mg/kg bw CP and 0.25 Gy + 0.25 mg/kg bw MMC for comet assay were used. For comet assay cells were graded by eye into five categories corresponding to amounts of DNA in the tail.

The combination of X-rays and CP enhanced the induction of MN in polychromatic erythrocytes, and slight decreased of DNA damage in lymphocytes of bone marrow by comparison with the effect observed after treatment to each agent given alone. Incidence of micronuclei at 24 h and 72 h were 1.5-2 times higher by comparison with the results after irradiation alone. Combined X-rays and MMC exposure enhanced effects on the induction of micronuclei at 72 h and did not induce any significant changes in DNA migration by comparison with results observed for each agent acting alone.

Although different mechanisms are involved when positive results appear in the micronucleus or the comet assay, presented results support the idea that both micronuclei and comet assay could complement each other in the investigation of genotoxic effects of physical and chemical agents. They could be helpful to assess the lowest effective doses.
IV.P.16. PROTECTIVE EFFECTS of VITAMINS A, C and E AGAINST GENOTOXICITY in MAMMALIAN CELLS

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Antioxidant vitamins are well-known as dietary components which can modify the process of carcinogenesis. Hydrophilic vitamin C is considered as a powerful reducing agent in the vicinity of DNA. Vitamins A and E, which are soluble in organic solvents and lipids, play a role in cellular membranes mostly. Protective effects of these vitamins against the genotoxicity of N-nitroso compounds (NNC), N-nitrosomorpholine (NMOR) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), were studied.

By the comet assay we proved that NMOR and MNNG caused an increase of DNA strand breaks. To compare the origin of DNA strand breaks induced by these NNC, the steps of unwinding and electrophoresis during the comet assay were performed at two pH values, i.e. pH=12.1 and pH>13. A dose dependent rise of breaks at pH>13 and lack of any break formation at pH=12.1 revealed that DNA breaks induced by both, NMOR and MNNG, have origin in alkali-labile sites.

To determine more exactly the nature of DNA damage induced by NMOR and MNNG, in further experiments the cells were pretreated with antioxidant vitamins A, C and E. Each vitamin significantly decreased the level of DNA strand breaks formed after NMOR treatment, however none of them had any protective effect on MNNG-induced genotoxicity. In order to elucidate the observed difference in protective effects of vitamins against NMOR and MNNG as well as to contribute to clear up the mechanism of their genotoxic effects, the kinetics of DNA repair after treatment were studied. Repair kinetics were determined for up to 4 h post-treatment time. A clear reduction of NMOR-induced DNA damage with increasing repair time was followed. In contrast, any decline of the level of DNA strand breaks in MNNG-treated cells was not observed.

The obtained results confirmed that although both, NMOR and MNNG, belong to the group of NNC, the mechanisms of their genotoxic effects are quite different. Vitamins A, C and E were ineffective against DNA methylation caused by MNNG but their antioxidant action was obvious in NMOR treated cells.
Resveratrol (3,4',5-trihydroxystilbene), a polyphenol found at high levels in a wide variety of plant species including mulberries and grapes, has been reported to exhibit a wide range of beneficial biological properties including modulation of multistage carcinogenesis. This compound inhibited 7,12-dimethylbenz[a]anthracene (DMBA)-induced preneoplastic lesion formation in mouse mammary organ culture and reduced the incidence and multiplicity of DMBA/TPA-induced papillomas in the two-stage mouse skin model. Antimutagenic activity of resveratrol was also demonstrated against the foodborne heterocyclic amine, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and 2-aminofluorene in Salmonella bacterial tester strains. These chemical carcinogens require metabolic activation by cytochrome P450 1A and 2E1 respectively in order to exert their genotoxic effect. In the present study we examined the effect of resveratrol on the activities of aryl hydrocarbon hydroxylase (AHH) and p-nitrophenol hydroxylase (PNPH), biomarkers of CYP1A1 and CYP2E1 respectively and the formation of benzo[a]pyrene (B[a]P) and DMBA-DNA adducts in mouse epidermis. Topical application of resveratrol at the doses of 8 or 16 µmol resulted in significant decrease of AHH activity. The effect of resveratrol on PNPH was less pronounced. However in additional in vitro studies with mouse liver microsomes the NADPH-dependent competitive inhibition of p-nitrophenol was observed (K_i = 2.1 ± 0.2 µM, IC_{50} = 21 µM). Application of the same doses of resveratrol one hour prior the treatment with the initiating dose of B[a]P (400 nmol) or DMBA (10 nmol) had no effect on B[a]P-DNA adduct formation as measured by SFS assay. ^32P-postlabelling analysis of DMBA-DNA adducts showed the significant decrease of all adduct levels. These results indicate that the modulation of carcinogen metabolism may be involved in the anti-initiating activity of resveratrol.
IV.P.18. EFFECT of CURCUMA on RADIATION-INDUCED APOPTOSIS in HUMAN CANCER CELLS


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There have been considerable efforts to search for naturally occurring substances for intervention in carcinogenesis. Many components from dietary or medicinal plants have been identified that possess substantial chemopreventive properties. Curcumin, a yellow pigment from Curcuma longa, exhibits anti-inflammatory, antitumor, and antioxidative properties. Although its precise mode of action has not been elucidated so far, studies have shown that chemopreventive action of curcumin might be due to its ability to induce apoptosis (or programmed cell death) in cancer cells. This original study was conducted in order to estimate whether curcuma enhances the radiation sensitivity of cancer cells. For this purpose, curcuma (concentrations ranging from 0 to 200 µM) was applied to human cancer cell cultures (HeLa, EOL-1 and IM-9) with or without X-irradiation (doses comprised between 0 and 8 Gy). Cell proliferation was monitored by trypan blue exclusion. For the estimation of apoptosis, changes in cell morphology and flow cytometry analysis (DNA content and presence of the sub-G1 peak) were performed.

Microscopic examination of the curcuma-treated cells (with concentrations above 100 µM) showed a characteristic morphology of apoptosis. Furthermore, cells treated with curcuma exhibited a sub-G1 peak the magnitude of which was proportional to the concentration of curcuma. X-irradiation alone induced polyploidisation and apoptosis of the three cell lines, proportional to the doses of irradiation with a marked difference in radiation sensitivity between the cell lines (IM-9 < EOL-1 < HEla). However, when radiation and curcuma were applied together, our results showed that in HEla, EOL-1 and IM-9 curcumin showed a radiation sensitising effect only at the dose of 200 µM. This result may open the prospect of synergical therapy provided that the intrinsic toxicity of curcuma on normal cells is also addressed.
IV.PO.19. ANTIGENOTOXICITY of *Lactobacillus fermentum* on PESTICIDE TREATED VEGETABLES with MICRONUCLEI *Allium cepa* TEST

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Several investigations have studied the potential health risks from pesticide residues in food, including toxicological and/or mutagenic activity. Many studies suggest that some lactic-acid bacteria and/or fermented milk products can either inhibit the genotoxic activity of alkylating compounds or reduce the incidence of gastro-intestinal cancer. The potential of dietary lactic-acid bacteria to prevent tumours of the colon is very promising and of great practical value. This study aimed to elucidate the potential antimutagenic property of *Lactobacillus fermentum* strain on the probable mutagenic activity of two pesticides (procymidone and chlorothalonil) and pesticide extracts of 15 fruit samples and 1 vegetable sample using the micronucleus test (MCN/test) in *Allium cepa*. The extraction of the samples was performed using dichloromethane, the extracts were analysed for pesticides by gas chromatography and HPLC. The samples containing pesticides were tested for the mutagenicity and antimutagenicity activities. For this, the sample extracts were divided into two parts: one part was tested for mutagenicity and one part for the antimutagenicity test. For the antimutagenicity test, *Lactobacillus fermentum* DSM 20075 (2x10^8 cells/ml) was incubated with the pesticides and the sample extracts dissolved in 1.5% DMSO, and their dilutions. Incubation was performed at 37°C for 3 hrs. The suspensions were then centrifuged at 4,000 rpm for 20 minutes and the supernatants were used for the MCN/test. Results showed that the pesticide residues were found in 13 samples and they showed a significant increase (P<0.05) of the MCN frequency in *Allium cepa* root tip cells, after 48 hrs of exposure to the extracts. The same result showed the procymidone. Chlorothalonil and 3 samples uncontaminated by pesticides showed no mutagenic activity. Pretreatment with *Lactobacillus fermentum* inhibited the genotoxic effect of the procymidone and pesticide extracts of 10 samples, whereas mutagenic activity was reduced in 3 samples.
IV.P.20. NATURAL ANTIOXIDANTS STIMULATED REDUCTION of DNA DAMAGE in HAMSTER CELLS TREATED with GENOTOXIC AGENTS

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Endogenous oxidative damage to DNA is thought to be an important etiologic factor in the development of chronic diseases such as cancer. Many products of the vegetable kingdom have been suggested to limit oxidative damage to DNA in humans. To this group belong all the natural compounds which were studied: 1. five types of lignins, the most abundant organic polymers on earth present in all plants; 2. rosemary, which manifests different effects important from the point of view of cancer prevention; 3. glucans, the high molecular polymers of glucose, which represent one of the natural skeletal constituents of the cell walls of yeast, fungi and plants. The aim of our study was to examine possible protective effects of these natural antioxidants against oxidative DNA damage induced by different oxidative agents in hamster lung V79 cells. The level of DNA damage (DNA strand breaks and oxidative DNA damage) was measured using the classical and modified single cell gel electrophoresis, the comet assay.

Antioxidant activity of water-soluble sulfur-free lignin obtained by fractionation of hardwood hydrolysate as well as four more lignin preparations obtained by chemical degradation of the original lignin was investigated in cells damaged by visible-light excited methylene blue. Our results showed that only the original lignin did not increase substantially the level of DNA damage and manifested antioxidative activity against DNA base modifications generated by visible light + photosensitizer. Ethanol extract from rosemary reduced after a long-term or short-term preincubation of V79 cells the genotoxic activity of both H2O2- and visible-light-excited methylene blue. The protective effect of glucan derivatives against oxidative DNA damage, studied in hamster V79 cells influenced by H2O2, visible-light-excited methylene blue, and ofloxacin was graded as follows: carboxymethyl-chitin-glucan>sulfoethyl-glucan >carboxymethyl-glucan. Obtained results confirmed the protective effect of lignin, rosemary and glucans against oxidative damage to DNA probably as a consequence of scavenging of both OH radicals and singlet oxygen.
The alkaline microgel electrophoresis assay, also known as Single Cell Gel or comet assay, is a technique whereby DNA single-strand breaks and/or alkali-labile sites are detected in individual cells. In this study we have employed this method to detect protection provided by an infusion of cedron in Mus musculus bone marrow cells.

Animals were separated in five groups: i. untreated (negative control), ii. treated with cisplatin 6 mg/ K.b.w., iii. treated with an infusion of cedron during 20 days at 5%, iv. pretreated with the infusion of cedron and then treated with cisplatin and v. treated with methylmethanesulfonate (positive control). We used four animals per group and cells were collected at 6 hrs after treatments. Each slide was analyzed by using a Zeiss epifluorescence microscope equipped with an excitation filter of 515-569 nm. For each animal, 100 cells were analyzed by an automatic digital analysis system comet assay (Metasystem Ltd., Germany) determining tail moment (tail length X tail %DNA/100)

Our results indicate that the pretreatment with infusion protects the animal against damage induced by cis-platinum. We detected a statistically significant lower level of damage in animals pretreated with the infusion of cedron and then treated with cisplatin. However, more detailed investigations in vivo are necessary to learn which of the infusion’s components is responsible for the effect we detect in this investigation.
IV.P.22. INVESTIGATION of the ANTIMUTAGENIC EFFECT of Cruciferae VEGETABLES on BACTERIAL TEST SYSTEMS and HUMAN CELL LINES

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Glucosinolates are a group of compounds widely distributed among the plants of the Cruciferae family. These compounds are easily hydrolysed by the enzyme myrosinase which becomes activated after mechanical destruction of the plant cells. Isothiocyanates, nitriles, thiocyanates and indole compounds are the most common products of such hydrolysis. In our previous report we have shown that there was no cytotoxic and mutagenic effect of indole-3-carbinol and cauliflower extract on two bacterial test systems, Salmonella typhimurium, strains TA98 and TA100. In this work we have examined potential inhibitory effect of both compounds against two standard mutagens, 2-aminoanthracene and 4-nitroquinoline-1-oxide. It was estimated that simultaneous treatment of bacterial cells with mutagen and indole compound leads to a significant inhibition of prototrophic growth.

Indole compounds didn't inhibit the activity of P-glycoprotein in parental laryngeal carcinoma cells (HEp2) and their cisplatin resistant subline (CK2). Indole compounds caused significant increase of glutathione level and glutathione-S-transferase activity in HEp2 cells. On the contrary, in CK2 cells which have increased basal level of glutathione, both drugs decreased the level of glutathione and glutathione-S-transferase activity. Indole-3-carbinol and cauliflower extract didn't cause any aberrant event in human lymphocytes.

IV.P.23. The BIOMASS of MEDICINAL PLANT CULTURED CELLS is a PROMISING SOURCE of the ANTIMUTAGENIC PREPARATIONS

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In the novel Escherichia coli – bacteriophage lambda system the antimutagenic properties of the aqueous-alcoholic extracts from the biomass of cultured cells of some valuable medicinal plants, Panax ginseng C.A.Mey, Polyscias filicifolia Bailey, Rhodiola rosea L. and Ungernia victoris Vved. ex Artjuschenko, were examined.

In E. coli bacteriophage lambda test-system the bacteriophage is represented as a test-object. The advantage of this system is the possibility to estimate quantitatively the effect of mutagen on extracellular phage, i.e., non-metabolising DNA protected solely by the protein envelope.

The principal applicability of the E. coli bacteriophage lambda system is its ability to estimate the protective effect of plant extracts on lethal and mutagenic activity of mutagens.

The protective activities of the extracts were suggested by their ability to increase the survival of the test-phage exposed to the mutagen (nitrous acid) extracellularly under in vitro conditions.

Phage exposition to mutagen under in vitro conditions makes it possible to discriminate between the protective and antimutagenic activities of the extracts. The protective effects of the investigated extracts greatly exceeded their antimutagenic activities. A high protective effect was exhibited by the extracts of U. victoris, P. ginseng and P. filicifolia. When nitrous acid treated phages were treated with these extracts, 679-, 712- and 10-fold increase in survival was observed, respectively; the extracts of Rh. rosea showed an 8-fold increase in antimutagenic activity whereas the extract of roots of Rh. rosea showed antiviral activity [1].

IV.PO.24. INVOLVEMENT of DNA REPAIR in ANTIMUTAGENESIS of SAGE

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Antimutagenic effect may be the consequence of multiple mechanisms involved in DNA replication and repair. Due to the variety of DNA lesions and the complexity of repair pathways it is difficult to identify the processes involved in antimutagenesis.

Results obtained with microbial tests: *E.coli* K12, *S.typhimurium* and *S.cerevisiae*, show that ethereal oil of sage (*Salvia officinalis* L.) and its fractions possess antimutagenic properties. Inhibition of spontaneous and UV-induced mutagenesis varies in the range of 30-70%, depending on the screening test and fractions content. Fractions containing mainly monoterpenoids (F1-F4) inhibit mutagenesis in both permeable (*rfα* and *lpcA*) and non-permeable strains while fraction with a high content of sesquiterpenoids (F5) inhibits mutagenesis only in permeable strains. Major sage monoterpenoids: camphor, α+β thujone, and 1,8-cineole also show significant antimutagenic effect.

The effect of sage terpenoids on DNA repair mechanisms was further tested with *E.coli* assay system (Simic *et al.*, 1997; 1998). In different genetic backgrounds, genetic endpoints such as spontaneous and induced reversions, recombination, and induction of SOS repair were monitored.

Ethereal oil of sage (EO) and fractions F1-F4 have no effect on spontaneous mutation frequency in both repair proficient and mismatch repair deficient strains, while fraction F5 reduces spontaneous mutagenesis only in permeable mutS strain. Antimutagenic potential of α+β thujone and 1,8-cineole against UV-induced mutations is diminished in excision repair deficient mutant. EO and α+β thujone reduce SOS induction following UV-irradiation while EO and camphor stimulate intrachromosomal recombination in recombination proficient strain.

IV.P.25. ANTIGENOTOXIC EFFECT of SAGE

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Ethereal oil of sage (Salvia officinalis L.) and its fractions show antimutagenic effect in microbial tests: E.coli K12, S.typhimurium and S.cerevisiae. Inhibition of spontaneous and UV-induced mutagenesis varies in the range of 30-70%, depending on the screening test and fractions content. Moreover, major sage monoterpenoids, camphor, α+β thujone and 1,8-cineole, also show significant antimutagenic properties by modulating DNA repair processes (enhancement of error free repair, inhibition of error prone SOS repair).

The antigenotoxic effect of ethereal oil of sage (EO) is further tested in mammalian tests: in vivo citogenetic test in mice and in vitro micronucleus test in human lymphocytes. In both tests EO induces no genotoxic effect. However, dietary exposure of mice suppresses mitomycin-C induced chromosome aberration. On the contrary, there is no effect on γ-ray induced micronuclei.
S.1. CELLS CULTURED at LOW DENSITY HAVE INCREASED FREQUENCIES of MICRONUCLEI

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The in vitro micronucleus assay offers a reliable and more cost effective alternative to the in vitro chromosome aberration assay, having the ability to detect clastogens and aneuploidy inducing chemicals. This assay is currently under discussion and validation with the view to issuing an OECD test guideline.

Our experience with using this assay with various cell lines has shown a direct relationship between pre-experiment seeding density and micronucleus frequency at harvest. We have seen the same pattern of effect when using two different cell lines (V79 and CHO cells). Our experiments have shown that a low seeding density prior to the commencement of treatment is associated with increases in spontaneous micronucleus frequency irrespective of the type of cell line used.

Further investigation as to the nature of these micronuclei (either via chromosomal breakage (clastogenesis) or whole chromosome loss (aneugenicity)) via the application of anti-kinetochore antibody staining has illustrated the mechanism of action to be chromosome breakage. This was verified further by chromosome aberration analysis of cultured V79 cells which showed increased chromosome aberration frequencies were also associated with low density cell seeding.

We conclude that when using cell lines such as V79 or CHO cells for use in in vitro micronucleus studies, pre-treatment seeding should be performed at medium to high density. Confluence at the time of treatment should be approximately 70-80% to ensure low background aberration frequencies.

The relevance of these observations to the interpretation of data from in vitro micronucleus tests is discussed.
VALIDATION STUDIES on COMET ASSAY for HIGH-THROUGHPUT USE


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The Single Cell Gel Electrophoresis or the comet assay is a simple, rapid and sensitive method to determine strand breaks, alkali labile sites, incomplete excision repair sites, and DNA crosslinks at the level of individual cells. Compared with other genotoxicity assays, the advantages of comet assay include: (1) its sensitivity for detecting low levels of DNA damage; (2) the requirement for small numbers of cells per sample; (3) flexibility; (4) low costs; (5) ease of application; (6) the ability to conduct studies using relatively small amounts of a test substance; and (7) the relatively short time period needed to complete an experiment.

In this preliminary work we validated the experimental conditions for performing large-scale in vitro comet assay, ideal for genetic toxicology laboratories.

Freezing of Samples: The effect of freeze-thaw cycle was compared to fresh sample on the comet assay. Slow freezing (with 10% DMSO and EDTA) of the treated cell samples had minimal effect on the DNA migration. However, rapid freezing increased the DNA migration. The duration of freezing did not affect the DNA migration. Therefore, it is recommended that cell samples can be stored for future comet assay use.

Lysis: The optimal DNA migration was seen after over night lysis in most cell lines. Storage of slides in lysis solution upto 2 weeks did not increase the DNA migration. However storage beyond 2 weeks produced variable results. Therefore, it is recommended that we can store slides in lysis solution for upto 2 weeks.

Staining and scoring of comets: We stained the comet slides with various concentrations of ethidium bromide. 1-2 µg/mL of ethidium bromide gave satisfactory response in terms of less image saturation and in defining the end of head and tail accurately.

Comet Parameters: Olive tail moment and Percent DNA in the Tail gave very good correlation with the DNA damage. Tail length also had a good correlation.

The above modifications will make comet assay more flexible in terms of handling large number of samples and stream lining the whole process, and thus help in developing this assay as a high throughput screen.
S.III. OPTIMISATION STUDIES on in vivo COMET ASSAY FOLLOWING INTRATRACHEAL DOSING

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The Single Cell Gel Electrophoresis or the comet assay is a simple, rapid and sensitive method to determine strand breaks, alkali labile sites, incomplete excision repair sites, and DNA crosslinks at the level of individual cells. Since comet assay is very sensitive and requires small number of cells for analysis, it is an ideal tool to investigate DNA damage in in vivo models. In the in vivo comet assay, oral gavage or injections are the usual routes of administration of the test article, and genotoxicity looked for in various target organs. Since there is a scarcity of literature on other routes of administration, we optimised the conditions for intra-tracheal dosing and have performed the comet assay from the isolated pneumocytes. In this method, rats are anaesthetised, trachea intubated and the test article sprayed in to the lungs. After 3 hours, the rats are sacrificed, lungs dissected, washed free of blood with merchant's solution, single cell suspension prepared, and comet assay performed (1 hr lysis; 20 minutes unwinding; 20 minutes electrophoresis at 35 V 300 mA).

Several parameters were identified for intratracheal dosing:

The dosing volume should not exceed 0.1 mL, as animal respiration will be affected.

The compound must be freely soluble to enable a fine spray to be administered into the lungs without clogging.

The solvent used should be a physiological solution to reduce the animals suffering.

Two positive controls N-nitrosodimethylamine (DMN), and methyl methanesulphonate (MMS), and inert solvents were tested by this method. The detailed methodology for intra-tracheal dosing and results obtained will be discussed.