Session 15

Free Radicals in Medicine III (Liver, Eye, Skin)

15.1 FREE RADICAL SCAVENGERS IN TOXIC LIVER LESIONS

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The nonenzymatic antioxidans can be natural and synthetic antioxidants. The authors extensively studied the effect of some natural and synthetic antioxidants in several models in vitro and in vivo. The aim was to prove the hepatoprotective effect of these drugs. The in vitro experiments carried out by pulse radiolysis proved that silibinin (the most important isomer of silymarin) are able to reduce superoxid radicals. In experimental hyperlipidaemic rats both the natural silibinin and other synthetic antioxidants produced a decrease of cholesterol and triglyceride contents of liver microsome treated with fat rich diet. The efficiency of silymarin treatment was investigated in double blind study, on patients with chronic alcoholic liver disease. The treatment lasted for 6 months. The elements of antioxidant protective system, the activity of serum glutation peroxidase (GPX) serum free - SH group, superoxide dismutase (SOD) of erythrocytes and lymphocytes were studied. The liver function tests showed a significant improvement in the therapy groups. Level of malondialdehide (MDA), marker of serum LPO, decreased significantly during the silymarin treatment ($p<0,02$). Following silymarin administration the serum glutation peroxidase (GPX) activity the free SH group level, and the superoxid dismutase (SOD) activities of erythrocytes and lymphocytes significantly increased. During a 6-month administration of placebo considerable changes in SOD expression of lymphocytes were not observed. Side-effects described to silymarin were not observed. Our studies show that in hyperlipidaemic cats and in patients with chronic alcoholic liver disease the extrahepatically detectable oxidative stress state - its typical biochemical parameters-were favourable influenced by silymarin treatment. Applied therapy decreased lipid peroxidation and improved the patients' antioxidant protection.

15.2 PROTECTIVE OR PHOTOSENSITISING ROLE OF VITAMIN E? LIGHT-INDUCED AND DARK REACTIONS OF VITAMIN E RADICAL IN LIVER, EYE AND SKIN

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1. To explain an extremely high efficiency of vitamin E as the major chain-breaking antioxidant a hypothesis was put forward on its possible regeneration by some intracellular reductants. Using direct ESR measurements we demonstrated that vitamin E chromanoxyl radicals can be reduced non-enzymically in membranes and LDL by ascorbate, ascorbate+reduced thiols (dihydrolipoate, GSH), ubiquinol. In the absence of ascorbate thiols are not efficient. In electron-transporting membranes (liver mitochondria and microsomes) we discovered enzymic NADPH-,NADH-, and succinate-supported reduction of chromanoxyl radicals. We found that ubiquinones mediate the enzymic regeneration of vitamin E by electron transport. Based on these data the functioning of the vitamin E cycle was postulated in which vitamin E acts as a harvesting centre collecting reducing equivalents from other water-and lipid-soluble antioxidants for its regeneration.

2. Vitamin E absorbance (lambdamax 295nm) extends well into the solar spectrum. We hypothesise that in UVB-exposed tissues (e.g. in skin and eye lens) chromanoxyl radicals may be generated by solar light. Reduction of these radicals by other antioxidants (e.g. ascorbate thiols) will regenerate vitamin E at the expense of their own depletion. We found that UVB generates vitamin E chromanoxyl radicals in mouse skin and rat lens homogenates. The radicals are reduced by ascorbate or ascorbate+dihydorlipoate. Hence vitamin E in skin and eye may act in two conflicting manners upon solar illumination: in addition to its antioxidant function as a peroxyl radical scavenger, it may act as an endogenous photosensitizer enhancing light-induced oxidative damage. This may explain very low concentration of vitamin E in UVB-exposed tissue (skin, eye, lens).

15.3 CORRELATION BETWEEN LIPOPEROXIDATION ADDUCTS AND NON ENZYMATIC GLYCATION IN COLLAGEN DURING AGING

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The role of oxidative stress during aging is mediated by the formation of protein adducts with lipoperoxidation side products. Furthermore during aging advanced glycation endproducts (AGE) accumulate in slow turnover tissues. Specific fluorescences characterize, the compounds from the two non enzymatic reactions. To evaluate the relevance of such protein modification during aging we investigated collagen-linked fluorescences in subcutaneous tissue of healthy aging Wistar rats (2-28 months). The fluorescence of AGE showed an exponential increase age-dependent, meanwhile the fluorescence derived from MDA and HNE adducts increased less and significant relationships were found. A significant correlation between Maillard-derived and oxidation dependent fluorescence was observed. In support of these results, "in vitro" experiments were performed by incubating collagen with specific precursors (ribose, MDA and HNE). The results showed typical maximum peaks in the expected area. An interconnection between glycation and oxidation reactions was strongly suggested supporting the hypothesis that both pathways, even if with different mechanisms, are significantly activated in aging and may play a leading role in tissue damage.

15.4 VITAMIN E AND RETINAL FUNCTION

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Ophthalmic studies in patients with severe vitamin E deficiency show characteristic visual changes including night blindness, loss of visual acuity and pigmentary abnormalities. Changes in visual electrophysiology indicating retinal dysfunction have also been described in these patients. In order to gain an understanding of the mechanism whereby vitamin E maintains normal retinal function we have carried out longitudinal (50 weeks) physiological and biochemical studies in vitamin E deficient and control rats.

Visual function was assessed by measuring flash electroretinograms (ERG), oscillatory potentials (OP) and cortical visual evoked potentials (VEP). The ERGs and OPs were significantly ($p<0.001$) reduced in the deficient rats after 20 weeks and undetectable at 50 weeks. By 30 weeks changes in the VEP secondary to retinal dysfunction were also recorded. These electrophysiological abnormalities are similar to those reported in patients with severe vitamin E deficiency. After 30 weeks of deficiency the retinal changes could not be reversed by vitamin E repletion.

These functional changes are being correlated with biochemical studies of the retina and associated neural pathways. These include the measurement of [1] vitamin E and A concentrations, [2] markers of free radical activity (dichlorofluorescein) and damage (malondialdehyde), [3] retinal fatty acid profiles and [4] retinal membrane fluidity.

15.5 **An In Vitro Model of Lipofuscinogenesis in Human Retinal Pigment Epithelium Cells.** MR Liles[1], MV Miceli[1,2], PD Oliver[1,3], and DA Newsome[1,2]. [1]Sensory and Electrophysiology Research Unit, Touro Infirmary and Department of [2]Ophthalmology and [3]Anatomy, Tulane Univ., New Orleans, LA, USA

Damage mediated by reactive oxygen intermediates in the retinal pigment epithelium (RPE) may contribute to the development of macular degeneration, which is accompanied by prominent lipofuscin formation. The RPE phagocytozes shed outer segments of the retina, which are rich in long chain fatty acids. During ß-oxidation of long chain fatty acids, H_2O_2 is generated in the peroxisome and is decomposed by catalase. Human RPE catalase activity decreases with age and macular degeneration (Liles et al, Arch Ophthamol 109, 1991). To develop an acatalasemic cell culture model, we treated cultured RPE with 3-amino triazole (3AT, 1mM) for 2 weeks, which inhibited catalase activity by > 95%. This treatment did not alter the activity of other antioxidant enzymes or affect phagocytosis of rod outer segments (ROS). Compared to controls, RPE cells treated with 3AT and fed ROS for 2 weeks had 64% more lipid peroxidation (TBARS) and 11% more lipofuscin-like autofluorescence. To demonstrate the presence of intracellular oxidation, we used a H_2O_2 sensitive probe covalently bound to ROS. 4 hours post ingestion of labeled ROS, we noted oxidative activity comparable to a murine macrophage cell line (RAW 264.7). In contrast to the macrophage, extracellular superoxide production by RPE was not detectable. In the RPE, catalase is essential for protection against intracellular production of hydrogen peroxide. The mechanism by which peroxisomally generated H_2O_2 contributes to RPE lipofuscinogenesis and macular degeneration is under investigation.

15.6 LASER SCANNING CONFOCAL MICROSCOPY (LSCM) AND DIGITIZED VIDEO MICROSCOPY (DVM) OF CULTURED HEPATOCYTES AFTER OXIDATIVE STRESS WITH *t*-BuOOH: PROTECTION BY 1,10,-PHENANTHROLINE.

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Here, employing LSCM and DVM we defined subcellular events leading to lethal injury in single cultured hepatocytes after exposure to 100-300 μM *t*-BuOOH, a lipid hydroperoxide analog which produces oxidative stress. 1-day cultured rat hepatocytes were labelled with rhodamine-dextran for lysosomes, rhodamine 123 for mitochondrial membrane potential, Fura-2 for Ca^{2+}, and propidium iodide for loss of cell viability. Lipid peroxidation was assessed by malondialdehyde (MDA) formation. After about 20 minutes of exposure to *t*-BuOOH, individual lysosomes broke down followed by mitochondrial depolarization. After 30 minutes cells lost their viability. At about the same time, cytosolic free Ca^{2+} rose sharply. In the presence of 10 μM phenanthroline, onset of cell death was delayed several fold. Lysosomal breakdown was also greatly delayed but mitochondrial depolarization was only slightly changed. Again, Ca^{2+} rose sharply at the onset of cell killing. Phenanthroline also decreased MDA formation induced by *t*-BuOOH. In conclusion, phenanthroline provided striking protection against lethal injury to hepatocytes after oxidative stress. Protection may be mediated by stabilization of lysosomal membranes and suppression of lipid peroxidation.

15.7 PROTECTION BY VITAMIN E AND SILIBININ PRE-AND POST TREATMENTS OF HEPATIC CELLULAR DAMAGE IN PARTIALLY HEPATECTOMIZED RATS

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The effect of Vitamin E and Silibinin pre-and post treatments were studied in 70% partial hepatectomia (lob.sin.lat.,lob.sin.med., lob. dext.med.) in young male Wistar rats. The animals were treated with 50 mg/b.w.kg Silibinin i.p. and 500 mg/b.w.kg Vitamin E mixed in food for 5 days before operation and for 2 days after lobectomia. The effect of antioxidant treatments on changes of lipid peroxidation, content of diene conjugates, thiobarbituric acid reactive products and natural scavenger capacity of rat liver homogenates were measured by spectrophotometric and luminometric methods. In the sera from partially hepatectomized rats after 72 h there were significant enhanced in the level of GPT, gamma GT, GOT, AP, cholesterol, triglyceride and bilirubin comparing to sham operated animals. The parameters measured in the serum and detected tissue injuries caused by free radical reactions were significantly decreased and the data were approached to those of sham operated animals during Vitamin E and Silibinin treatments. It was established, that antioxidants favourably influenced on regeneration of rat liver.

15.8 LIVER PROTECTING AND LIPID LOWERING EFFECTS OF SEMPERVIVUM TECTORUM IN THE RAT

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Sempervivum species are wellknown plants in folk medicine for treatment of inflammations. Phytochemical screening of the plant extract proved the presence of a notable quantity of low molecular mass, potentially antioxidative compounds (flavonol glycosides: 0,7%; polyphenols: 4.2%; phenolcarboxylic acids; polysaccharides; etc).
Superoxide scavenging activity of liophylized extract was determined by the electron paramagnetic resonance spin trapping methods. The extract inhibited the ascorbic acid induced and iron stimulated lipid peroxidation in the liver microsomal fraction of rats. Liver protecting activities of the extract were examined on experimental hyperlipidemia, in the pathomechanism of fatty liver. The effect of antioxidant treatment on changes of fatty acid composition of liver homogenate was determined by capillary gaschromatography. Thiobarbituric acid reactive products and diene conjugates were measured by spectrophotometric methods and the changes of natural scavenger capacity of the liver homogenate were detected by luminometry. Serum lipid level lowering and HDL-cholesterol enhancing activities of Sempervivum tectorum were observed in experimental hyperlipidemia.

15.9 PREVENTIVE EFFECT OF EGB 761 ON CHLOROQUINE RETINA FUNCTIONAL DISORDERS

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Toxicity of xenobiotics are known to be associated to free radical release. Chloroquine, a drug widely used for chronical treatement of malaria, induces retinal disorders, specifically directed towards pigmentary epithelium and photoreceptors. The aim of this study was to test the effect of a free radical scavenger, the Ginkgo biloba extract (EGB 761) on the chloroquine-induced retinal impairments. Experiment was realized on isolated albinos rat retinas. Retinal function was assessed by electroretinogram (ERG) obtained after standard stimulation of retina, by flashes of white light (300 lux, 1 ms). In this study, two groups of animals were analysed, untreated rats and rats treated with EGB 761 (100 mg/kg/po) during 15 days. Results showed that in untreated rats (n = 10), chloroquine (10^{-5} M) added to the solution perfusing, induced rapid and severe decrease in ERG b wave amplitude. In rats pretreated with EGB 761 (n = 10), the ERG b wave amplitude decrease was not so marked and retina survival was increased.

These experiments confirm the toxic effect of chloroquine on retinal function. The protective effect of EGB 761 may be due to its free-radical scavenger property.

15.10 HEPATIC ANTIOXIDANTS AND LIPID PEROXIDATION DURING FETAL LIVER DEVELOPMENT. ROLE OF MATERNAL ADRENAL STEROIDS.

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The aim of present work was to study the effect of early maternal adrenalectomy on Cu/Zn-SOD and GPx activities, GSH and GSSG levels, as well as, endogenous and "in vitro" lipid peroxidation in fetal liver on day 20 and 21 of pregnancy. The experimental model was as follows: (a) maternal bilateral adrenalectomy (day 1 of gestation) and (b) control pregnant rats. Maternal adrenalectomy produce a significant diminution on SOD activity and GSH concentration, both in day 20 and 21. It is noteworthy a higher GSSG/GSH ratio in day 20 control respect to adrenalectomized group, which had significantly increased total GPx activity. The "in vivo" lipid peroxidation was equal in both groups on day 20 and 21. However, "in vitro" was higher in control than adrenalectomized group on day 20. The absence of maternal glucocortcoids affects in a different way to the studied fetal liver antioxidants defences on day 20 or 21.

15.11 PRODUCTION OF O_2^- *IN VITRO* AND TOXICITY *IN VIVO* OF ADRIAMYCIN (Adr) AND $Fe(Adr)_3$

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Fe_3Adr (Quelamycin) had been proposed as a less toxic substitute for Adr. In preliminary studies (i) $Fe(Adr)_3$ too was less toxic than Adr, when administered as a fresh solution to tumor-transplanted C57 female mice, and (ii) the toxicity of Adr decreased while that of $Fe(Adr)_3$ increased with aging of the solutions (unpublished data). As the toxicity of Adr has been attributed to excessive production of superoxide anion radical (O_2^-), we compared the ability of fresh and aged solutions of the two compounds to modulate the production of O_2^- - measured as SOD-inhibited succinoylated cytochrome *c* reduction - by liver microsomes from phenobarbital treated rats. A linear, dose-dependent increase in O_2^- production was measured in the presence of Adr (7.2 ± 0.1, 8.7 ± 0.3, 14 ± 0.6, 20.3 ± 0.6 and 28.7 ± 0.4 nmol/mg protein/min, with 0, 7.7, 20, 40 and 74 μM Adr), but not in the presence of $Fe(Adr)_3$ at concentrations up to 21 μM. When O_2^- production in the presence of fresh, 24 and 48 hr old solutions of the two compounds and in their absence (controls) were compared, O_2^- production decreased with aging, for both Adr (30.1 ± 1.0, 20.6 ± 2.2 and 13.5 ± 1.2, respectively) and $Fe(Adr)_3$ (8.7 ± 0.1, 7.2 ± 0.4 and 5.4 ± 0.3, respectively), control values being 7.0 ± 0.7 and 7.6 ± 0.6 for Adr and $Fe(Adr)_3$, respectively. These results may suggest a role for O_2^- production in the toxicity in mice of both fresh and aged Adr solution, and, possibly, fresh $Fe(Adr)_3$ solutions, while the greater toxicity of aged $Fe(Adr)_3$ solutions might be unrelated to O_2^- production.

(Adr was kindly supplied by Farmitalia-C.Erba)

15.12 INCREASED SUPEROXIDE ANION PRODUCTION IN DERMAL FIBROBLASTS OF PSORIATIC PATIENTS

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Psoriasis is an inflammatory skin disorder with keratinocyte hyperproliferation as an incomplete differentiation of their cells.

Dysregulation and overproduction of cytokine release of reactive oxygen species by many types of cells are described.

Fibroblasts are ubiquitous mesenchymal cells and generate some inflammatory citokynes. They can play an important role in inflammatory skin diseases such as psoriasis. We have therefore investigated the liberation of superoxide anions in dermal fibroblasts of psoriatic patients.

Superoxide anion production was measured with the technique of Pick and Mizel in fibroblast cultures of psoriatic and normal patients.

The liberation of superoxide anion is significantly increased in fibroblast culture of psoriatic skin: the augmentation of liberation is respectively of 83% and 122% in uninvolved and involved psoriatic fibroblasts.

In conclusion, fibroblasts are a source of superoxide anion production in psoriatic lesions in vivo and can participate in the recruitment of polymorphonuclears in psoriatic skin.

15.13 ALLANTOIN AND URIC ACID AS A MARKER FOR OXIDATIVE MECHANISMS IN CATARACT DEVELOPMENT.

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Recent population and clinical studies have indicated that oxidative mechanisms may play a role in the pathogenesis of the so-called senile cataract. Since the lens is challenged by a number of oxidative insults either endogenous (hydrogen peroxide) and exogenous (photoperoxidation), oxidative stress is likely to be a causative cataractogenic mechanism. In this study we measured levels of uric acid and allantoin, its major oxidation product in aqueous humor and plasma from cataract patients.

Allantoin and uric acid were measured simultaneously by an improved HPLC technique. Levels of allantoin in the aqueous ranged from 10.5 - 23 µmol/L, (mean ± SEM = 15.14 ± 1.2). Uric acid is generated in the human body by the oxidation of purines but no enzyme is present to oxidize it further, thus allantoin might be a "marker" *in vivo* for the extent of oxidative stress. Because the depletion of uric acid is associated with increase in allantoin, a calculated index from the ratio of their concentrations would be a more sensitive marker. The index in aqueous humor from cataract patients was three times the value in plasma, suggesting accelerated oxidative depletion of uric acid. Oxidative damage appears to be a likely mechanism in the development of cataract.

15.14 HEME OXYGENASE INDUCTION BY UVA IRRADIATION OF SKIN FIBROBLASTS IS FOLLOWED BY AN INCREASE IN FERRITIN

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Heme oxygenases 1 and 2 (HO-1 and HO-2) are the rate limiting enzymes in the degradation of heme proteins. Whereas HO-2 is a constitutive protein, HO-1 messenger RNA levels are transiently elevated by a number of oxidative challenges including UVA light (320 - 380 nm). For example mRNA levels are increased approximately 10 fold 3 h after exposure to 250 kJ m^{-2} of UVA light. In this study we show that HO enzymatic activity increases to a maximum of approximately 4 fold 18 h after irradiation with 250 kJ m^{-2} of UVA light. Elevated HO levels would increase heme degradation thereby increasing intracellular free iron levels. It would be expected that an increased level of iron would derepress the iron responsive elements involved in ferritin translation inhibition. This effect was confirmed by measuring a 42 ± 10% increase in ferritin levels 22 h after irradiation. An increase in ferritin will contribute to the iron scavenging capacity of the cell thus limiting damage during subsequent oxidative challenges.

15.15 LIVER ANTIOXIDANT ENZYMES AND MAXIMUM LONGEVITY IN SEVEN VERTEBRATE SPECIES

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There is considerable interest in clarifying if antioxidant enzymes have been used as longevity determinants in animal evolution. A previous study showed a positive correlation between SOD activity/metabolic rate and longevity in various mammalian species. Nevertheless no correlations were found for CAT and GPx or for SOD/protein. Another study did not find correlations with maximum life span for these enzymes in other mammals. We have measured liver SOD, CAT, Se and non-Se GPx, glutathione reductase and cytochrome oxidase in seven adult vertebrates with well defined maximum life spans: two mammals (guinea pig and rat), two birds (canary and pigeon), two amphibians (*Rana perezi* and *Xenopus laevis*) and a fish (*Salmo trutta*). The results did not show significant correlations between these enzymatic antioxidants and species-specific maximum life span. It is concluded that enzymatic antioxidant defense is not a longevity determinant in vertebrate evolution.

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15.16 HEPATIC DAMAGE WHEN THE LIVER WAS PERFUSED WITH A RADICAL INITIATOR AND ITS INHIBITION BY VITAMIN E

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One of the most basic characteristics of hepatic failure is the decrease in energy charge levels and mitochondria has been increasingly recognized as a critical site in the pathogenesis of hepatic failure because of its central role in ATP-synthesizing ability. Using an azo compound, AAPH (*2,2'-azobis(2-amidinopropane)dihydrochloride*), which is well known to generate peroxyl radicals in water phase at a constant rate, therefore, we tried to elucidate the injurious actions of free radicals on hepatic mitochondrial function under controlled conditions. In liver mitochondria, acetoacetic acid (AcAA) undergoes reduction to β-hydroxybutyric acid (β-OHBA) by β-OHBA dehydrogenase localized in the mitochondrial cristae and they can freely penetrate the cell membrane into the blood stream. The equilibrium between their concentrations reflects the ratio of NAD/NADH in the mitochondria (mitochondrial redox potential). In this study, we catheterized the portal vein and inferior vena cava of the rats and infused Ca-free Hanks-HEPES buffer containing AAPH via portal vein. Aliquots of perfusate coming out of inferior vena cava were taken at specific times to determine the constitution of ketone bodies and hepatic cytosolic enzymes. The ketone bodies in the perfusate decreased rapidly and β-OHBA disappeared much faster than AcAA, indicating the change in mitochondrial redox potential. Such a rapid depletion of mitochondrial NADH may be an initial event induced by free radicals. In the case of rats injected of α-tocopherol previously, the change in mitochondrial redox potential was slow, dependently on doses of the tocopherol administration. The similar effect was obtained by the addition of Trolox, a water-soluble analogue of vitamin E, into the perfusate. The leakage of cytosolic enzymes (GOT, GPT) into the perfusate was a subsequent event to the change in mitochondrial redox potential, suggesting that hepatic cell membrane damage may be caused by increased cytosolic Ca due to NADH depletion. These results show that the liver was damaged exactly depending on the amount of radicals generated in the circulation and hepatic α-tocopherol inhibited their injurious effects efficiently.

15.17 *Oxidative damage to β–crystallin and reaction with* transglutaminase.

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In our previous work we showed that the attack by oxidising free radicals on β-crystallin predisposes it to transglutaminase (TGase) activity and we suggested that this synergistic effect might be involved in the pathogenesis of cataract. The previous study utilized the incorporation of ^{14}C-putrescine and effectively measured the reactivity of glutamine residues that act as amine-acceptor sites. We have extended this work by utilizing a synthetic probe for the lysine residues that can function as amine-donors in the transglutaminase reaction as well as studying the effects of oxidising free radicals on TGase-mediated cross-linking of crystallin subunits.
The experiments measuring lysine-site reactivity mirror the ^{14}C-putrescine results and confirm that oxidising free radicals make both types of site more available to TGase. Electrophoretic analysis (1D and 2D) demonstrates that these probes are incorporated into whole crystallin subunits, not radiolytically-produced fragments. These data suggest that the effect is due to a conformational change in the crystallin following free radical attack. The effect of oxidising radicals on TGase-mediated cross-linkings is difficult to ascertain against a background of radical-mediated, TGase -independent cross-linking.

15.18 ROLE OF LIPID PEROXIDATION AND POLYMORPHONUCLEAR LEUKOCYTES-DERIVED OXYGEN RADICALS IN DUODENAL ULCERS INDUCED BY MEPIRIZOLE IN RATS

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We have reported that active oxygen species may play an important role in the pathogenesis of duodenal ulcers induced by mepirizole(MEP). In this study, we investigated the role of active oxygen species from polymorphonuclear leukocytes(PMN).

Male Wistar rats each weighing 190-210 g were used. We administrated to them MEP(200mg/kg) p.o.. In one study, the myeloperoxidase (MPO) activity which is an index of PMN's accumulation were measured by Krawisz's method after administration of MEP. After administration of MEP, we found the increase in MPO activity in the duodenal mucosa before the appearance of the ulcers. In another study, we administrated anti-PMN antibody i.p. at 18hr before administration of MEP. And we measured the total area of duodenal ulcers and TBARS in the mucosa. The increase both in the total area of the ulcers and in TBARS in the mucosa was significantly inhibited by treatment with anti-PMN antibody.These results suggest that active oxygen species from PMN may play an important role in the duodenal ulcers induced by MEP.

15.19 EFFECT OF MERCURY AND N-ETHYL-MALEIMIDE ON PRIMARY CULTURE HEPATOCYTES.

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Sulphydril reagents are frequently involved in environmental or professional toxicology. Their mechanism(s) of action may differ in relation to the ability of the agent to penetrate the cell. A cell-penetrating agent, like NEM, interacts with surface proteins as well as with cytosolic or organular proteins, triggering an integrated cell reaction which may lead to cell disorganization and necrosis. Hg++ do not penetrate immediately the cell, interacting mainly with surface sulphydril groups; once plasmamembrane damage is established, cell may progress further to disorganization. Primary culture hepatocytes, in the presence of high concentration Hg++ (500 uM), rapidly form cytoplasmic blebs and progress to necrosis, allowing Hg to specifically compartimentalize into the mitochondria or associated to glycogen particles and smooth membranes of endoplasmic reticulum. Primary culture hepatocytes treated with NEM also form cytoplasmic blebs, with a much slower progression through disorganization and necrosis. The role of cytoskeleton, Ca++ and Hg++ will be discuss.
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Plenary Session on Pharmaceutical Intervention in Free Radical-Based Pathology

D.2 METAL-CATALYZED FREE RADICAL INJURIES IN CHILDHOOD DISORDERS AND PHARMACEUTICAL INTERVENTION

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Transition metals, copper and iron, are well known to catalyze the generation of free radicals in vitro which probably cause pathological conditons in diseases. In this paper, such conditions in childhood will be discussed.

Wilson's disease is an inherited disorder of copper metabolism characterized by progressive copper accumulation in the liver and subsequent overflow to extrahepatic tissues. Then, non-ceruloplasmin copper increased and ferroxidase activity decreased in plasma, although copper incorporated into ceruloplasmain is non-toxic. The copper released from the liver into blood stream catalyzes the generation of free radicals, which consume radical trapping antioxidants including urate and vitamins C and E in plasma. The finding was obtained that the radical trapping capacity was impaired due to increased non-ceruloplasmain copper in Wilson's diease, while the radical trapping capacity was improved in response to the copper chelating therapy. In addition, the antioxidant effect of the Wisonian plasma against the ferrous salts-stimulated lipid peroxidation was reduced, and zink inhibited the peroxidation. This finding coincided with amelioration of clinical course of Wilson's disease with zink therapy.

Homocystinuria, an inborn error of methionine metabolism characterizes elevated level of homocysteine in the body fluids which causes premature atherosclerosis and thrombosis. Transition metal-catalyzed oxidation of homocysteine thiols is thought to lead to the reduction of oxygen with the generation of superoxide and hydrogen peroxide. The active oxygen species generated in plasma must attack lipoproteins to produce oxidatively modified LDL, which may contribute to onset of atherosclerosis. We examined the possibility of homocysteine-induced oxidation of LDL in the presence of metals and the role of alpha-tocopherol in LDL in preventing its oxidative modification. Additionally, oxidatively modifeid LDL in the patients' plasma was demonstrated, acommapnied by decreased plasma alpha-tocopherol levels. Vitamin E therapy should possibly be required to prevent the premature atherosclerosis.

D.3 PHARMACEUTICAL INTERVENTION FOR THE PREVENTION OF POST ISCHEMIC REPERFUSION INJURY.

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In many organs a substantial proportion of the injury sustained as a consequence of ischemia is actually caused by a cascade of events which are initially triggered by the generation of the superoxide free radical by activated xanthine oxidase at the time of reperfusion. This mechanism, first elucidated in the feline small intestine, has been found to be operative in the heart, lung, stomach, liver, kidney, skin, skeletal muscle, and other organs. Its ubiquity is based upon the ubiquity of endothelial cell xanthine oxidase in the microvasculature. Pre-clinical studies in animal models of human disease have demonstrated a variable contribution of this reperfusion injury mechanism to the total injury sustained consequent to ischemia. While ill-conceived and poorly designed clinical trials which have failed to take this into account, and therefore have included heterogeneous and unstratifiable patient populations, have been predictably unenlightening, a number of well designed trials, particularly in renal transplantation in cardioplegia have confirmed the efficacy of free radical ablation for the prevention of reperfusion injury in man.

D.4 ANTIOXIDANT ACTIVITY OF SILYMARIN

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Silymarin contains silybin, isosilybin, silydianin and silychristin, a class of natural products, namely 3-hydroxy-4-chromanone derivatives. Silymarin has an effective antioxidant activity, which could explain its antihepatotoxic efficacy. The antioxidant activity has been evaluated by the RANCIMAT methodology in the autoxidation of fatty acids, esters (soybean oil, palm oil, palmitic acid, methyl linolenate) and cumene and expressed by the ratio of the induction periods of the substrate autoxidation in the presence and in the absence of silymarin. This drug acts as scavenger of peroxy radicals and shows a secondary antioxidant activity higher than BHT and slightly lower than alpha tocopherol. The structure of the chromanones and some previous results in the presence of iron salt suggest that silymarin could also exert a primary antioxidant effect, due to the chelating properties towards metal salts. The study of the mechanism is in progress.

D.5 SILYMARIN AS A FREE RADICAL SCAVENGER IN TOXIC LIVER LESIONS

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Sylimarin is an active flavolignan wich owning to its free radical scavenger activity a membrane stabilizing agent and an effective antioxidant. Silymarin is an isomeri mixture of silibinin, silidianin and silichristin, silibinin being the most active component The therapeutic application of silymarin (Legalon) for tratment of patients with live disease remains to be established. Recently a water-soluble form of silibinin (th dihemisuccinate sodium salt) has been developed. Silymarin and silibinin hemisuccinat sodium salt proved to be effective in animal experiments in the prevention of carbo tetrachloride, galactosamine and ethanol hepatoxicity as well as Amanita phalloide intoxication. Their noncompetitive lipoxygenase and prostaglandin synthetase inhibiting activity and cellular immunoreactivity reducing effect is favourable in the therapy o chronic liver diseases. In clinical trials these compounds were effective in chroni hepatitis, drug-induced liver toxicity (CCl4, galactosamine, ethanol, tuberculostatics A. phalloides, hypnotics, insecticides). The effect of silibinin, 1.0-10.0 g/ml on phytohaemagglutinin (PHA-) and Concanavalin-A (CON-A)-induced lymphocyte blast transformation in normal individuals and patients with chronic alcoholic liver disease. With a suboptimal dose of PHA, silibinin exerted no significant effect on blastogenesis in a concentration range corresponding to the usual in vivo therapeutic concentration. However, with an optimal stimulatory dose of PHA, silibinin significantly decreased thymidine 3H incorporation at a concentration of 10.0 g/ml Con-A, silibinin inhibited in vitro lymphocyte blast transformation of the patients in a dose-dependent fashion. Atherogenetic diet significantly inhibited the Con-A-induced blast transformation of rat spleen cells in vivo, Silibinin reversed this reaction. A similar effect of silibinin was found in lysosomal examinations of rat liver. Silymarin and its water-soluble derivative probably exert their cytoprotective effect in two ways (a) owing to their free radical scavenger activity they increase the tissue glutathione content and are potent antioxidants; and (b) by their interaction with cytochrome P-450 they block the biotransformation of drugs whose metabolites are cytotoxic. Our studies show that in hyperlipidaemic rats and in patients with chronic alcoholic liver disease the extrahepatically detectable oxidative stress state-its typical biochemical parameters were favourable influenced by silymarin treatment. Applied therapy decreased lipid peroxidation and improved the patients antioxidant protection.

Session 16

Antioxidant Defences in Eukaryotic Cells

16.1 ANTIOXIDANT MECHANISMOF VITAMIN E AND β-CAROTENE

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Vitamin E is an excellent trap for peroxyl radicals and is the major lipid soluble antioxidant present in mammalian cells. It therefore occupies a unique position in the arsenal of natural antioxidants providing protections against various diseases. Studies carried out with deuterium-labelled α-tocopherol have shown that there is a wide range in rates of turnover of vitamin E in tissues. The tissues that show the lowest rates of turnover (e.g., neural tissue) also are found to be those most susceptible to the effects of a deficiency of vitamin E. The cytosolic tocopherol-binding protein, which is found only in the liver, appears to play a central role in regulating levels of vitamin E in plasma.

There is much current interest in β-carotene because eat appears to have anticancer activity. It is believed that the anticancer activity derives from the antioxidant properties of β-carotene. However, the nature of the antioxidant action is unclear. Although β-carotene is a superior quencher of singlet oxygen, there is a question as to the importance of singlet oxygen in most tissues. β-Carotene can trap peroxyl radicals but only under low partial pressures of oxygen. This fact is difficult to reconcile with the epidemiological evidence that β-carotene is most effective in the lung.

16.2 ORAL ANTIOXIDANT ADMINISTRATION PARTIALLY PREVENTS EXERCISE-INDUCED GLUTATHIONE OXIDATION IN RATS AND HUMANS

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Exhaustive physical exercise generates free radicals (1). This causes oxidative stress (2). The glutathione system constitutes a major defence against oxidative stress (3). An oxidation of the ghlutathione pool in various tissues was previously reported (4). We have found that a widely used method for the measurement of glutathione which is adequate for use in various tissues is not adequate for blood because of high rates of oxidation of GSH. This may lead to erroneous conclusions when trying to evaluate oxidative stress using the GSSG/GSH ratio. A new method to measure GSSG and GSH in blood will be described. Using this method, we have found that exhaustive concentric physical exercise causes an oxidation of GSH in blood. Furthermore, changes in GSSG/GSH ratio are parallel to changes in the lactate/pyruvate ratio indicating that when the anaerobic threshold is reached, glutathione oxidation occurs. Oral glutathione results in an increase in glutathione in tissues, because it increases the plasma levels of cysteine and the rate of intracellular glutathione synthesis (5). Thus oral glutathione might serve to protect cells against oxidative stress caused by exercise. Indeed, administration of antioxidants such as glutathione, vitamin C, vitamin E or mixtures of these three antioxidants partially prevent the oxidation of glutathione caused by exercise.

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16.3 PHOSPHOLIPID HYDROPEROXIDE GLUTATHIONE PEROXIDASE IS THE MAJOR SELENOPEROXIDASE IN NUCLEI AND MITOCHONDRIA OF RAT TESTIS

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The selenoenzyme Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPX) was detected enzymatically and immunohistochemically in rat testis. Subcellular fractionation revealed an unusual activity distribution pattern, since a specific activity five times higher in nuclei and mitochondria than in cytosol was measured. Furthermore, PHGPX increased during testis maturation. In fact, while neither immunodetectable protein nor activity were present in weaning rats, both rose up from the 20th to the 60th day after birth. This rise was prevented by hypohysectomy. In adult animals the specific activity was 102 and 94 nmoles/min/mg protein in nuclei and mitochondria, respectively. These values were the highest so far measured in mammalian tissues. In adult animals also hypophysectomy led to disappearance of PHGPX activity, which was partially restored by gonadotropin treatment. By contrast, classical Glutathione Peroxidase (GPX) specific activity was not affected by aging, hypophysectomy and gonadotropins. Specific testis immunolabelling by antiserum against PHGPX appeared microscopically as a finely granular brown end product localized throughout the cytoplasm in spermatogonia, but confined to the peripherals regions of the cytoplasm, the nuclear membrane and mitochondria in maturating primary spermatocytes of adult normal testis. Spermatozoa were not immunostained with this technique, but Western blot analysis showed the presence of PHGPX in mature spermatozoa. A possible identity of PHGPX with the previously described 15-20 Kd selenoprotein in the mid-piece of spermatozoa involved in their maturation (*Gamete Res.* 4, 139) is proposed.

16.4 INVOLVEMENT OF FREE RADICALS CATALYZED BY METALS IN WILSON'S DISEASE AND ANTI-OXIDANT DEFENSE.

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Wilson's disease is an inherited disorder of copper metabolism characterized by progressive copper accumulation in the liver and subsequent overflow to extrahepatic tissues. The chemistry with transition metals has been supposed to be involved in the copper toxicosis, however, the mechanisms of their injurious actions still remain unclear. Recently a mutant strain of Long-Evans rats (LEC) has been found to bear a very close similarity to Wilson's disease:
1) Acute onset of fluminent hepatitis associated with hemolytic crisis at the age of 4 months; *2)* Progressive accumulation of copper in the liver leading to hepatic damage; and *3)* Defects in biliary excretion of copper and incorporation into ceruloplasmin. In this study, using this animal model, we speculated on the involvement of copper in free radical-mediated injuries of Wilson's disease.

In LEC rats, copper was found to accumulate in the hepatic cytosol in very high concentration and its majority was included in increased metallothionein. At the age of 4 months when hepatic metallothionein was supersaturated with copper, the liver was severely damaged and hepatic copper was released into the blood stream. If copper released from the liver catalyzes the generation of free radicals, the consumption of plasma antioxidants and hemolysis would be the likely consequences. We observed that nonceruloplasmin copper in plasma increased and radical-trapping capacity was impaired as the leakage of hepatic cytosolic enzymes was getting significant. At that time, antioxidant defense systems in the liver and RBC were impaired and their membrane constituents showed the chages due to lipid peroxidation. Thus our scenario that hemolytic crisis was brought by copper released from damaged liver has been substantiated and the evidencefor the involvement of copper-mediated radical reaction in the pathogenesis has been obtained.

16.5 Oxidative Stress Status (OSS) in HIV seropositive patients. Possible improvement by antioxidant therapy.

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Introduction :
- A growing body of evidence concurs to an antioxidants deficiency in HIV seropositive patients. In cultured cells oxidative stress increases HIV replication and antioxidants decreases it. Vitamin E improves the antiviral action of AZT.
- We studied :
* the action of Dithiocarbamate (DTC) versus placebo on OSS in 29 HIV seropositive patients.
* the action of antioxidants therapy with several antioxidants including Superoxide Dismutase (SOD) in 3 HIV seropositiv patients, also treated with AZT.

Results :
- After 2 years of DTC administration, there was no difference in OSS between treated (16) and placebo (13) groups. The clinical evolution in the 2 groups was also the same.
- In 3 patients treated with several antioxidant (Vit E, Vit A, selenium, N acetyl cysteine) and Cu Zn SOD the OSS improved well as the biological markers monitored.(CD4, immunoglobuline,ß2microglobuline). One patient returns to work for one year after a one year interruption in very good health.

Conclusion :
The antioxidant therapy associated with antiviral treatment deserves to be tested in controlled trial monitored by OSS and biological markers of HIV infection.

16.6 REDOX CYCLE OF ASCORBATE IN THE LIVER: A STUDY OF DEHYDRASCORBATE-REDUCTASE ACTIVITIES

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A number of antioxidant systems protect cells against oxidative stress. Among these, ascorbic acid (AA) is generally believed to play an important role both by scavenging aqueous radicals and by regenerating vitamin E. Following interaction with free radicals, AA undergoes one-electron oxidation to the semi-dehydroascorbic radical from which, by dismutation, dehydroascorbic acid (DHAA) is produced. DHAA is then further metabolized and lost by the cell. In order to restore the antioxidant potential offered by AA, cellular systems capable to reduce back its oxidized forms are clearly of particular importance. However, available data concerning DHAA reduction are controversial.
The present study deals with a re-evaluation of liver DHAA-reducing enzymatic activities. Several DHAA-reductases were observed in the (dialysed) cytosolic compartment, which were dependent either on glutathione (GSH) or NADPH as cofactors. The measured apparent Km's for the substrates indicate that one GSH- and one NADPH-dependent activity can efficiently work in the range of physiological concentrations of GSH, NADPH and DHAA. Thus, an efficient reduction of oxidized forms of AA can be accomplished in the liver cell. This may represent an efficient defence system, as it has been observed in isolated hepatocytes challenged with various prooxidants.

16.7 IDENTIFICATION OF PRODUCTS FROM OXIDATION OF URIC ACID INDUCED BY HYDROXYL RADICALS

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The aim of the present study was to separate and characterise products formed by oxidation of uric acid by hydroxyl radicals with a view to probing for these products *in vivo* in clinical contexts thought to be associated with free radical production, especially alcohol-related liver injury and rheumatoid disease.

Aerated solutions of 200 µM urate, or its oxidation products, allantoin or parabanic acid were exposed to gamma radiolysis, (570 Gy/min), as a source of HO· radicals at pH 3.4 and 7.4. Aliquots were taken every 5 minutes for 20 minutes and oxidation products were analysed by HPLC with a diode array detector. Chromatographic conditions were as follows:Column: 250 x 4.6mm Activon Gold-Pak (reverse phase, 5 µm particle size); Mobile Phase: 5mM NH_3HPO_4/H_3PO_4, pH 3.4 (for pH 3.4 irradiation) or 10mM KH_2PO_4/H_3PO_4, pH 3.4 (for pH 7.4 irradiation). Identities of oxidation products were made on the basis of similarity of retention time and absorbance spectrum with known standards. Hydroperoxides were measured by tri-iodide formation in the 20 minute sample.

Exposure of urate to such HO· fluxes produced a nett loss of the parent compound with formation of a complex mixture of products with allantoin and parabanic acid being the predominant products at pH 3.4. When irradiated at physiological pH, the rate of urate loss was lower and the distribution of products slightly different. A small but significant amount of urate hydroperoxide was detected at both pH's. A mechanism for urate oxidation under these conditions will be presented.

16.8 The importance of the three antioxidant enzymes in cellular defense.

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Aerobic cells contain various amounts of the three main antioxidant enzymes: superoxide dismutase (SOD), catalase and GSH peroxidase. These three enzymes are necessary for cell survival since inhibition of their activity from 21 % for GSH peroxidase and 55 % for catalase leads to the arrest of cell mitosis and to cell death. Amongst them, GSH peroxidase was shown to be more efficient than catalase and much more than SOD. This result was obtained by comparing the cell protection against oxidative stress after their microinjection in the cytoplasm. A solution containing 2000 times more SOD than GSH peroxidase and 100 times than catalase was necessary to obtain the same protective effect.
In order to evaluate the effect of free radicals on cells, we have to distinguish between their respective effects on cell mitosis, cell death and cell ageing. The effects on mitosis and cell death are well described and the results clearly show a threshold response The level of this threshold is determined by the level of anti-oxidant enzyme concentration indicating that there is a dynamic balance between the defense system and the free radical production.This dynamic interaction can also be seen in a modelisation of the general system of free radical production and elimination where bifurcation point between stable and unstable domains was found. There is now evidence that short free radical stresses can also speed up the ageing of in vitro cultured human fibroblasts. This was demonstrated by looking at the evolution of the cell types which evolve much faster under oxidative stresses.However this accelared ageing process is not restricted to the free radicals and is dependant on the energetic level of the cells.

16.9 IRON MEDIATED INCREASES IN MITOCHONDRIAL AND LYSOSOMAL ACTIVITIES AND INDUCTION OF HSP70 AND UBIQUITIN mRNA IN *IN VITRO* CULTURE

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Following heat shock (42.5°C for 30 minutes), differentiated C1300 N2A neuroblastoma cells increase the synthesis of mRNA's for the inducible form of hsp70 and for ubiquitin. Incubation of C1300 cells with iron (1-100mM) also induces an elevation in mRNA's coding for ubiquitin and hsp70 which can be blocked by preincubation of the cells with alpha-tocopherol. Iron was shown to increase mitochondrial and lysosomal enzyme activies in these cultures as measured by the MTT and neutral-red uptake cytotoxicity assays. These changes were not initially asssociated with any loss in viability as assessed by the lactate dehydrogenase release assay. The results suggest that there is production of cytoprotective heat shock proteins in response to iron-mediated cell damage in neuronal cells, possibly involving free radical generation. The apparent stress response by vulnerable neurones in neurodegenerative disease, particularly Parkinson's disease, may be induced by iron-mediated free radical production in degenerating neurones.

16.10 EFFECT OF HYDROGEN PEROXIDE ON HUMAN EPITHELIAL CELLS (WISH).

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Hydrogen peroxide (H_2O_2) is one of the many active species produced during normal cell metabolism. When H_2O_2 is overproduced, it can cause damage of DNA and proteins directly or indirectly by generating oxygen free radicals. We studied the effect of H_2O_2 on cultured epithelial cells derived from human amnios with respect to cell functions: in addition, the morphology and the cell structure were also investigated. The presence of low H_2O_2 concentrations in the culture medium, completaly inhibited cell growth, although WISH contained catalase and glutathione-peroxidase activities. After 1 h incubation with 5 mM H_2O_2 the majority of cells were still alive, but the reincubation with normal medium, dramatically decreased the cell viability. Cell adhesion was dose-dependently reduced by H_2O_2 treatment (0.1-0.5 mM). The incubation with 1.5 mM H_2O_2 caused blebs formation on cell surface, mitochondrial swelling and cytoskeleton modifications both in microtubules and microfilaments. WISH treated with 1.5 mM H_2O_2 showed decreased levels of GSH compared to control cells: even the glutathione-transferase activity was reduced while the other enzymes of the glutathione-cycle were unchanged.

16.11

Differentiation-induced manganous superoxide dismutase is enhanced by TNFα and phagocytosis of *Staphylococcus aureus* in U937 cells. S. Kantengwa, H. Stubbe and B. S. Polla Allergy Unit, University Hospital of Geneva, Switzerland

Superoxide dismutase (SOD, EC 1.15.1.1) is a key enzyme for the scavenging of superoxide anion. SOD exists in at least two isoforms: the constitutive, containing copper and zinc as metal core, and the inducible mitochondrial enzyme (MnSOD) which has been found associated with differentiation and development in eukaryotes. As a protective enzyme against oxidative injury, SOD is a candidate member of the stress protein families. We investigated the expression of SODs during differentiation and activation of the human premonocytic line U937. Total SOD activity was determined in a xanthine-xanthine oxidase system and the two isoforms were characterized by immunoblotting with polyclonal antibodies. Cellular functions such as respiratory burst and phagocytosis are acquired during differentiation of U937 cells. In 1,25-dihydroxyvitamin D3-differentiated cells we found a threefold increase in total SOD activity. This increase was enhanced by rhTNFα (5 ng/ml) (+30%) and further so by phagocytosis of inactivated *S. aureus* (+70%). By Western blot analysis MnSOD was not detected in undifferentiated U937 cells but was induced in differentiated and activated cells, whereas CuZnSOD remained unchanged. Our results on MnSOD suggest that during differentiation, monocytic cells acquire in parallel the ability to kill bacteria (phagocytosis and respiratory burst) and to protect themselves against oxidative injury.

16.12 A NOVEL METHOD FOR DETERMINING BOTH Mn- AND CuZn-SUPEROXIDE DISMUTASE ACTIVITIES IN GASTRIC MUCOSA

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In the present study, a highly sensitive chemiluminescence method for determining Mn- and CuZn-SOD activities in human gastric mucosa obtained by endoscopic biopsy was reported.

METHODS: SOD activity was measured by the inhibition of the cypridina luciferin analog(CLA)-dependent chemiluminescence induced by superoxide radicals. Mn-SOD, which is sensitive to sodium dodecyl sulfate, was determined and calculated by subtraction of SOD activity in tissue extract treated with this detergent (CuZn-SOD) from that in untreated tissue extract (total SOD). Both SOD activities were expressed as units of SOD activity/mg protein.

RESULTS: CuZn- and Mn-SOD activities could be measured in only one biopsied specimen. CuZn-SOD activities in the fundic gland area were significantly higher than those in the pyloric gland area. Both CuZn- and Mn-SOD activities in the gastric mucosa showed no significant differences among subjects in their 20s to 70s.

CONCLUSIONS: Using a CLA-dependent chemiluminescence method, Mn- and CuZn-SOD activities in the human gastric mucosa obtained by endoscopic biopsy were measured. These SOD activities showed no significant aging-related changes in healthy volunteers' stomach.

16.13 ANTIOXIDATIVE PROPERTIES OF GLUTATHIONE ARE POTENTIATED BY NITECAPONE

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Oxygen derived free radical mediated reactions contribute to a wide variety of tissue injuries including duodenal and gastric ulcers. Glutathione (GSH) dependent systems reduce lipid peroxidation especially when adequate amount of vitamin E is present in the cell membranes. GSH itself is a poor scavenger of free radicals, but it has a role in recycling vitamin E. Nitecapone (NC) is a gastroprotective agent with multifactorial mechanisms of action. Interestingly, under oxidative stress NC spares GSH better than ascorbate, which may enhance cellular defense mechanisms. In this study the effect of nitecapone alone or combined with GSH on inhibition of lipid peroxidation was tested *in vitro*. Lipid peroxidation was induced in guinea-pig liver microsomes by azobisamidinopropane (ABAP) or t-butylhydroperoxide (tBHP) and quantitated as MDA by HPLC and TBARS by spectrophotometry. NC inhibited ABAP and tBHP induced lipid peroxidation with IC_{50}-values of 8 μM and 80 μM, respectively. The corresponding values for GSH were 72 μM and 350 μM. Furthermore NC reduced lipid peroxidation more effectively in nondenatured microsomes than in boiled microsomes (IC_{50} = 32 μM / ABAP). In contrast GSH had similiar effect in both assay conditions. NC in combination with GSH protected the nondenaturated microsomes significantly longer than GSH or NC alone.The results indicate that NC can either spare cellular GSH or additively contribute to GSH-related protection against lipid peroxidation.

16.14 HYDROGEN PEROXIDE IS NOT MORE CYTOTOXIC AT 37°C THAN AT 4°C

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H_2O_2 cytotoxicity as measured by cell survival was claimed to vary depending on the temperature at which cells had been exposed to it. The lowest surviving fraction was found at 37°C, whereas H_2O_2 at the same concentration did not affect the cells at 4°C (s.f.=100%). However, we show this interpretation to be faulty, and the difference being due to a significantly lower rate of H_2O_2 degradation by cells at 4°C than at 37°C. An equal consumption of H_2O_2 at either temperature (achieved by extending the incubation time with H_2O_2 at 4°C) resulted in the cell survival lower by 20% at 4°C than at 37°C. This allows the conclusion that temperature does not affect H_2O_2 cytotoxicity but determines the rate of H_2O_2 consumption by cells exposed to it while having no or little influence on cell survival.

16.15 ANTIOXIDANT DEFENSE SYSTEM IN THE ERYTHROCYTES AND PLASMA IN VARIOUS STRAINS OF THE RATS

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The extent of the oxidant damage to the erythrocytes could be a suitable biomarker of the oxidant damage, generally. We examined superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in the erythrocytes and glutathione-S-transferase (GST) in the plasma of the rats.The males of outbred rats (Wistar and Mill Hill hybrid hooded), inbred rat (DA) and the Belgrade laboratory rat (two phenotypes : anemic and nonanemic) were used. The animals bred under similar breeding conditions. Superoxide dismutase activity was found to be markedly lower in the erythrocytes of the Belgrade anemic rats in comparison with other strains. Catalase activity was lower in the erythrocytes of the Wistar strain than in other investigated rat strains. Glutathione peroxidase activities were clearly increased in the Belgrade rats,especially in nonanemic ones.However, glutathione-S-transferase activities in the plasma of two Belgrade phenotypes were clearly depressed in comparison with other strains. It was concluded there are the phenotype and strain specific differences in the blood antioxidant system of examined animals.

16.16 ANTIOXIDANT DEFENCE SYSTEM IN THE HIBERNATORS EXPOSED TO LOW ENVIRONMENTAL TEMPERATURE

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Male ground squirrel (Citellus citellus) were acute exposed to low environmental temperature (7°C) 3,6 and 24 hr in different seasons:summer,autumn and winter.All animals were at 30°C before the experiment.The following antioxidant enzymes:superoxide dismutase (SOD),glutathione peroxidase (GSH-Px),catalase (Cat),glutathione S transferase (GST) and glutathione reductase (GR),as well as the nonenzyme components: vitamins C and E ,and GSH were examined in the liver and interscapular brown adipose tissue (IBAT).

The ground squirrels kept at 30°C examined in different periods of year,showed different activity of AOS components,with season dependent response after short exposure to low temperature. Small changes in the activity of AOS components were found after cold exposure in summer.Exposure to cold produced changes in more components of antioxidants defence in autumn. Nevertheless the intensity of changes was the greatest in the winter.

Very slowly answer to cold exposure was characteristic for the summer,and fast for the period when ground squirrels were prepared for the hibernation.

16.17 ACTIVITY AND EXPRESSION OF ANTIOXIDANT ENZYMES IN RABBIT ARTICULAR CHONDROCYTES TREATED BY SUPEROXIDE ANIONS

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Oxygen reactive species play an important role in rhumatoïd arthritis and arthrosis.

We have studied in a cellular model the antioxidant enzymes activity following an oxidative stress, in order to appreciate its threshold.

Rabbit articular chondrocytes cultures have been exposed to superoxide anions generated by the hypoxanthine-xanthine oxidase system. The activities of glutathion peroxidase and copper-zinc superoxide dismutase have been measered after the treatment.

The enzymatic kinetics study has revealed an increase in their activity. In the treated cells, SOD activity enhanced earlier than glutathion peroxidase activity.

To elucidate the regulation of their activity, we have studied the expression of their mRNA, but no difference between these mRNA levels have been observed.

We postulated that the increase of this antioxidant enzymes activity could be explained by the activation of an enzymatic inactive pool.

16.18 FAST SPECTROPHOTOMETRIC ASSAY OF SOD ACTIVITY BASED ON THE ACTIVATION OF BXT-01050 AUTOXIDATION

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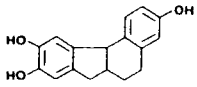
This new assay method is based on the SOD-mediated increase in the rate of autoxidation of BXT-01050 (I) in aqueous alkaline solution.

(I)  (λmax=282 nm)

This autoxidation yields a chromophore with a maximal absorbance wavelength of 525 nm. Two mechanisms are proposed. The first one involves two reversible steps of one-electron transfer to oxygen, producing 2 equivalents of $O_2^{\cdot-}$. Subsequent deprotonation of the resulting orthoquinone yields the chromophoric product. The second hypothesis is consistent with a one-electron oxidation of BXT-01050 into the corresponding semiquinone, which upon deprotonation of its five-membered ring, adds oxygen to form a tertiary peroxyl radical which then extrudes superoxide. In either case, equilibrium of $O_2^{\cdot-}$ production are driven to the right by SOD activity. The assay is carried out at pH 8.8, 37°C, in air-saturated buffer. The kinetic measurement of 525-nm absorbance is performed upon addition of BXT-01050. This reagent is stabilized in its stock solution by the means of boric acid. The SOD activity is determined from the difference in autoxidation rates, measured over 30 s, in the presence and in the absence of the sample. Another reagent with vinylpyridinium structure directly eliminates interferences due to sample mercaptans such as glutathione, by the means of a very fast alkylation reaction. A fast, reproducible and specific measurement of SOD activity only requires a single determination per sample. At pH 8.8, an optimal assay sensitivity is achieved without inactivation of Cu/Zn-, Mn- or Fe-SOD.

16.19 FAST AND SPECIFIC COLORIMETRIC ASSAY OF MERCAPTANS WITH NON-ENZYMATIC DISCRIMINATION OF GLUTATHIONE

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A large number of halogeno-N-alkylquinolinium structures were assessed as potential and chromogenic reagents of mercaptans. The rate of formation and chromophoric features of thioether adducts were studied from halogen substitution in 2-, 4-, 5-, 6-, 7- or 8-position on the quinoline ring.

Substitution of good leaving groups, in 4-position on the quinoline ring, was always characterised by fast kinetics and intense absorbance of the thioether adducts in the 340- to 370-nm range, around neutral pH. For instance, the reaction of 0.05-10 mM GSH with BXT-03015 (i.e. 4-chloro-7-trifluoromethyl-1-methyl-quinolinium) in 10-fold excess, is completed in less than 5 minutes at pH 7.3, and it yields a 4-thioetherquinolinium with strong absorbance at 356nm. In biological samples, a mixture of different mercaptans yields thioether adducts wich cannot be resolved by the means of colorimetry. In most cases however, fast HPLC separation of such adducts provides a convenient tool for measurement of individual mercaptans.

When the GSH-adduct of BXT-03015 is further incubated at pH>13 and ambient temperature, a fast reaction of ß-elimination yields 100% of the corresponding 4-mercaptoquinolinium with a strong absorbance around 400nm. In a survey of more than twenty other mercaptans, including mercaptoethanol, dithiothreitol, cysteine derivatives and various drugs, ß-elimination was never observed, in the above conditions. The latter, therefore, provides a chemical signature of GSH.

A fast, reproducible and sensitive measurement of total mercaptans in general and GSH in particular, requires only one sampling and two colorimetric determinations.

16.20 SYNERGISTIC ANTIOXIDANT EFFECTS OF ß-CAROTENE AND α-TOCOPHEROL IN A MEMBRANE MODEL

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Rat liver microsomal membranes were incubated at 37°C under air with either NADPH/ADP/Fe^{+3} or with 2,2'-azobis(2-amidinopropane) (AAPH), and the antioxidant activities of ß-carotene and α-tocopherol, individually or in combination, were studied. Lipid peroxidation was monitored by MDA formation. ß-carotene, added individually at the concentration of 10 nmoles/mg protein, although consumed, was not effective as an antioxidant using 25 mM AAPH, while it produced a significant inhibition of MDA formation using NADPH/ADP/Fe^{+3}. α-Tocopherol added individually at the concentration of 6 nmoles/mg protein, effectively suppressed MDA formation and produced a clear lag time using either NADPH/ADP/Fe^{+3} or AAPH as prooxidants. The addition of both the prooxidants also resulted in the loss of added α-tocopherol. The combination of ß-carotene and α-tocopherol, at the same concentrations used individually, resulted in an inhibition of MDA formation that was significantly greater than the sum of the inhibitions observed individually. The presence of both the antioxidants induced an α-tocopherol consumption which was higher than the consumption of α-tocopherol alone. ß-carotene consumption did not change significantly in the presence of α-tocopherol. These data provide the first evidence that ß-carotene can act synergistically with α-tocopherol as an effective radical-trapping antioxidant in a membrane model.

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16.21 EFFECT OF OXIDATIVE STRESS ON SYNTHESIS OF EXTRACELLULAR-SOD AND OTHER SOD ISOENZYMES IN HUMAN DERMAL FIBROBLASTS.

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Oxidative stress was induced by a variety of agents; by xanthine oxidase-hypoxanthine, via redox-cycling and autoxidation of paraquat and pyrogallol, via GSH depletion by buthionine sulfoximine and diethylmaleate, by Fe and Cu ions in excess, by tertbutylhydroperoxide and cumene hydroperoxide and by 90% oxygen. Protection was also enhanced by selenite, citiolone, SOD, and catalase. The effect of the factors on cultured fibroblasts was tested in a wide range of doses for periods up to 4 days. With EC-SOD and to less extent CuZn-SOD, depressions of synthesis were seen at high doses, apparently because of toxic effects. No stimulating effects were observed. With some of the agents at intermediate doses; xanthine oxidase, paraquat, pyrogallol, and high PO2 about 2-fold increases in Mn-SOD activity were seen. Compared with inductions in Mn-SOD produced by IL-1 and TNF, 30- to 50- fold, the effects are minor and may be due to unspecific interferences with the transscriptional machinery, which may involve a labile protein depressor. We have previously shown that EC-SOD synthesis is fundamentally influenced by inflammatory cytokines (S. L. Marklund (1992) J. Biol. Chem., in press). We suggest, based on the present data and the previous study, that in mammals EC-SOD and Mn-SOD are regulated by cytokines on the tissue level and not directly on the cellular level by oxidative stress, and that none of these influences affect the CuZn-SOD activity. The regulation is thus different from that of SOD's in unicellular organisms, which respond directly to oxidative stress.

16.22 CHARACTERIZATION OF CHO CELL VARIANTS RESISTANT TO HYDROGEN PEROXIDE*

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Hydrogen peroxide resistant sublines of Chinese Hamster Ovary (CHO) cells were selected by continuos in vitro exposure to the oxidant. Stepwise increase in hydrogen peroxide concentration produced variants which were progressively more resistant to the growth inhibitory effect elicited by the oxidant. These cells displayed a different morphology when compared to the parental cell line and the higher cell volume and total protein content were accompained by an increased ratio nucleus/cytoplasm. Cytoscheletal proteins were also higher and actin was overexpressed (about 10 fold) in resistant cells. Light and electron microscopy analysis has suggested that cell variants had an increased number of nucleoli. Wild type cells were slightly more sensitive to the DNA damaging action elicited by the oxidant, although the different sensitivities to the induction of DNA single strand breakage appeared related to the differential depletion of H_2O_2, rather than being dependent on different velocities in DNA repair processes. On the other hand, catalase activity and NPSH levels did not show large variations in resistant sublines with respect to the parental cell line. The differences in cell size and the amount of total cell proteins of the sublines seemed to account for the small changes in catalase activity and NPSH levels observed in the resistant cells. Results obtained in this study suggest that resistance to hydrogen peroxide is associated to marked variations in cell morphology and certainly cannot be fully explained on the basis of an increased capacity of cell variants to metabolize the oxidant.

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16.23 CHARACTERIZATION OF CUPROZINC SUPEROXIDE DISMUTASE FROM PLANT PEROXISOMES

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In previous works we carried out with isolated peroxisomes from watermelon cotyledons, the presence of a soluble Cu,Zn-SOD was demonstrated (1,2), and this enzyme has been recently purified and partially characterized (3).

The determination of the amino acid composition of peroxisomal Cu,Zn-SOD showed a lower histidine and a higher tyrosine content than most Cu,Zn-SODs. The amino acid sequence of the N-terminal region of peroxisomal Cu,Zn-SOD was determined and compared with that of Cu,Zn-SODs from different cellular loci. A polyclonal antibody against Cu,Zn-SOD from watermelon peroxisomes was raised in rabbits. The IgG fraction was purified by ammonium-sulfate precipitation and FPLC with a Mono Q column. The antibody had a high affinity and was monospecific by Western blotting. The immunological cross-reactivity of peroxisomal Cu,Zn-SOD antibody to different cell-free extracts and pure SODs was studied by SDS-PAGE and Western blotting.

(1) J. Plant Physiol. 127, 395-409 (1987)
(2) Plant Physiol. 88, 1215-1218 (1988)
(3) Plant Physiol. 98, in press (1992)

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16.24 DI- AND POLYAMINES AS ANTIOXIDANTS

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Antioxidative properties of putrescine, spermidine and spermine were tested with in vitro models at three concentrations.

/i/ First it was studied, how the polyamines mentioned influence the filtration parameters /Fi/ and biochemical parameters in human red blood cells /RBCs/.

/ii/ The question also arose how these polyamines influence the initiated LP of pig brain homogenate.

/iii/ Moreover it was studied how diamine and polyamines influence guinea pig hearts LP.

16.25 GLUTATHIONE-S-TRANSFERASE SUBUNITS IN CELLS OF EPITHELIAL ORIGIN ISOLATED FROM RAT LIVER. CONSTITUTIVE AND INDUCIBLE ISOZYME PROFILE.

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Cytosolic glutathione transferases (GSTs) are a family of multifunctional enzymes involved in phase II metabolism of xenobiotics. In this paper we present a complete analysis of GST subunits in cytosolic fractions obtained directly from hepatocytes and biliary epithelial cells (BEC) isolated from rat liver. This approach, that avoids contamination by GSTs from liver cells of mesenchymal origin, gives the following major information: a) hepatocytes exhibit the complete pattern of subunits belonging to alpha and mu classes but almost undetectable levels of subunit 7 (pi class); b) BEC have a distinctive constitutive pattern comprising high levels of subunit 7 (pi class), subunits 2 (alpha class) as well as 3 and 4 (mu class); c) hepatocytes show a clear induction profile of alpha and mu class subunits after phenobarbital and β-naphthoflavone; induction is much less evident in BEC; d) BEC and hepatocytes show a clear induction of all constitutive subunits after dietary ethoxyquin supplementation with special reference to subunit 7.

A NUCLEAR POOL OF GLUTATHIONE IN EUKARIOTIC CELLS 16.26

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Using the compound monochlorobimane (BmCl) that specifically (via glutathione transferases) interacts with glutathione (GSH) to form a highly fluorescent adduct, the intracellular distribution of GSH has been investigated in living cultured rat hepatocytes. Image analysis of BmCl-labelled hepatocytes revealed a predominant localization of the fluorescence in the nucleus with a nuclear-cytoplasmic concentration gradient of approximately three. This concentration gradient was collapsed by treatment of the cells with ATP-depleting agents, suggesting the existence of an active (ATP-dependent) mechanism regulating the accumulation of GSH in the nucleus. In time-course experiments using BmCl-labelled hepatocytes, a progressive redistribution of the fluorescence (and hence of the BmCl-GSH adduct) from the nucleus to the cytoplasm was detected. Using hepatocyte couplets, where a specialized canalicular space is retained, a subsequent extrusion of the fluorescence in this compartment occurred. Taken together these results suggest the existence of a multicomponent defense system within the nucleus composed by (1) GSH, (2) GSH-transferase(s) and (3) a GSH-conjugate exporting process. This system and the plasma membrane GSH-conjugate translocator would cooperate in protecting DNA and other nuclear structures from oxidative and chemical injury.

Session 17

Inflammation

17.1 ROLE OF NEUTROPHIL-DEPENDENT OXYGEN RADICAL IN INFLAMMATORY REACTIONS AND CANCER TREATMENTS

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In response to activation of neutrophils by particular or specific soluble mediators, these cells undergo a respiratory burst. Generation of reactive oxygen species from activated neutrophils are modulated by several chemical mediators (arachidonic cascade products, PAF, TNF, IL, G-CSF, etc.), and these active species are important mediators of both normal tissue injury during inflammation and tumor cell killing during cancer treatments. Lipid peroxidation induced by oxygen radical is believed to be one of the major causes of cell damage and injuries of cell membranes. Oxygen radical scavengers attenuate acute inflammatory gastric mucosal injuries and TNB-induced colitis which is a model of chronic inflammatory bowel diseases, and both injuries are significantly inhibited in neutrophil-depleted rats administered with anti-neutrophil serum (ANS). These scavengers and ANS treatment also inhibited the increase in lipid peroxides in the gastric and colon mucosa. These result indicates that oxygen radicals generated from neutrophils and lipid peroxidation are implicated in these inflammatory disease. In addition, we report that neutrophil-dependent oxygen radicals and lipid peroxidation play an important role in the antitumor effect of intra-arterial injection of the transient embolus, anticancer drugs, and hyperthermia.

REDOX MECHANISMS IN T CELL ACTIVATION 17.2

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A number of antioxidants (here defined as agents that inhibit the formation of, or preferentially react with, radical species) inhibit T cell proliferation in vitro. These include cysteamine, desferrioxamine, ferricyanide, nordihydroguaiaretic acid (NDGA) and ebselen. In T cells loaded with dichlorofluorescin, addition of PMA leads within an hour to the formation of dichlorofluorescein, presumably indicating intracellular peroxide formation. This is prevented by cysteamine. These data, together with others, have been taken to indicate the necessity for an oxidative step in the early stages of T lymphocyte activation. The nature of this oxidative signal, and its integration with other biochemical processes and with "activation genes", is now under investigation in this laboratory.

The anti-oxidants do not interfere with the delivery of mitogenic signals to the cell. However, they do inhibit (but not completely block) cell surface expression of receptors for interleukin 2 and transferrin. Desferrioxamine, ferricyanide and NDGA strongly inhibit the increase in ornithine decarboxylase (ODC) activity that follows mitogenic activation of T cells but do not prevent the increase in ODC mRNA levels, suggesting that their effect is exerted post-transcriptionally. Whether this inhibition of ODC activity is critical in the anti-proliferative effects of these agents in this system is not clear.

Recent reports of antioxidant-inhibitable activation of transcription factors by H_2O_2 suggest a mechanism through which our observations can be explained.

17.3 EXPRESSION OF HUMAN EC-SOD IN PANCREATIC β-CELLS OF THE NON-OBESE DIABETIC (NOD) MOUSE

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To study the putative role of EC-SOD in inflammatory processes we wanted to in vivo increase the local concentration of EC-SOD in an organ affected by inflammation. The non-obese diabetic (NOD) mouse which is established as a model of human type 1, insulin dependent diabetes, is an attractive experimental system for such studies. The NOD mice spontaneously develop diabetes and the underlying cause is the destruction of the pancreatic β-cells. Overt diabetes is preceded by a period in which lymphoid cells infiltrate the pancreas but remain outside the islets (periinsulitis) and by a period of insulitis, i.e. infiltration of mononuclear cells into the islets of Langerhans and destruction of insulin producing β-cells. In addition, the NOD mouse shows a tendency for inflammation of certain exocrine glands because salivary, lacrimal, peribronchial and vaginal glands are consistently affected by cellular infiltrates. Thus the NOD mouse constitutes a model not only for diabetes but also for inflammation in general. The β-cells are also unusually sensitive to oxygen radicals and experimental diabetes induced by alloxan can be prevented by administation of Cu/Zn SOD. To create a NOD mouse expressing EC-SOD in pancreatic β-cells, we linked the EC-SOD cDNA to the insulin 5' regulatory region. Due to the heparan sulphate binding of EC-SOD this should render high local concentrations of EC-SOD in the islets of Langerhans. We now have a transgenic line of mice that by elisa shows human EC-SOD in plasma and the level of EC-SOD is increased upon injection of heparin. Immunostaining of pancreas also shows expression of human EC-SOD restricted to the islets of Langerhans. It is now possible to study the role of oxygen radicals in the development of periinsulitis, insulitis and diabetes with or without induction by alloxan.

IDENTIFICATION OF THE REACTION PRODUCTS OF UNSATURATED FATTY ACIDS WITH THE NEUTROPHIL OXIDANT, HYPOCHLOROUS ACID. 17.4

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Hypochlorous acid (HOCl) is a highly reactive oxidant and is thought to play an important role both in microbial killing and inflammatory tissue injury by neutrophils. Although it has been observed that HOCl inhibits neutrophil-mediated lipid peroxidation, suggesting that it causes lipid modification other than peroxidation, surprisingly little attention has been given to its reaction with membrane lipids.

We have studied the reaction of HOCl with oleic acid (18:1), linoleic acid (18:2), and arachidonic acid (20:4), both as free fatty acids (in micellar form) and esterified in phosphatidylcholine (PC) (in vesicular form). The reaction mixtures were analyzed by gas chromatography-mass spectrometry of the methylated and trimethylsilylated derivatives. The reaction products were identified as chlorohydrin derivatives of the corresponding fatty acids. Addition of HOCl to a fatty acid double bond yielded a mixture of stereoisomeric products. At high HOCl : fatty acid ratios (> 0.5), two or more double bonds were found to be modified in the case of 18:2 and 20:4.

Experiments with PC vesicles containing equimolar amounts of 18:1, 18:2, and 20:4 as the sn-2 fatty acid of PC revealed that these three fatty acids have comparable reactivities towards HOCl. When HOCl was produced *in situ* by the neutrophil enzymatic system myeloperoxidase/H_2O_2/Cl^-, the same fatty acid chlorohydrins were formed as when HOCl was added as such. Lipid chlorohydrins are more polar than native fatty acids, and if formed in cell membranes could cause disruption of membrane structure. Since cellular targets for HOCl appear to be membrane constituents, chlorohydrin formation from unsaturated fatty acids could be significant in neutrophil-mediated cytotoxicity.

17.5 OXIDATION OF PLASMA CONSTITUENTS BY HYPOCHLORITE

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Hypochlorous acid is produced by myeloperoxidase. Halliwell et al. have previously suggested that albumin and ascorbate are important antioxidants protecting against HOCl, but the integrated defenses against this molecule have not been explored. Freshly prepared human plasma was incubated with 0, 50, 100, 200 and 500 μM NaOCl at 37°C for 30 min with shaking. Lipid hydroperoxide and protein SH were determined immediately following incubation. Remaining samples were frozen at -20°C for determination of ascorbic acid, uric acid, protein carbonyls and thiobarbituric acid reactive materials (TBARS). NaOCl caused a dose-dependent decrease in plasma SH with 75% loss of SH at 500 μM NaOCl and a 50% decrease at approximately 200 μM NaOCl. Ascorbic acid decreased with increasing NaOCl concentration with 78% loss at 500 μM NaOCl. Uric acid was only slightly decreased (11% at 500 μM NaOCl). There was no increase in protein carbonyls over the control levels. There was no detectable lipid hydroperoxide as determined by the HPLC post-column chemiluminescence technique (detection limit 30 nM hydroperoxide) even at 500 μM NaOCl. NaOCl also did not affect TBARS. The results indicate that NaOCl oxidizes proteins -SH groups rather than lipids. That ascorbic acid but not uric acid was decreased by NaOCl suggests that ascorbic acid may be protective against NaOCl toxicity.

17.6 COLLAGEN BREAKDOWN BY HYPOCHLOROUS ACID AND N-CHLOROAMINES: POSSIBLE ROLE IN SYNOVITIS

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We have tested the effects of the neutrophil/macrophage products, hypochlorous acid (HOCl) and N-chloroamines on the structural integrity and proteolytic susceptibility of collagen to determine if these agents could play a role in inflammatory joint destruction. Rates of HOCl reaction with collagen, and collagen gelation were monitored by spectrophotometric methods. Direct fragmentation, and degradation by collagenase were measured by the release of acid-soluble counts from [^{3}H]collagen, electrophoresis gels, thin-layer chromatography, and fluorescamine reactivity. Physiologically relevant concentrations of HOCL (5-50μM) reacted rapidly and quantitatively at several sites in the collagen polypeptide chain, causing extensive protein fragmentation and preventing collagen gelation. In contrast, reaction with (5-50μM) N-chloroamines induced little or no direct collagen fragmentation. N-chloroalanine and N-chloroleucine did, however, increase the susceptibility of collagen to degradation by collagenase by as much as three-fold. Interestingly, N-chlorotaurine had no effect on the proteolytic susceptibility of collagen. HOCl treatment of collagen produced a mixed profile of effects on proteolytic susceptibility; probably because some of the products were improved substrates for proteolysis and some were inhibitors of collagenase.

It is known that HOCl can directly activate neutrophil procollagenase. We now report that N-chlorotaurine (but not other N-chloroamines) directly inhibits bacterial collagenase. Inhibition of collagenase by N-chlorotaurine may provide a mechanism for minimizing inflammatory joint damage if the mammalian enzyme mimics its bacterial cousin.

17.7 REACTIVE OXYGEN FORMATION IN MURINE AND HUMAN T LYMPHOCYTES.

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Oxidation of dichlorofluorescin (DCFH) to the fluorescent compound dichlorofluorescein (DCF) has been used previously to demonstrate reactive oxygen formation in neutrophils (Bass *et al.*, 1983 J. Immun. 130, 1910-1917). By flow cytometry we have shown that, in human and murine lymphocytes loaded with DCFH, phorbol myristate acetate (at mitogenic concentrations) induced an increase in DCFH oxidation as demonstrated by an increase in DCF fluorescence. By combining this procedure with cell-surface labelling of lymphocyte antigens, we have demonstrated that T lymphocytes from both species possess the capacity to oxidise DCFH. Certain antioxidants that inhibit lymphocyte proliferation (ebselen, diphenylene iodonium, nordihydroguaiaretic acid, and desferrioxamine) have been tested for their ability to block the increase in DCF fluorescence. Of these, only the iron chelator desferrioxamine did not interfere nonspecifically with the fluorescent signal. Desferrioxamine was found to block DCFH oxidation in murine but not human lymphocytes.
The observation that a T cell mitogen can induce intracellular reactive oxygen formation provides further support for the existence of a positive role for oxidative events in T lymphocyte activation.

17.8 SUPEROXIDE GENERATION AND SCAVENGING ACTIVITY BY LEUKOCYTES IN RHEUMATOID ARTHRITIS AND BEHCET'S DISEASES

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The superoxide ($O_2^{\cdot-}$) generation of polymorphonuclear cells (PMN) and the superoxide scavenging activity (SSA) of PMN, mononuclear cells (MNC) & plasma were measured in 45 patients with rheumatoid arthritis (RA) and 23 patients with Behcet's disease (BD) by using the highly sensitive and specific MCLA-dependent chemiluminescence and electron spin resonance/spin trapping methods. Since many, slow-acting anti-rheumatic drugs (SARDs) used in RA and BD, may alter the $O_2^{\cdot-}$ metabolism, the effects of SARDs on SSA were also studied. The $O_2^{\cdot-}$ generation by PMN was significantly increased, while the SSA of PMN and plasma was decreased both in RA and in BD patients as compared those from healthy controls. A negative correlation was also found between the SSA of plasma and erythrocyte sedimentation rates in both diseases. The SSA of plasma and PMN were significantly higher in patients treated with SARDs than those without. None of the SARDs examined could scavenge $O_2^{\cdot-}$ at concentrations reported in patients' plasma. The results indicate that the decreased levels of SSA in plasma and PMN are not disease specific and associated with the disease activity and/or the enhanced $O_2^{\cdot-}$ generation by PMN. SARDs examined, express their anti-inflammatory effects indirectly, by blocking the $O_2^{\cdot-}$ generation of PMN.

17.9 ALTERED OXIDATIVE METABOLISM IN PMNLs OF ELDERLY AND PATIENTS SUFFERING FROM ARTERIOSCLEROSIS

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The respiratory burst (RB) plays an important role in the defense mechanism of the organism against invaders. We know that in some pathological state as well as in aging the incidence of infections and tissue destruction are increasing. We measured the production superoxide anion and hydrogen peroxide in sera, in resting and in stimulated PMNLs of healthy young /< 25yrs/, of healthy elderly /> 65yrs/, and of middle-aged /35-59yrs/ and elderly /> 60yrs/ arteriosclerotic subjects. In resting state the RB was increased with aging and in arterisoclerosis independently of the patient age. Under stimulations (opsonized zymozan, FMLP, carbachol) through various receptors we found a clear decrease in the stimulation of the RB with aging and in arteriosclerosis, while it remained unchanged on the effect of non-specific stimulation (A23187, PMA) comparing to young subjects. To elucidate the tranmembrane signalling mechanism involved in respiratory burst stimulation, various inhibitors were used as follows: neomycin (for phospholipase C enzyme), mepacrine (for phospholipase A_2 enzyme) and pertussis toxin (for Gi protein). The results suggest that phospholipase C as well as phospholipase A_2 could be involved in the transmembrane signalling mechanism, depending on the stimulus, but the impairment of the pertussis toxin sensitive GTP binding G protein with aging and with arteriosclerosis might explain, at least in part, the decreased response of the RB after stimulating the different receptors. Our results suggest that we assist to similar tranmembrane signalling alteration in aging and in arteriosclerosis, the arteriosclerotic process might appear as an early aging process.

17.10 CHEMILUMINESCENT TECHNIQUES IN STUDIES OF THE RADICAL REACTIONS IN LABORATORY AND CLINICAL INVESTIGATIONS.

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There are a variety of instruments and experimental procedures for chemiluminescence (CL) measurements of model biochemical systems, blood components and urine. Cell-electroporating luminometer (EP-CL) has been designed by "BIKAP" group (Moscow) and used in our laboratory. The following series of experiments have been carried out by using this instrument and its predecessors:

1. Studies of CL kinetics in mitochondria and liposomes. Rate constants of Fe^{2+}-induced lipid peroxidation reactions (LPO) have been determined.

2. Discovery of a series of "sensitizes", i.e. compounds enhancing CL intensity in above mentioned systems, including Eu^{3+}-tetracycline, rhodamine G and several laser dyes. Some of these compounds increase CL intensity during LPO reactions by 3-4 orders of magnitude.

3. Diagnostics of early infarction, inflammatory diseases and drug allergy by measuring luminol-dependent CL in blood cells and whole blood after chemical or electrical stimulation.

17.11 NONSTEROIDAL ANTI-INFLAMMATORY AGENTS: IS INHIBITION OF SUPEROXIDE/PEROXIDE GENERATION OR RADICAL SCAVENGING INVOLVED IN THEIR MODE OF ACTION ?

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Nonsteroidal antiinflammatory agents are polycompetent substances that are able to inhibit or modulate more than one molecular or cellular event. Reactive oxygen species (ROS) are presumed to play an essential part in producing tissue damage and inflammation, and there is a large body of papers suggesting an inhibition of generation or scavenging of ROS mainly by nonsteroidal anti-inflammatory agents Thus, these effects might be part of the mode of anti-inflammatory substance activity. There are, however, some unexplained discrepancies which cause some doubt of this view:

-Some substances which are potent inhibitors of the respiratory burst cause only weak or no anti-inflammatory activity (ascorbic acid, tocopherol, D-penicillamine and others).

-We found indeed an inhibition of ROS (generation or scavenging) by nonsteroidal anti-inflammatory agents (Table). However, taken together, we can hardly suggest that this activity is part of their mode of anti-inflammatory action.

Substance	AOA*) ED_{50} mol/l	H_2O_2 IC_{50} mol/l	Carrag.edema ED_{40}; mg/kg 3h
Diclofenac-Na	1.8×10^{-4}	1.1×10^{-5}	1.4 p.o.
BW 755 C	3.5×10^{-6}	9.0×10^{-6}	35 p.o.
Ascorbic acid	5.0×10^{-5}	5.0×10^{-6}	>2000 p.o.
$MnCl_2$	2.3×10^{-4}	35% at 10^{-4}	20 i.v.
Propyl gallate	2×10^{-6}	5×10^{-6}	>250 p.o.

*)Antioxidative activity, ESR technique

17.12 EFFECT OF ZINC-CARNOSINE CHELATE COMPOUND (Z-103) ON GASTRIC MUCOSAL INJURY INDUCED BY HYPOXANTHINE-XANTHINE OXIDASE INFUSION IN RATS

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Z-103 is a chelate compound consisting of zinc ion and L-carnosine. It is known that zinc and carnosine have anti-oxidative effects. In this study, the protective effect of Z-103 on gastric mucosal injury induced by hypoxanthine-xanthine oxidase(HX/XO) infusion was studied in rats. HX was infused through the jugular vein, XO was infused through the left gastric artery at the rate of 40 μl/min for 30 min. Z-103 was administrated 1 hr before a start of infusion. At 30 min after HX-XO infusion, rats were sacrificed. The extent of any gastric mucosal lesions was expressed in a score 0;normal, 1;mild, 2;moderate, 3;severe erosions. Erosions and bleeding were revealed in the gastric mucosa. The score of any lesions was significantly decreased by treatment with Z-103. TBARS in serum and the gastric mucosa after HX/XO infusion showed slight increase, and that tended to be decreased by treatment with Z-103. α-Tocopherol in the gastric mucosa significantly decreased. Z-103 did not inhibit the decrease. GSH-Px and SOD didn't show any changes after HX/XO infusion. GSH and ATP were significantly decreased by HX/XO infusion. Z-103 markedly restored the decrease. Therefore, Z-103 is effective against free-radical induced gastric mucosal injury.

17.13 ROLE OF POLYMORPHONUCLEAR LEUKOCYTES(PMN) AND ACTIVE OXYGEN SPECIES IN HYPERTHERMIA ---ANTITUMOR EFFECT OF HYPERTHERMIA COMBINED WITH G-CSF---

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Cancer is still in the top of the cause of death in Japan. Therefore, it is a dream for clinicians of the free radical damage to tissues can be applied for treatment of cancer. We have reported that polymorphonuclear leukocytes(PMN) and active oxygen species from PMN may play an important role in the mechanism of the antitumor effect of Hyperthermia. In this time, we would focus our experimental studies of rat AH109A carcinoma treated with Hyperthermia combined with arterial injection of G-CSF. Rats with transplantable AH109A carcinoma at the hind leg received Hyperthermia. These tumor showed a mild suppression for the further development only by Hyperthermia. However, when arterial injection of G-CSF was applied in the combination of Hyperthermia, marked suppression on the development of the tumor was observed. Our data suggest that Hyperthermia combined with G-CSF is closely related to the generation of free radical-mediated tumor cell killing, and this treatment can be the effective treatment for cancer.

17.14 ROLE OF POLYMORPHONUCLEAR LEUKOCYTES(PMN) AND ACTIVE OXYGEN SPECIES IN HYPERTHERMIA --- AUGMENTATIVE EFFECT OF G-CSF ON SUPER-OXIDE GENERATION FROM PMN ---

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We examined,in vitro,the effect of G-CSF and temperature on superoxide(O_2^-) generation from PMN. <*Materials and Methods*> 1) Preparation of PMN. PMN were isolated from the peripheral blood of healthy volunteers by dextran sedimentation followed by Ficoll-Paque separation and hypotonic lysis of contaminating erythrocytes. 2) Measurements of superoxide. A Luminescence Reader was used for the assay of CLA dependent chemilumines-cence. The standard reaction mixtures contained $2x10^5$ PMN/ml, 1 μM CLA, 0.1 mM DETAPAC, plus a stimulus in a total volume of 2ml HBSS. A stimulus was added after 3min. preincubation of PMN with G-CSF. The reaction was initiated by the addition of G-CSF at a final concentration of 0.1-1000 ng/ml and at 37 to 45°C temperature. <*Results*> 1) O_2^- generation from PMN was re-markably augmented ,stimulating by both OZ and PMA, at 41°C as compared with 37°C. 2) PMN generated O_2^- at the concentration of G-CSF 25ng/ml or more at 37°C. 3) O_2^- generation was enhanced both at the concentration of 25 ng/ml and at 41°C compared with at 41°C alone. <*Conclusion*> The significant augmentation of O_2^- generation from PMN was observed at 25ng/ml G-CSF and 41 °C.

17.15 PROTECTION BY EBSELEN AGAINST ENDOTOXIN-INDUCED INTESTINAL MUCOSAL INJURY IN RATS

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We have reported that active oxygen species may play an impor-tant role in the pathogenesis of the experimental model of endotoxin shock. Ebselen is a seleno-organic compound which exerts novel biological activities, i.e., gluthathione peroxidase like activity, inhi-bition of NADPH oxidase activity. In present study, the effects of Ebselen on small intestinal mucosal injury induced by endotoxin were investigated in rats. Male Wistar rats weighning 200g were used. Ebselen was orally administered 60 mins before intravenous injection of endotoxin. The small intestinal mucosa was carefully examined macroscopically 45 mins after injection of endotoxin, and alpha-tocopherol, superoxide dismutase, glutathione peroxidase, thiobarbituric acid reactive substances in tissue were measured. Severe intestinal mucosal damage was macroscopically found after injection of endotoxin, and this damage was significantly inhibited by pretreatment with Ebselen. And the levels of alpha-tocopherol, or antioxidant enzymes showed the possibility that Ebselen pro-tected against this injury through its antioxidant function.

17.16 EFFECT OF HISTAMINE ON XANTHINE OXIDASE

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Xanthine oxidase (EC 1.1.3.22) derives from a NAD^+-dependent dehydrogenase (EC 1.1.1.204) by limited proteolysis or by oxidation of thiol groups essential for the dehydrogenase activity. During the oxidation of substrates the enzyme in its O_2-dependent forms generates super-oxide anion and H_2O_2, which in the pre-sence of chelated iron are converted into hydroxyl radical by the Haber-Weiss and the Fenton reactions. These oxygen products aggravate cell damage when xanthine dehydrogenase is converted to an oxidase during several pathological conditions. It has been reported that histamine can enhance xanthine oxidase activity in vitro as well as in vivo. We investigated about the effect of hista-mine on the dehydrogenase and oxidase activity of the enzyme. Xanthine de-hydrogenase was partially purified from rat liver and converted to an oxidase either reversibly by treatment with oxidized glutathione or irreversibly by proteolytic cleavage. Physiological con-centrations of histamine induced a 26% increment of the dehydrogenase activity of native enzyme, a 36% increment of the reversibly converted xanthine oxidase activity and a 67% increment of the irreversibly transformed enzyme.

17.17 HYDROGEN PEROXIDE GENERATION IN HUMAN PLATELETS

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Different effects of hydrogen peroxide on platelet function have been reported: inhibition and activation are both described. Moreover it was shown that human platelets stimulated by particulate stimuli or collagen generate H_2O_2. By means of a sensitive fluorimetric assay we have measured hydrogen peroxide formation, following the oxidation of dichlorofluorescin (DCFH) entrapped into platelets. We have demonstrated that resting platelets produce nanomolar concentrations of DCF, proportional to platelet number and steady during 60 minutes of incubation. A significant increase of basal DCF was generated by stimuli as arachidonic acid, A23187, thrombin or PMA. The effect of these agonists was also tested in the presence of 3-amino-1,2,4,-triazole (AT) or N-ethylmaleimide (NEM), inhibitors of catalase and glutathione peroxidase respectively. A further significant enhancement of DCF produced in stimulated platelets was observed only when NEM was present. Moreover DCF correlates with the externally added hydrogen peroxide or the oxidizing species formed by xanthine oxidase and acetaldehyde. The yield, not modified by superoxide dismutase, is higher in the presence of AT or NEM. A cooperative effect of both inhibitors was shown.

17.18 ACETAMINOPHEN AS A PUTATIVE SCAVENGER OF PEROXYL RADICALS

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Non steroidal anti-inflammatory drugs have been developed mostly directed at pathways of eicosanoid synthesis. However it is possible that such drugs exert multiple actions, namely antioxidant effects, in inflammation processes. Acetaminophen is a widely used non-steroidal anti-inflammatory drug that has been already shown to possess antioxidant activity in vitro. This action was developed on the basis of the thiobarbituric acid test that lacks sensitivity and specificity. The aim of our study attempts to ascertain the action of low concentrations of acetaminophen on membrane lipid peroxidation investigating its potential role as a peroxyl radical scavenger. Sarcoplasmic reticulum membranes (SR) and liposomes of their total lipid extract were used as model membranes to study the drug effect on lipid peroxidation induced by the free radical generating system Fe^{++}/ascorbate. The initial stages of the degradative process were continuously monitored by the fluorescence quenching of *cis*-parinaric incorporated into membranes. Furthermore, lipid peroxidation was evaluated by the degradation of phospholipid aliphatic chains by gas-chromatographic analysis and the production of thiobarbituric acid reactive substances. In order to clarify its antioxidant activity, the putative direct reaction of paracetamol and peroxyl radical involved in lipid peroxidation was assayed by exposing a SR lipid extract to the lipid soluble radical initiator 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN), in the presence of different concentrations of the drug. Lipid peroxidation was quantitatively monitored by the *c,t* and *t,t* hydroperoxidienes formed using second-derivative spectrophotometry, and the peroxyl radicals scavenging activity of paracetamol was assessed as compared with that of α-tocopherol. The observed strong chain breaking activity of paracetamol is likely to contribute to its anti-inflammatory activity.

17.19 AN INVESTIGATION INTO THE MECHANISM OF LYSIS OF RED BLOOD CELLS BY HOCl

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In response to inflammatory stimuli, neutrophils produce HOCl, which can be both microbicidal and cytotoxic. This study was carried out to determine the mechanism by which HOCl causes cell lysis, using red cells as a model.
Reagent HOCl lysed red cells in a dose dependent manner. Lysis was inhibitible by the addition of scavengers such as methionine and taurine, when these were added prior to mixing of the red cells and HOCl. Loss of membrane protein sulphydryls occurred even at sublytic doses of HOCl, and was almost complete before lysis was significant. It is unlikely that -SH oxidation in itself was the cause of lysis since N-ethylmaleimide- or diamide- treated cells lysed only very slowly. SDS-PAGE of the membrane proteins showed that HOCL caused the formation of, firstly reversible, and then irreversible crosslinked material.
Haemolysis was preceded by cell swelling and passive K^+ leak, suggesting that the membrane had lost its impermeability to ions. Analysis of the red cell fatty acids by TLC showed the formation of a more polar species. This may be a chlorohydrin derivative, formed by reaction of HOCl with an unsaturated fatty acid. Our hypothesis is that the formation of such a species leads to a destabilisation of the lipid bilayer, and consequent loss of its barrier function.

17.20 MACROPHAGE DEACTIVATING FACTOR: PURIFICATION AND PARTIAL BIOCHEMICAL CHARACTERIZATION OF A NOVEL CYTOKINE.

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The release of superoxide anion by inflammatory macrophages, multinucleated giant cells and epithelioid cells, obtained by insertion of round glass coverslips into the subcutaneous tissue of mice, was investigated. Superoxide anion was spontaneously released by cells on coverslips implanted up to 7 days, but not 14 to 21 days. When 14 day coverslips were incubated for 30 minutes *in vitro* , the medium so conditioned (**MCM**) inhibited superoxide release by cells of 5-day preparations. This indicates release by cells on longer term coverslips of a substance that inhibits superoxide anion production by cells of 5-day coverslips. This inhibitory factor (**MDF**) was sensitive to proteases, but was heat stable and exerted its effects even when the test cells were exposed to PMA. It is able to regulate superoxide release by neutrophils as well as macrophages. **MDF** is also not species specific and acts on guinea pig, human and mouse neutrophils and macrophages. PMA, all-trans-retinal or fMet-Leu-Phe do not reverse this "deactivation". The factor was purified by ultrafiltration, gel chromatography and reverse phase HPLC. **MCM** shows two peaks of activity when subjected to gel filtration corresponding to molecular weights of 3 and 11kD respectively. Extracts of the cells themselves had only the former peak. Fractions corresponding to a Mw of 3 kD were combined and subjected to HPLC. **MDF** activity then appeared in a single peak. **MCM** thus contains a factor that is able to modulate the cidal activity of phagocitic cells that utilize reactive oxygen species for cytocidal activity.

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17.21 EFFECTS OF COPPER COMPLEXED NIFLUMIC ACID ON RAT POLYMORPHONUCLEAR RESPONSIVENESS

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Previous studies have demonstrated that some copper complexes of non-steroidal anti-inflammatory drugs (NSAIDs) are more effective than their parent drugs as anti-inflammatory and inhibiting agents of migration and oxidative metabolism of polymorphonuclear (PMN) leukocytes. These studies were intended to compare the effects of niflumic acid with those of its copper complexe Cu $(II)_2$ $(niflumate)_4$ on rat PMN reponsiveness. Experiments were performed *in vitro* and *in vivo*. PMN migration was measured with the Boyden chamber using N-formyl-methionyl-leucyl-phenylalanine (fMLP) as the chemoattractant. PMN oxidative metabolism was assessed by O_2^- generation (reduction of ferricytochrome C) and chemiluminescence using opsonized zymosan as stimulus.
A dose related decrease of these parameters was observed with both drugs as well in *in vitro* as *in vivo* experiments. However copper complex was found more effective than its parent drug or Cu gluconate.
It is concluded that modulation of the PMN responsiveness by copper complexes offers an accounting for the anti-inflammatory activity of these compounds.

17.22 CIMETIDINE AND OTHER H_2 RECEPTOR ANTAGONISTS AS POWERFUL HYDROXYL RADICAL SCAVENGERS

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H_2 Receptor antagonists are beneficial in treating gastric and duodenal ulcers by acting as inhibitors of the histamine-stimulated gastric secretion. In this study we report another characteristic of H_2 receptor antagonists, viz. powerful hydroxyl radical scavenging activity.

Hydroxyl radicals are generated in a reaction mixture containing: 100 µM $FeCl_3$, 100 µM EDTA, 100 µM ascorbic acid, 1 mM H_2O_2 and 2.8 mM deoxyribose as the detector molecule. After incubation for 1 hour at 37 °C thiobarbituric acid reactive fragments of deoxyribose, caused by hydroxyl radicals can be measured. Rate constants for reaction of H_2 receptor antagonists as scavengers with hydroxyl radicals are obtained in this assay.

We found that the H_2 receptor antagonists like cimetidine, burimamide, ranitidine, famotidine and tiotidine are good hydroxyl radical scavengers. We observed rate constants ranging from 12.9×10^9 $M^{-1}s^{-1}$ to 14.8×10^9 $M^{-1}s^{-1}$. For the widely applied drug cimetidine we found a rate constant of 14.8×10^9 $M^{-1}s^{-1}$. For comparison, the well-known hydroxyl radical scavenger mannitol had a much lower rate constant i.e. 1.7×10^9 $M^{-1}s^{-1}$.

Using fragments of the cimetidine molecule it was found that the cyano guanidine group is not necessary for the hydroxyl radical scavenging activity. A methylated imidazole with a side chain containing a sulfur and amino group, had a rate constant comparable to cimetidine, i.e. 14.7×10^9 $M^{-1}s^{-1}$. Therefore it was concluded that the imidazole part of cimetidine molecule is responsible for the potent hydroxyl radical scavenging effect. The radical scavenging activity of H_2 receptor antagonists might also contribute to their established pharmacological profile.

17.23 THE EFFECTS OF NIFLUMIC ACID ON POLYPHOSPHOINOSITIDE AND OXIDATIVE METABOLISM IN POLYMORPHONUCLEAR LEUCOCYTES FROM HEALTHY AND THERMALLY-INJURED RATS

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Thermal injury in rats leads to an impairment of polymorphonuclear leucocyte (PMN) functions, particularly oxidative metabolism and phosphoinositide turn-over. As prostaglandin E2, which has immunosuppressive properties, is released in high levels after burn trauma, we investigated the *in vitro* and *in vivo* effects of a non-steroidal antiinflammatory drug, niflumic acid, on oxidative and phosphoinositide metabolism in PMN from healthy and burned rats. Given the role of fluoride ions on PMN, the influence of niflumic acid was compared with that of sodium fluoride (NaF) at equivalent doses of F^-.

In vitro, niflumic acid and sodium fluoride had no effect on oxidative metabolism in stimulated (by fMLP or opsonized zymosan) or non-stimulated PMN from healthy and burned rats. Niflumic acid slightly increased the production of inositol phosphate by non-stimulated PMN from healthy and burned rats. Niflumic acid and NaF partly restored the stimulating effect of fMLP on inositol phosphate production by PMN from burned rats.

In vivo treatment with niflumic acid (0.1 mg to 10 mg/kg) and NaF (0.05 to 5 mg/kg) increased the oxidative metabolism of PMN from burned rats but not healthy rats. Niflumic acid, more than NaF, restored the activity of both stimulants on phosphoinositide metabolism in PMN from burned rats.

In conclusion, at non antiinflammatory doses, yet inhibiting cyclooxygenase activity, niflumic acid exerts a complex effect on the burn-induced depression of PMN functions. The fluoride anion induces similar but generally weaker effects and seems to be involved in the restoring effects of niflumic acid on PMN functions in burned rats.

17.24 MYELOPEROXIDASE-DEPENDENT PROTEOLYSIS AS A CAUSE OF INFLUENZA PNEUMONIA DEVELOPMENT

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The proteolytic cleavage of surface influenza virus glycoprotein - haemagglutinin (HA) is nesessary for virus infection, but the cause of proteolysis activation is unknown. We found out, that bronchoalveolar fluid (BAF) of infected mice contains considerable amounts of myeloperoxidase (MPO) and different proteases. The release of MPO leads to inactivation of antiproteases via generation of HOCl and, therefore, to increasing of proteases in addition to secreted proteases. The treatment of mice with human superoxide dismutase leads to a considerable survival of infected mice and a decrease in proteolytic and MPO activities in BAF. We assume the following steps of reactive oxygen species (ROS) influence on the development of influenza pneumonia:
virus---→ ROS generation by neutrophils (especially, HOCl) ---→ oxidative inactivation of antiproteases---→ proteolytic activity increase ---→ cleavage of HA ---→ virus replication in the host cells.

17.25 DIFFERENT SENSITIVITY OF BOVINE AND HUMAN Cu, Zn SOD TO OXIDATIVE INACTIVATION

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Superoxide dismutase (SOD) is considered as an effective antiinflammatory agent. However, contradictive results were obtained about SOD efficiency from various sources (human, rat, bovine, etc.). Since a high concentration of oxidants into the inflammation site is observed, we studied oxidative inactivation of bovine and human Cu,Zn SOD by hypochlorite (HOCl). HOCl may be considered as the main oxidant in inflammatory locus. We found out that hSOD was more active than bSOD after HOCl incubation. For example, hSOD residual activity was 40% and bSOD one was only 5% after treatment with 100 μM HOCl during 30 minutes. In addition, structural HOCl-induced disorder was developed in a higher degree in the case of bSOD. The advantage in oxidative stability of hSOD could lead to the more effective application of hSOD versus bSOD.

17.26 ACTIVATION OF HUMAN PMN BY 4-HYDROXYNONENAL

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The lipid peroxidation product 4-hydroxynonenal (HNE) is chemokinetic for human PMN leukocytes. The possibility that this activity is mediated by specific binding sites on PMN was investigated. Results showed that [3H]-HNE binding to PMN is a complex process. HNE binds nonspecifically to many SH-groups of the cells. When SH-groups are completely blocked with NEM, it is possible to show the presence of saturable, specific binding sites (Kd=319 nM). The binding is partly reversible. Competition tests with various aldehydes showed the following order of potency as displacers: 2-nonenal > HNE > 4-hydroxyhexenal > nonanal. Under the same experimental conditions (NEM preincubation) the chemokinetic activity of the same aldehydes was measured. The biological potency of the aldehydes as chemokinetic agents correlated with their potency as displacers.

17.27 EFFECTS OF 4-HYDROXYNONENAL (HNE) ON RAT NEUTROPHILS: SECOND MESSENGERS MEDIATION

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HNE is a reactive aldehyde derived from the non enzimatic brea kdown of arachidonic acid. It has been identified at micromolar concentrations in exudates formed during various experimental inflammatory reactions. "In vitro" studies showed that HNE exerts different effects on rat neutrophils (PMN) according to the concentrations tested. 10^{-4} M HNE inhibits PMN functions without affecting cell viability; whereas in the micromolar range it induces PMN morphological polarization and oriented migration. At chemotactic concentrations (10^{-5}-10^{-7} M) no stimulation of respiratory burst is observed. The aim of our study was to investigate more closely the effects and mechanisms of HNE action. Previous data showed that HNE stimulates phospholipase-C activity; present data evidence also an increased adenylate cyclase activity in PMN plasmamembranes treated with micromolar HNE concentrations. These results suggest that HNE chemotactic activity might be mediated, like that of other more well known chemoattractants, by the stimulation of second messangers production.

17.28 THE INVOLVEMENT OF OXYGEN RADICALS IN ACUTE PANCREATITIS

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Purpose of the study was to assess the involvement of oxygen radicals in [illegible] and hemorrhagic pancreatitis. Acute pancreatitis (AP) was induced in rats by cerulein (5μg/kg/h) and by retrograde injection of 5% sodium- taurocholate for 30 min, 3.5 hours, and 12 hours. At these time points serum enzymes, conjugated dienes (CD), and malondialdehyde (MDA) in the tissue were measured and the tissue samples underwent light-microscopical examination. In both models of AP the serum enzyme levels, CD and MDA, increased already at an early phase followed by severe histological damages. Treatment with superoxide dismutase (100.000 U/kg/h) and catalase (400.000 U/kg/h) prevented increase in CD and MDA, reduced the histological damages and improved the survival rate of the rats. It is concluded that oxygen radicals seem to be instrumental for the development of AP.

17.29 STUDY OF THE ANTI-INFLAMMATORY ACTIVITY OF SOME FLAVONOIDS FROM CLERODENDRUM INDICUM L

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In continuation of our work on new plant drugs, we have isolated 4 active principles: hispidulin (6-OCH3-apigenin), nepetin (6-OCH3-luteolin), scutellarein (6-OH-apigenin) and scutellarein-7-glucuronide, from an Indian medicinal plant Clerodendrum indicum L. (Verbenaceae) using DCCC and their structures were characterized by spectral methods. Flavonoids were assayed in two different models: carrageenan mouse paw oedema (1) and tetradecanoylphorbol acetate (TPA) -induced mouse ear oedema (2). Flavonoids or vehicle were administered orally at a dose of 150 mg/kg, 1 hr before induction of inflammation. Mouse ear oedema was induced by TPA (2.5 µg/ear) dissolved in acetone. Drugs were administered topically at a dose of 1 mg/ear in acetone or in 80% EtOH. Subplantar injection of carrageenan in mouse caused a time-course increase in paw swelling which was maximum at 3 hr. Oral administration of nepetin and scutellarein produced an inhibition higher than 50%, quite similar to that produced by indomethacin (56%). The percentage inhibition of ear oedema was found to be over 70% for all drugs assayed. In this model, the same behaviour has been observed as nepetin and scutellarein have manifested the highest anti-inflammatory activity. Based on the results obtained with scutellarein and scutellarein-7-glucuronide, it seems that glucuronic acid substitution reduces the inhibitory effects. On the other hand, catechol or pyrogallol groups are structural features relevant for the anti-inflammatory activity.

(1) Sugishita, E., Amagaya, S., Ogihara, Y. J. Pharmacobiodyn. 4, 565 (1981). (2) De Young, L.M., Kheifets, J.B., Ballaron, S.J., Young, J.M., Agents Actions, 26, 335 (1989).

17.30 ANTI-INFLAMMATORY EFFECTS OF SOME FLAVONOIDS ISOLATED FROM INDIAN MEDICINAL PLANTS

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Three flavonoids isolated from Indian species: hibifolin from *Hibiscus vitifolius* (Malvaceae), kaempferol-3-galactoside from *Pterospermum suberifolium* (Sterculiaceae) and morelloflavone from *Garcinia spicata* (Clusiaceae) have been tested to evaluate their possible anti-inflammatory activity. These compounds have been isolated using different chromatographic techniques including DCCC. Identification of these flavonoids have been carried out by UV-Vis, ^{13}C-NMR, and ^{1}H-NMR spectral analysis. Flavonoids were assayed on carrageenan mouse paw oedema (1) and on tetradecanoylphorbol acetate (TPA) -induced mouse ear oedema (2). Flavonoids or vehicle were administered orally at a dose of 150 mg/kg. Indomethacin was used as reference drug at a dose of 5 mg/kg. Oedema was measured at 1, 3, and 5 hr after induction of inflammation and was found to be maximum at 3 hr. Mouse ear edema was induced by TPA (2.5 µg/ear) dissolved in acetone. Drugs were administered topically at a dose of 1 mg/ear in acetone or in 80% EtOH. A reference group was treated with indomethacin (0.5 mg/ear). Oedema was measured 4 hr after TPA delivery. Kaempferol-3-galactoside and morelloflavone have shown the highest effect on the paw oedema, with percentages of inhibition over 50%. However, it is noteworthy that on TPA-induced ear oedema all compounds manifested a powerful inhibition with percentages close to 100% and thus, these flavonoids can be considered as effective topical anti-inflammatory agents.

(1) Sugishita, E., Amagaya, S.; Ogihara, Y. *J. Pharmacobiodyn. 4*, 565 (1981). (2) De Young, L.M., Kheifets, J.B., Ballaron, S.J., Young, J.M. *Agents Actions, 26*, 335 (1989).

17.31 "OXIDATIVE DAMAGE TO CONNECTIVE TISSUE: THE ROLE OF FREE RADICALS, COPPER(II) IONS, HYDROGEN PEROXIDE AND SALT"

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Copper(II) ions are diversely reported both to enhance and inhibit oxidative damage to biological systems. Superoxide radicals are produced in living tissues and are likely initiators of oxidative damage. Hyaluronic acid (HA) is a major constituent of connective tissue and in certain disease states undergoes free-radical mediated degradation. Copper(II) ions could influence free radical-mediated damage of HA by (a) complex formation with superoxide anion radicals, (b) oxidation of hyaluronic free radicals and (c) thermal reaction with hydrogen peroxide. Absorption spectrophotometry was used to provide binding constants for Cu(II)-HA complexes (apparent K=1200 M^{-1}) Pulse radiolysis studies showed that an intermediate, CuO_2^+, is formed when $O_2^{\cdot-}$ reacts with Cu(II). CuO_2^+ subsequently decays unimolecularly (k= 2.5 x $10^4 s^{-1}$) presumably to form Cu(I) and O_2. When HA free radicals are formed by $^{\cdot}OH$ attack in pulse radiolysis studies, Cu(II) ions were shown to reduce the yield of strand breaks by a factor of 2 relative to a system containing no Cu(II). Finally, the reaction of hydrogen peroxide with HA was shown to occur in the presence of Cu(II) leading to chain breakage of HA. The rate was considerably enhanced in the presence of chloride ions.

17.32 XANTHINE OXIDASE-DERIVED FREE RADICALS IN CYTOKINE-MEDIATED PATHOLOGIES

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Proinflammatory cytokines, particularly interleukins and tumor necrosis factor (TNF) are pathogenetic mediators of infection and inflammation, where free radicals are also overproduced. We found that a glutathione precursor (N-acetylcysteine) can inhibit endotoxin-induced TNF production in vivo and protect against endotoxin-induced pulmonary edema. On the contrary, inhibitors of glutathione synthesis potentiate TNF production. This suggests that free radicals constitute a second messenger system in the production of TNF. Furthermore endotoxin administration induces xanthine oxidase (XO), a potent free radical producer, in most tissues. This induction is mediated by another cytokine, interferon, and is induced by administration of recombinant interferons. Allopurinol, a XO inhibitor, protected against endotoxin-induced pulmonary edema in vivo, and against the increase in endothelial cell permeability induced by endotoxin in vitro.

In conclusion, free radicals play a role as second messengers of cytokine action, in addition to being involved in their production.

Session 18

Reactive Species in Metabolic Disorders

18.1 FREE RADICAL PRODUCING REACTIONS OF Fe2+ IONS IN BIOMEMBRANES AND LIPOPROTEINS

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Fe^{2+} ions are known to react with hydrogen and lipid peroxides to produce free radicals. Fe^{2+} autooxidation by dioxygen is accelerated by many compounds including desferrioxamine and was found to be followed by formation of superoxide. In reaction of Fe^{2+} ions with hypochloric acid, hydroxyl radicals were formed as indicated by spin traps and chemiluminescence measurements. In hydrophobic phase of biomembrane lipid layer free radicals mediate chain peroxidation reactions, which kinetics and mechanism can be studied by measuring chemiluminescence. Fe^{2+} ions are prooxidants or antioxidants depending on iron concentration. The rate of interaction of Fe^{2+} dissolved in aqueous phase with NO-radicals of spin-labelled fatty acid chains inside the lipid layer was measured by monitoring the EPR signal disappearance. This rate decreased in the presence of cholesterol and increased after lipid peroxidation in a close parallelism with membrane polarity rather than membrane fluidity. Apparently Fe^{2+} ions can penetrate inside lipid layer and interact there with either lipid hydroperoxides (producing new radicals) or with lipid radicals (so inhibiting chain peroxidation reactions).

18.2 OXIDATIVE STRESS IN ISCHEMIA-REPERFUSION.

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Oxidative stress, defined as an increase in the physiologic steady-state concentrations of active oxygen species ($O_2^{\cdot-}$, H_2O_2, $HO^{\cdot}$, $ROO^{\cdot}$ and $^1O_2$), occurs upon reperfusion of ischemic tissues. Organ chemiluminescence *in situ* showed overshoots of 20 to 60% over the normal stable photoemission 2 to 5 min after reperfusion in rat liver and intestine and in rabbit heart. The phenomenom was also recorded in isolated cells of the amoeba A castellanii. Organ ischemia lasted from 15 min (heart and intestine) to 60-180 min (liver). Tissue chain-breaker antioxidants (retinoids and tocopherols) determined by hydroperoxide-initiated chemiluminescence, decreased after reperfusion in rat liver and human heart, indicating oxidative stress and correlating with mitochondrial structural damage. In rat liver, ischemia-time correlated with the decrease in mitochondrial state 3 respiration and with LDH release, morphological changes and edema (water content). Intracellular H_2O_2 steady-state levels determined in liver slices were increased after ischemia-reperfusion. Allopurinol treatment did not alter the increase in intracellular H_2O_2 concentration following ischemia-reperfusion. Phenobarbital treatment did not alter H_2O_2 concentrations, but increased cytochrome P-450 level, *in situ* organ chemiluminescence and LDH release, indicating a role of cyt. P-450 in H_2O_2-mediated cytosolic injury. Oxidative stress during reperfusion seems a consequence of the increase in the rate of $O_2^{\cdot-}$ and H_2O_2 production by inhibited mitochondria. Conversion of xanthine dehydrogenase to oxidase, inactivation of antioxidant enzymes and PMN infiltration appear as amplifying events that follow to the primary mitochondrial phenomena.

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18.3 THE EFFECT OF ASCORBATE AND DESFERRIOXAMINE TREATMENT ON OXIDATIVE STRESS IN DIABETES

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Increased oxidative stress may contribute to the development of complications in diabetes mellitus. In the streptozotocin diabetic rat untreated diabetes is characterised by increased lipid peroxidation as shown by increased levels of plasma malondialdehyde (MDA) [diabetic 1.92 ± 0.21 μmol/l (n = 11, mean ± SEM) vs control 1.03 ± 0.23 μmol/l (n = 9), $p < 0.01$] and conjugated dienes (CD) [0.595 ± 0.007 U vs 0.540 ± 0.010 U, $p < 0.01$]. In addition, there were reduced levels of ascorbate (40.6 ± 5.6 vs 67.2 ± 5.7μmol/l, $p < 0..01$), retinol (0.98 ± 0.12 vs 1.39 ± 0.14μmol/l, $p < 0.05$) and tocopherol (18.0 ± 1.5 vs 28.4 ± 1.6 μmol/mmol cholesterol, $p < 0.01$). Insulin treatment returned all of these parameters to normal. Ascorbate supplementation restored antioxidant vitamin status to normal but failed to reduce lipid peroxidation [MDA 2.10 ± 0.25 μmol/l, CD 0.620 ± 0.008 U (n = 9), p = NS vs untreated diabetes], probably due to a balance between antioxidant and pro-oxidant effects mediated by interactions with transition metals. In support of this, the addition of daily subcutaneous desferrioxamine to oral ascorbate treatment significantly reduced lipid peroxidation [MDA 1.27 ± 0.21 μmol/l, CD 0.559 ± 0.008 U (n = 11), $p < 0.05$ vs untreated diabetes] although desferrioxamine alone was without effect [MDA 1.67 ± 0.21 μmol/l, CD 0.595 ± 0.007 U (n = 12), p = NS vs untreated diabetes]. Due to the presence of increased transition metal availability in diabetes, the use of ascorbate supplementation as an antioxidant treatment may not be effective.

18.4 OXYRADICAL GENERATION AND IRON SEQUESTRATION BY A NOVEL MICROSOMAL PROTEIN: A DOUBLE EDGED SWORD ?

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An Mr ~ 66,000 Microsomal Iron Protein (MIP) with 17 atoms Fe (III)/molecule has been identified and purified (1). An iron-free apoMIP has also been prepared and shown to incorporate Fe (III) directly or *via* the oxidation of Fe (II) (1,2). In either case, apoMIP cannot be loaded with more than the iron present in native MIP, which should, therefore, be considered as iron saturated (1,2). MIP can shuttle electrons from NADPH-cyt. P-450 reductase to oxygen *via* the reduction-reoxidation of its bound iron. MIP can, therefore, stimulate the oxidation of NADPH and the formation of $O_2^{\cdot-}$, with each iron center being involved in electron transfer. Although native MIP cannot accomodate any extra iron, microsomes from iron-loaded animals have higher Fe (III) contents and NADPH oxidation activities. This suggests that the cell can adapt to iron overload by synthetizing apoMIP for the formation of new MIP. Both $O_2^{\cdot-}$ and free iron can be toxic to the cell, hence MIP behaves as a double edged sword. On one hand, MIP may disrupt cell homeostasis by producing $O_2^{\cdot-}$. On the other hand, MIP may assist ferritin in sequestering free iron. Pathophysiological ramifications can be envisioned in tissues (e.g., heart) with low superoxide dismutase content and ferritins with low iron-sequestering activity. (NATO CRG 901102).

1. Minotti, G. and Ikeda-Saito, M. (1991) J. Biol. Chem. 266, 20111-20117
2. Minotti, G. and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, in press.

18.5 EFFECT OF PROTOPORPHYRIN ON THE SUSCEPTIBILITY OF HUMAN ERYTHROCYTES TO OXIDATIVE STRESS.

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Protoporphyrin (PP) is accumulated in all body tissues of patients with erythropoietic protoporphyria. Exposure to light can lead to severe skin damage as a result of the formation of reactive oxygen species, but in the dark the accumulation of PP can also induce profound toxicity. However, not much is known about the biochemical background of these dark effects of PP. We used erythrocytes as a model system to investigate the toxic dark effects of PP, with special emphasis on membrane damage. Red cell membrane damage can be reflected by lipid peroxidation and increased permeability of potassium ions. Incubation of erythrocytes with hydrogen peroxide, *tert*. butyl-hydroperoxide and cumene hydroperoxide resulted in K^+-leakage and lipid peroxidation. The presence of PP affected peroxide-induced lipid peroxidation and K^+-leakage in different ways. Lipid peroxidation was inhibited in all cases, whereas the presence of PP enhanced the K^+-leakage considerably. Another porphyrin, namely uroporphyrin, had no effect on either lipid peroxidation or K^+-leakage. Subsequently it was determined that protoporphyrin binds to membrane proteins, whereas the hydrophylic uroporphyrin does not. These results might be explained as follows: due to its localization in the membrane protoporphyrin is able to scavenge lipid radicals, resulting in inhibition of lipid peroxidation, but also in the formation of porphyrin radicals. The formation of these porphyrin radicals exerts a toxic effect, which in the erythrocyte results in an enhanced K^+-leakage and cell lysis.

18.6 IRON RELEASE AS A MECHANISM OF MEMBRANE DAMAGE IN ERYTHROCYTES EXPOSED TO OXIDANT AGENTS.

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Iron is released from intracellular stores in an active form when the cells undergo an oxidative stress; such a release can induce cellular damage. Mouse erythrocytes have been incubated together with oxidant agents such as acrolein, phenylhydrazine, divicine, and isouramil. Methemoglobin formation and free iron release (Desferrioxamine (DFO)-chelatable iron) can be observed during the incubations. When the erythrocytes are depleted of glutathione (directly by acrolein or after a short incubation with diethylmaleate in the other cases), iron release induces lipid peroxidation (LP) in erythrocyte membranes and lysis.LP and lysis are completely prevented in erythrocytes properly loaded by DFO. It seems, therefore, that intracellular iron chelation prevents erythrocyte membrane damage suggesting a primary role of iron initiating oxidative reactions. Further studies show that iron, released from the erythrocyte lysate by oxidant agents, induces damage (LP and protein aggregation in red cell membranes) in the own ghosts separated from the lysate by a dialysis bag. These findings could be of interest in the study of the alterations induced in tissues by hemorrhages.

18.7 ANTIOXIDANT STATUS IN HEREDITARY HAEMOCHROMATOSIS

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Iron overload may increase free radical production and oxidative stress in haemochromatosis. We have therefore assessed antioxidant status and lipid peroxidation in 15 patients with hereditary haemochromatosis and 15 healthy age and sex matched controls. Groups were compared using the unpaired t test or Mann-Whitney U test and results are given as mean with 95% confidence limits. Plasma iron was increased in haemochromatosis patients (24.8 ± 3.7 vs. 17.8 ± 1.7 μmol/l, $p < 0.05$), as were iron saturation (51.8 ± 9.9 vs. 38.1 ± 6.0%, $p < 0.05$) and ferritin (111.7 ± 48.3 vs. 50.1 ± 12.9 μg/l, $p < 0.05$). Plasma malondialdehyde, measured by an HPLC technique, was increased in the haemochromatosis group (0.59 ± 0.10 vs. 0.46 ± 0.06 μmol/l, $p < 0.05$). In addition tocopherol was reduced (5.89 ± 0.73 vs. 7.24 ± 0.77 μmol/mmol cholesterol, $p < 0.05$), as was retinol (1.78 ± 0.24 vs. 2.46 ± 0.24 μmol/l, $p < 0.01$). Total ascorbate was reduced in haemochromatosis (51.3 ± 18.8 vs. 89.1 ± 23.8 μmol/l, $p < 0.01$), but the ratio of dehydroascorbate to total ascorbate was increased (22.1 ± 9.0 vs. 11.4 ± 3.7%, $p < 0.05$). There was no change in thiol groups (506 ± 85 vs. 448 ± 95 μmol/l, p = NS). These results provide further support for the idea that increased oxidative stress is important in the pathophysiology of hereditary haemochromatosis.

18.8 MEASUREMENT OF THE RESPIRATORY BURST AND PROTEASES ACTIVITY IN SERA AND PMNLs OF PATIENTS SUFFERING FROM ARTERIOSCLEROSIS

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We know that in some pathological state the incidence of infections and tissue destruction are increasing. We measured the production of superoxide anion and hydrogen peroxide, the elastase and myoeloperoxidase activities in sera, in resting and in stimulated PMNLs of healthy young /< 25yrs/, elderly /> 65yrs/, and of middle-aged /35-59yrs/ and elderly /> 60yrs/ arteriosclerotic subjects. In resting state the RB was increased with aging and in arterisoclerosis independently of the patient age. Under stimulations through various receptors we found a clear decrease in the stimulation of the RB in arteriosclerosis, while it remained unchanged on the effect of non-specific stimulation comparing to young subjects. In the sera of patients suffering from arteriosclerosis the RB was not increased. The elastase and myeloperoxidase activities increased in sera and PMNLs of arteriosclerotic patients. Our results suggest that we assist to similar tranmembrane signalling alteration in aging and in arteriosclerosis, the arteriosclerotic process might appear as an early aging process and the increased RB and proteases activity could contribute to the pathogenesis of arteriosclerosis.

18.9 LIPOXYGENASE-CATALYZED MODIFICATIONS ON HDL3

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Lipoprotein oxidative modifications may be the result of a concerted interplay between the non enzymatic and enzymatic lipid peroxidation. We examined the effects produced by a purified soybean lipoxygenase (SLO) on HDL3 in comparison with those found in extensively oxidized HDL (Cu-HDL), prepared by a Cu-catalyzed reaction [1]. The level of SLO-catalyzed HDL oxidation was controlled by varing the SLO concentration (2000-36000 U SLO/μmol PL for 24 h). The presence of phospholipase A2 (0.15 μg/μmol PL) did not increase the rate of oxidation. Biochemical and NMR analysis of SLO oxidized-HDL (SLO-HDL) revealed modifications of lipoprotein structure compared to control HDL (c-HDL) as well as to Cu-HDL. In particular we observed in SLO-HDL (using 36000 U SLO/μmol PL):

	SLO-HDL	Cu-HDL	c-HDL
LPO(μmol/mmol PL)	338±34	5±5.6	nd
TBARS(nmol/mg TC)	9.3±0.03	38±6.4	nd
EM on agarose(cm)	1.8-2.2	2.5-3	1.8-2
Apo AI MW (kDa)	25.4	28-200	22.3
Oxysterol(% on TC)	nd	30	nd

We suggest that SLO-HDL is an example of minimally modified HDL.

1]Bradamante S et al. FABM 1992 in press

FREE RADICALS INJURIES IN AGING: A SURVEY FROM THE RELIABILITY THEORY POINT OF VIEW 18.10

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The results of our measurements of production of oxygen free radicals and ESR signals in tissues of rats of different age, as well as the analysis of the data available from literature, evidence that any intensification of free radical chain processes such as combustions or outbreaks in living systems with age seems to be incredible. However, the kinetics of mortality rate growth for human beings and animals follows the exponential law with age. The reliability theory approach to the free radical theory of aging has been developed. In terms of this approach it becomes possible to explain how the linear kinetics of accumulation of free radical injuries in the cellular targets (chromosomes) leads to the exponential mortality rate growth, as well as to explain the nature of the well-known empiric correlations between the maximum life-span values and the metabolic factors and estimate species-specific life span potentials (120 years for men). It has been also estimated that the life-span of primates could run up to 250 years but for the injuries due to the oxygen free radicals.

18.11 INCREASED HYDROGEN PEROXIDE FORMATION IN STIMULATED PLATELETS OF PATIENTS WITH ESSENTIAL THROMBOCYTHAEMIA (ET)

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In platelets of patients affected with ET, metabolic, structural and functional abnormalities have been described. A non controlled oxygen radical production seems to be implicated in pathological and aging processes. Since some of the alterations described in ET could be related to their modified oxidative state, the basal levels of oxygen species (superoxide anion and hydrogen peroxide) and their modification induced by agonists have been assayed. In activated platelets of patients lower concentrations of superoxide anion and higher quantities of hydrogen peroxide than in controls have been measured. The assay of the most important enzymes implicated in the reactive oxygen species metabolism put in evidence that the specific activity of NADPH oxidase and that of superoxide dismutase are increased. Catalase on the contrary is decreased in platelets of ET patients.

The abnormal hydrogen peroxide production observed in platelets of patients, consequence of the modified ratio between the producing and the scavenging oxygen radical enzymes could be involved in the numerous platelet alterations described in ET.

AN ASSOCIATION BETWEEN TRANSFERRIN SATURATION AND MORTALITY IN MALNOURISHED JAMAICAN CHILDREN 18.12

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Mortality amongst malnourished children is associated with high hepatic stores of iron (Fe). This excess Fe has been implicated in the actiology of oedematous malnutrition. To examine the possibility that unbound Fe may be present in the plasma of malnourished Jamaican children, Fe and its transport protein, transferrin (Tr) were measured. Transferrin saturation (%TS) was calculated. A group of 23 healthy children served as controls. Plasma Fe levels (μg/dl) in children with marasmic-kwashiorkor (MK; n=59: 70±4 -meant±SEM), kwashiorkor (K; n=37: 76±4), and in those who died (D; n=24: 111±15) were not significantly different from the control group (C; 79±8). In the marasmic group Fe levels (M; n=63: 66±4 μg/dl) were significantly lower ($p<0.05$) than normal. In malnutrition, Tr levels (mg/dl) were significantly lower ($p<0.001$) than normal: C- 233±10; M-170±15; n=66: MK-110±10; n=61: K-85±13; n=41: D- 77±15; n=24. In the marasmic group transferrin levels were significantly higher ($p<0.001$) than the other malnourished groups. %TS was lowest in the marasmic group and highest in the group of children who died. At a %TS of <30%, mortality was 5% compared with a mortality of 24% when %TS was >90%.It is concluded that oedematous malnourished Jamaican children have normal plasma levels of iron, but significantly reduced levels of circulating transferrin. The latter were more abnormally decreased in children with kwashiorkor and those who died. Elevated %TS was associated with increased mortality.

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18.13 5-AMINOLEVULINIC ACID: A POSSIBLE ENDOGENOUS SOURCE OF REACTIVE OXYGEN SPECIES IN SATURNISM AND ACUTE INTERMITTENT PORPHYRIA

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5-Aminolevulinic acid (ALA) - a heme precursor accumulated in saturnism and acute intermittent porphyria (AIP) carriers - has been shown to yield $O_2^{\cdot}$, H_2O_2, and $HO^{\cdot}$ by iron-catalyzed aerobic oxidation. Upon incubation of ALA (mM) with rat liver mitochondria (RLM), transmembrane potential collapse, calcium flux alterations, and state-4 respiratory rate increases can be observed. RLM swelling can also be verified even at the ALA concentrations (50-100 μM) expected to occur in the liver of AIP patients. Removal of contaminant Ca^{2+} (10 μM) from the RLM suspension medium by added EGTA abolishes the ALA-induced potential collapse and swelling. Prevention of ALA-induced swelling by addition of ruthenium red prior to RLM energization demonstrates the deleterious coadjuvant role of internal Ca^{2+}. Addition of 1-5 mM Mg^{2+} completely prevents the ALA-induced swelling, potential collapse and Ca^{2+} efflux. The RLM damage promoted by ALA/internal Ca^{2+} is partly repaired by addition of either catalase, o-phenanthroline or dithiothreitol, suggesting mitochondrial injury involves oxidation of membrane SH-proteins. These data might be relevant with regard to the biochemical mechanisms (oxidative stress?) involved in the clinical manifestations of both diseases. Acknowledgements: FAPESP, CNPq, and BID/USP and Atlantis Brasil Com. Ind. Ltda..

18.14 CHANGES OF INTRACELLULAR IONS INDUCED BY SUPEROXIDE : REAL TIME OBSERVATION IN SINGLE LIVING CELLS

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The effects of superoxide on intracellular ions were investigated in human amnion cells using digital imaging microscope and fluorescent dyes sensitive to intracellular Ca^{2+} ($[Ca^{2+}]i$), intracellular pH (pHi) and intracellular Mg^{2+} ($[Mg^{2+}]i$). Superoxide increased $[Ca^{2+}]i$. The increase was partially dependent on extracellular Ca^{2+}. Superoxide also induced increase of pHi and decrease of $[Mg^{2+}]i$, which preceded the $[Ca^{2+}]i$ change. These changes were inhibited by anion channel blocker DIDS and SOD injected into cells by cell fusion technique. Changes of pHi and $[Mg^{2+}]i$ manipulated by NH_4Cl or $[Mg^{2+}]i$ ionophore affected the $[Ca^{2+}]i$ increase induced by superoxide. These results suggest that superoxide, transported through anion channels into cells, directly induced pHi and $[Mg^{2+}]i$ changes and that the changes may regulate biological functions of the cells via the $[Ca^{2+}]i$ increase.

18.15 TOXICITY OF SINGLET OXYGEN GENERATED THERMOLYTICALLY IN *ESCHERICHIA COLI*

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Much interests have been focused on reactivities of singlet oxygen ($^1O_2$) not only in the field of organic chemistry, but also in that of biological chemistry. The effect of $^1O_2$ on living cells has been examined mainly using a class of dyes known as photodynamic sensitizers. However, $^1O_2$ generated from the photosensitizer-light-O_2 system or from the NaOCl-H_2O_2 system is often accompanied by other reactive species whose toxicities should be difficult to distinguish from that of $^1O_2$ in living cells. Generally, photosensitizers with irradiation often react with biological components in dependent of $^1O_2$. In photodynamic systems it is often uncertain to what extent an effect can be ascribed to $^1O_2$. Thus the photosensitizer-light-O_2 system should be used only with special precaution. We have applied a water-soluble $^1O_2$ source, which is generated thermolytically, but not photochemically, for examining biological damages by $^1O_2$. This $^1O_2$ generating system using naphtalene endoperoxide derivatives appears to overcome the difficulty and provide significant results for elucidation of $^1O_2$ toxicity. Our interest was focused on the toxicity of the water-soluble naphthalene-derived endoperoxide showing temperature-dependent $^1O_2$ release on *Escherichia coli* growth and survival. At this conference we report that *E. coli* growth was inhibited by $^1O_2$ from the endoperoxide without induction of superoxide dismutase and that the toxicity was well related to half-lives of naphthalene derivatives.

18.16 SIMULTANEOUS MEASUREMENT OF ALLANTOIN AND URIC ACID IN DIABETIC PATIENTS AS A MARKER FOR OXIDATIVE STRESS.

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Glucose and glycosylated proteins have been shown to promote the production of oxygen free radicals. Oxidative stress may therefore contribute to the development of diabetic complications. Several oxidation products were proposed to correlate with the extent of tissue pathological damage. In this study we determined the levels of uric acid and its major oxidation product allantoin in diabetic patients, in relation to their glycaemic control. Allantoin and uric acid were measured simultaneously by an improved HPLC technique in plasma ultrafiltrate from fifty diabetic patients and eighteen healthy individuals. Allantoin levels in the two groups were 19.8 $\pm$ 0.9 and 16.5 $\pm$ 1.3 respectively, uric acid levels were 290 $\pm$ 13 and 340 $\pm$ 7 (µmol/L, mean $\pm$ SEM).
Uric acid is generated in the human body by the oxidation of purines but no enzyme is present to oxidize it further, thus allantoin might be a "marker" *in vivo* for the extent of oxidative stress. However a calculated index from the concentration ratio between allantoin and uric acid in the two groups was 70 $\pm$ 3 versus 50 $\pm$ 4 (mean $\pm$ SEM) ($p < 0.005$), suggesting this may be a better indicator for oxidative stress than allantoin levels per se. The correlation between this marker and the degree of glycaemic control is still under investigation.

18.17 INDUCIBLE BILIRUBIN- AND PORPHYRINOGEN-OXIDIZING SYSTEM OF LIVER MICROSOMES.

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We have recently described a bilirubin-degrading system, in the hepatic and renal microsomal fraction, that can be induced by treatment with either TCDD, ß-naphthoflavone or 3-methylcholanthrene in vivo and markedly stimulated by addition of a planar polychlorinated biphenyl (PCB) to the microsomes in vitro. Addition of the biphenyl also caused a dose-dependent inhibition of the EROD activity, suggesting tight binding of the chemical to the active site of the induced cytochrome, leading to production of a bilirubin-degrading species.
We have now compared bilirubin and hexahydrouroporphyrin (uroporphyrinogen) as substrates for this inducible oxidizing system, using liver microsomes from ß-naphthoflavone-induced chick embryos and 3-methylcholanthrene-induced male rats. With chicken microsomes the rate of oxidation of both substrates was stimulated by planar PCBs (3,3',4,4'-tetrachlorobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl), while their non-planar di-orthosubstituted congeners were virtually inactive. Microsomes obtained from induced rats behaved in most cases as described above for chicken microsomes. However in the case of 4 out of a total of 14 rats, the basal rate of oxidation of both bilirubin and porphyrinogen (the rate, that is, without addition of a PCB) was significantly higher and the planar PCBs failed to stimulate further the oxidation of the porphyrinogen and were less effective at stimulating bilirubin oxidation. These findings help substantiate the hypothesis that interaction of a polyhalogenated aromatic compound with the induced cytochrome may initiate an oxidative mechanism leading to oxidation of target molecules in the cell. However we cannot yet provide an explanation for the anomalous behaviour of the microsomes obtained from some rats.

Session 19

DNA Damage and Repair

19.1 Oxidation reactions of the purine and pyrimidine moities of DNA and model compounds.

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The main available reactions of reactive oxygen species including OH radicals, superoxide radicals, singlet oxygen, ozone and hydrogen peroxide with the base moieties of 2'-deoxyribonucleosides and DNA are critically reviewed. In addition, information is also provided on the oxidation reactions that arise from the formation of a purine or a pyrimidine radical cation as the result of initial electron transfer processes. Emphasis is placed on the role of the DNA structure and external factors such as reducing agents on the reactions of purine radicals. A survey of the main approaches (HPLC separations associated with various spectroscopic detections, GC-MS, postlabeling assays, ...) involving either initial acid hydrolysis or enzymic digestion of DNA which were recently developed for monitoring the formation of oxidative DNA base damage in cells and tissues are also presented.

19.2 DNA BASE DAMAGE AND DNA-PROTEIN CROSS-LINKS IN CHROMATIN OF γ-IRRADIATED OR H_2O_2-TREATED CULTURED HUMAN CELLS

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The induction of DNA base damage and DNA-protein cross-links (DPCs) in chromatin of γ-irradiated or H_2O_2-treated cultured K562 human cells was investigated using GC/MS. Eleven modified DNA bases were identified and quantitated in chromatin of γ-irradiated cells. Hydroxyl radical involvement in product formation was inferred from the pattern of lesions. Linear dose-yield relationships of most modified DNA bases were observed from 42 up to 200 Gy, with no increases observed in the yields above 420 Gy. The yields of guanine-derived bases amounted to ≈45% of the total net yield of modified bases. Both 8.7-82 Gy of γ-rays and 0.5-10 mM H_2O_2-treatment of cells caused formation of a thymine-tyrosine cross-link. Its amount was increased up to ≈4-fold above the control level. The yield of this DPC reached a plateau above 82 Gy or 10 mM H_2O_2. Pretreatment of cells with KCN or ascorbic acid increased the amount of the Thy-Tyr cross-link, whereas dimethylsulfoxide and *o*-phenanthroline partially inhibited its formation.

19.3 OXIDATIVE STRESS IN HUMAN SPERM: DNA DAMAGE AND ANTIOXIDANTS.

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The sperm cells are subjected to oxidative damage under normal physiological conditions. Oxidative stress arising from an increase in the production of active oxygen species or insufficient antioxidant levels could lead to modifications of normal sperm cell functions. This oxidative stress may produce cells containing highly oxidized DNA, potentially increasing the probability of mutations and birth defects. The extent of oxidative damage and antioxidant protection in human sperm was determined by measuring the endogenous levels of: a) 8-oxo-2'-deoxyguanosine (oxo^{8}dG) in DNA; b) antioxidants in seminal plasma; and c) the spontaneous chimiluminescence (CL) of isolated sperm cells. The following results were obtained: a) steady state level of oxo8dG = 14±2 fmoles/μg of DNA (=2.3 oxo^{8}dG/10^5 dG = 27.000 oxo^{8}dG/sperm); b) antioxidants in seminal plasma: α-tocopherol = 1.1±0.1 μM; ascorbate = 399±55 μM; uric acid = 368±42 μM. The concentrations of ß-carotene, ubiquinol-10 and lycoperne were not detected (<50 nM); c) isolated spermatozoa CL=430 cps/5x10^7 cells (=2.0x10^4 photons/s/cell).
The results indicate: a) endogenous oxidative damage to sperm cells is extensive; b) this damage is increased in conditions of oxidative stress; c) adequate levels of antioxidants would protect the spermatozoa against irreversible oxidative damage. The inverse relationship observed between the level of oxidative damage to sperm DNA and seminal plasma ascorbic acid levels indicate that dietary ascorbic acid protects sperm form endogenous oxidative DNA damage. The steady state level of oxidative damage to sperm DNA is important in that it could lead to improper sperm function, infertility, and birth defects.
This work was supported by grants: NCI CA 39910 and IEHS ES 01896 to BNA; Rockfeller Foundation RF 89033 to CFG; and AHA 90-28 to PM.

19.4 MUTAGENECITY OF DOXORUBICIN IN A FREE RADICAL GENERATING SYSTEM

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Doxorubicin, an effective anticancer agent, has dose limiting cardiotoxicty and has been described as genotoxic with strong mutagenic activity. The capability of doxorubicin to intercalate DNA is the usually cited mechanism of mutagenic activity. There has, unfortunately, been but scant attention directed to its capability of producing free radicals in the mutagenic context yet studies using isolated DNA have correlated increased DNA damage with the doxorubicin Fe(III) complex (1,2). In this study, the mutagenicity of doxorubicin in a free radical generating system was tested. The free radical mutagen sensitive Salmonella strain TA102 was used in conjuction with two sources of iron $FeCl_3$ and Ferritin, the biological source of Iron. Comparisons also included experiments with GSH and rat liver S-9. The concentrations of Ferritin, inorganic iron, GSH and rat liver S9 used were non-mutagenic and non-cytotoxic to the tester strain bacteria. Ferritin alone increased the mutagenecity of doxorubicin by 45% while a 1:1 stoichiometric combination of doxorubicin with inorganic iron alone showed a 20% increase. Furthermore, the mutagenecity of doxorubicin was increased 100% with ferritin in the enzymatic environment as compared to the mutagenecity of doxorubicin in the enzymatic system alone. It is suggested from these and other results that ferritin may, enhance the mutagenecity of doxorubicin by providing ferric iron and thus potentiating its capability to produce doxorubicin free radicals and oxygen derived free radicals.
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19.5 DNA OXIDATIVE DAMAGE AND MUTAGENESIS IN *E. COLI*

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Although superoxide radicals do not directly attack DNA, they favor the formation of oxygen species, such as hydroxyl radicals produced via a Fenton reaction, which damage DNA. Superoxide dismutase as removing superoxide protect the cells from such damage. Indeed SOD deficient mutants were shown to have increased spontaneous mutagenesis. We show here that similarly an increase of iron intracellular concentration created by the derepression of the system of iron assimilation (in *E. coli fur* mutants) favors an *in vivo* Fenton reaction and leads to DNA damage. *fur* mutants show increase oxygen dependent spontaneous mutagenesis, and *fur recA* double mutants are viable in anaerobiosis, but died upon shift to aerobiosis. Addition of ferrosin (iron chelators), DMSO or thiourea (supposedly radical scavengers) completely or partially protect the cells from death upon shift to aerobiosis., suggesting that oxidative damage (presumably double strand breaks) are responsible for the cell death. This observation provides a biological significance to the regulation of MnSOD by *Fur*. Fur protein need reduced iron as a cofactor to be active. In sufficience of iron, Fur limites intracellular iron concentration and potential oxidative damage.by repressing iron assimilation, it also partially represses MnSOD expression. When iron becames scarce, the iron assimilation derepression produces an oxidative burst which is compensated by the derepression of MnSOD.

19.6 MUTAGENESIS BY OXYGEN FREE RADICALS

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Reactive oxygen species (ROS) produced in mammalian cells have been implicated in DNA damage and malignant transformation. In order to assess the contribution of ROS to mutagenesis, we have determined the effect of ROS damage to DNA on the fidelity of purified mammalian DNA polymerases β and α. Metal catalyzed oxidation systems containing $Fe(II)+H_2O_2$ or $Cu(I)+H_2O_2$+ascorbic acid were used to damage a single-stranded *lacZα* sequence in M13mp2 DNA. Following damage, the DNA was copied by purified recombinant rat DNA polymerase β (pol β) or calf thymus DNA polymerase α (pol α). Mutants with reduced β-galactosidase activity were isolated and sequenced. Oxidative damage to DNA increased the error frequency of pol β 3.4 to 4.2 fold and that of pol α 2.9 to 4.1 fold. Analysis of the mutational spectra suggests the mutants resulting from oxidative damage fall into four categories: randomly dispersed single base substitutions or deletions; "hotspots", where a single base substitution occurs frequently at the same site in *lacZα*; multiple mutations in which a single mutant contains 2 or more closely spaced base substitutions; and an exacerbation of certain mutational hotspots that are specific to each polymerase. The latter two categories suggest that in addition to miscoding by damaged nucleotides, ROS damage may induce conformational changes in DNA that reduce the fidelity of polymerases in the vicinity of a lesion.

19.7 DNA SCISSION MEDIATED BY ASCORBATE AND BY THIYL RADICALS

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It is established that molecules such as glutathione, ascorbic acid, tocopherol protect against damage to DNA by quenching reactive radicals. We suggested that although these molecules do have a protective effect when the body is exposed to reactive free radicals, they could in principle also damage DNA in the presence of certain chemicals. The purpose of this project is to identify under what circumstances this happens.

The model study was performed by allowing **ascorbate** to act as an electron donor to compounds capable of being converted into reactive species by this process. Arene diazonium salts were found to undergo very rapid reaction leading to scission of DNA from ΦX 174. The mechanism of the reaction was shown to involve electron transfer. The nature of the chemical reactions was further investigated by examining the reaction of diazonium salts with models of deoxyribosides in the presence of electron donors.

The reactions of thiyl radicals [related to **glutathionyl**] with alkynes were investigated. Here the product vinyl radicals also reacted with models of deoxyribosides by reactions which lead to DNA cleavage.

This study shows that it is now possible to predict that glutathione and ascorbate in the presence of co-factors can indeed lead to cleavage of DNA.

19.8 MENADIONE INDUCES AN INCREASE IN INTRACELLULAR Cu AVAILABLE FOR OXIDATIVE DNA DAMAGING REACTIONS.

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Transition metals are important mediators of oxidative DNA damage. Therefore, it is important to evaluate whether the cellular pool of "free" Fe and Cu is modified in cells under oxidative stress. When V79 Chinese hamster cells are exposed to menadione, Cu is released from storage sites and becomes available for oxidative DNA damaging reactions. This can be probed by 1,10 phenanthroline (phen), which forms a strong clastogenic ternary complex with Cu and DNA. Concentrations up to 1 mM of phen causes no DNA strand scission (dss). However, if cells are previously exposed to menadione, dss increases dramatically with increasing concentrations of phen. These dss are completely prevented by neocuproine, a specific Cu chelator. Possible mechanisms of Cu release have been considered. The redox cycle in which menadione is involved causes GSH depletion and it has been recently shown that GSH is involved in Cu transport. Metallothionein seems to be important in Cu storage and we determined that menadione causes a reduction in its content. Therefore, the depletion of two major intracellular Cu chelators causes an increase in the pool of "free" Cu to an extent that it may become significant as a mediator of oxidative DNA damage.

19.9 PHOTOSENSITIZED FORMATION OF 7,8-DIHYDRO-8-OXO-2'-DEOXYGUANOSINE (8-HYDROXY-2'-DEOXYGUANOSINE) IN DNA BY RIBOFLAVIN

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7,8-Dihydro-8-oxo-2'-deoxyguanosine (oxo^{8}dG, 8-hydroxydeoxyguanosine) is one of the major forms of DNA damage induced by oxygen radicals. Recent studies have shown that 1) oxo^{8}dG is produced in cellular DNA by oxygen radical forming carcinogens.; 2) *E.coli* and mammalian cells have repair mechanism for oxo^{8}dG; 3) oxo^{8}dG in DNA induces mutations. In this presentation we wish to report that riboflavin, an endogeneous cellular photosensitizer, was found to efficiently photoinduce the formation of oxo^{8}dG in DNA. Exposure of calf thymus DNA in phosphate buffer containing riboflavin to visible light was found to generate oxo^{8}dG whose formation was quantified the HPLC-ECD method. Irradiation in 80 % D_2O aerated solution was found to lead to a slight decrease in the yield of oxo^{8}dG, ruling out any significant involvement of singlet oxygen. In contrast, about five-fold enhancement in formation of oxo^{8}dG was observed when the photoreactions were performed in oxygen-free aqueous solutions. A reaction mechanism involving guanine radical cation and hydration reaction was proposed. This hypothesis was confirmed by the incorporation of [^{18}O]-atom within guanine moiety in an isotopic experiments using [^{18}O] H_2O. Formation of oxo^{8}dG in cellular DNA was also observed, when mouse lymphoma L5178Y cells or human lung fibroblast WI38,VA13 cells were irradiated in the presence of riboflavin.

19.10 RADICAL OXIDATION REACTIONS OF THE PURINE MOIETY OF 2'-DEOXYRIBONUCLEOSIDES AND DNA BY IRON-CONTAINING MINERALS

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The radical oxidation capability of several classes of iron rich minerals and asbestos including biotite, hematite, magnetite, mineite, nemalite, pyrite, vivianite and two chrysolites was investigated by using a double experimental approach. One involved the ESR-spin trapping measurement of oxygen reactive species released upon incubation of the minerals in phosphate buffered solutions. In addition, the formation of mineral-mediated oxidation purine decomposition products including 8-oxo-7,8-dihydro-2'-deoxyadenosine and 8-oxo-7,8-dihydro-2'-deoxyguanosine was searched within nucleosides and DNA. This was achieved by using specific and sensitive HPLC-electrochemical assays. Emphasis was placed on the mechanistic aspects of the radical oxidation reactions involved in the formation of the two C(8) hydroxylated purine decomposition products.

19.11 MODULATION OF OXYGEN RADICAL-INDUCED DIABETOGENESIS IN TRANSGENIC MICE.

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We have investigated the role of active oxygen in diabetogenesis through the use of transgenic mice which selectively augment normal CuZnSOD activity. Transgenic strains RIPSOD 11 and 389 carry a fusion transgene made of CuZnSOD cDNA of *Drosophila melanogaster* under the transcriptional control of the rat insulin promoter and selectively over-express CuZnSOD in pancreatic beta cells. Transgenic strain TgHS-SF carries an intact human CuZnSOD transgene and over-expresses CuZnSOD in a wide variety of tissues including the pancreas. We have measured the effect of CuZnSOD over-expression in these strains on diabetogenesis induced by the radical-generating diabetogenic drug, streptozotocin (STZ). Both RIPSOD strains are markedly hypersensitive to single acute doses of STZ and, in contrast to their nontransgenic littermates, develop rapid hyperglycemia within 24 hours indicative of a rapid failure of beta cell function. TgHS-SF, on the other hand, exhibits a marked resistance to the effects of acute STZ exposure and, in contrast to nontransgenic littermates and to both RIPSOD strains, TgHS-FS exhibits a marked delay in the onset of hyperglycemia indicative of significant beta cell protection. None of the transgenic strains show a differential hyperglycemic response to repeated subacute doses of STZ. These results demonstrate unequivocally that *in vivo* genetic manipulation of CuZnSOD expression can affect susceptibility to diabetogenesis, and that active oxygen has important diabetogenic potential.

19.12 Induction of heat shock protein synthesis appears to be independent from DNA strand breaks

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The induction of stress/heat shock (HS) proteins (HSPs) is suggested to be mediated by the presence, in a given cell, of abnormal, unfolded or misfolded proteins. Oxidative injury, one of the many inducers of HSPs, induces both DNA and protein alterations. Furthermore, HSPs relocate to the nucleus during cellular injury. We thus asked the question whether DNA alterations could represent the final common pathway for HSP induction. After exposure of the human premonocytic line U937 to HS, hydrogen peroxide, iron, bleomycin (BLM), and erythrophagocytosis, DNA strand breaks were measured by alkaline unwinding and determination of ethidium bromide fluorescence according to Birnboim (1981), and protein synthesis analyzed by metabolic labeling and SDS-PAGE. We found that both heat shock and H2O2 induced DNA strand breaks, and that these alterations were potentiated by iron. HS, but not H2O2, also induced HSP synthesis. The antitumor drug BLM, which intercalates into DNA molecules and causes DNA strand scission, did not induce HSP synthesis in U937 cells. In contrast, erythrophagocytosis, a physiological process which is in 1,25-dihydroxyvitaminD3-differentiated cells associated with high level induction of HSPs, did not induce DNA strand breaks. Taken together, these results indicate that DNA strand breaks are neither required nor sufficient for HSP induction. HSPs however appear to play a role in protection from oxidation-induced DNA strand breaks.

19.13 CHEMILUMINESCENCE AND DNA STRAND BREAKAGE DURING HORSERADISH PEROXIDASE CATALYZED OXIDATION OF DNA ADDUCTS WITH 2,4-DECADIENAL

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Lipid peroxidation in biological systems is believed to be a toxic and destructive sequence of reactions leading to cell damage. The interaction of DNA with degradation products formed from lipid hydroperoxides gives rise to a fluorescent product which is supposed to be implicated in cancer, inflamation and aging process (Frankel et al, Biochem. Biophys. Acta, 919, 239, 1987).
In the present work, adduct formation between 2,4-decadienal and DNA was followed by fluorescence emission (excitation 315 nm, emission 420 nm). Addition of 2 μM horseradish peroxidase (HRP) to the DNA-aldehyde adduct at physiological pH is accompanied by chemiluminescence which is dependent on adduct concentration. Furthermore, pBR322 plasmid DNA strand breaks were detected when exposed to this system.
Knowing now that DNA-2,4-decadienal adducts generate electronically excited species in the presence of HRP, one may speculate that these excited species may act as the toxic agents in the deleterious processes mentioned above.
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19.14 DNA DAMAGE TO MAMMALIAN CELLS UNDER OXIDATIVE STRESS.

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The central role of oxygen derived free radicals in inducing damage to various cellular components is well established. However, most studies reported in the literature have been conducted under conditions where the cellular growth environment was not controlled. In our studies, hybridoma cells were exposed to various elevated oxygen tension levels (200, 300 and 476% with respect to air saturation at 1 atmosphere) while growing in a bioreactor. Using a modified fluorometric assay for DNA unwinding, we show that there is a direct relationship between the extent of DNA strand breakage and oxygen tension. In addition, hyperoxia affects other metabolic functions such as the glucose consumption rate, lactate production rate and cell growth. When hyperoxia induced DNA strand breakage was compared to that induced by exposure to hydrogen peroxide, a semi-quantitative relationship was observed. Exposure to a dissolved oxygen level of 200% induced DNA strand breakage comparable to a bolus of 4.2 µM hydrogen peroxide. However, the kinetics were quite different with peroxide treated cells showing strand breaks within 10 minutes versus 180 minutes for hyperoxia exposed cells.

19.15 FREE RADICAL DAMAGE TO NUCLEIC ACIDS

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Damage to DNA can be produced either by direct ionization of the bases or via reactions of $HO^{\cdot}$, $e^{-}aq$ and $H^{\cdot}$ produced from water molecules. Evidence suggests that damage to the sugar-phosphate backbone of DNA is responsible for cleavage of the nucleotide chain, though only a small percentage of $HO^{\cdot}$ radicals react directly at this site; it has therefore been postulated that initial attack on the bases can lead to the formation of sugar radicals and hence strand-breakage.

E.s.r. spectroscopy, in conjunction with a rapid-flow system and spin-trapping has been used to study the reaction of $HO^{\cdot}$ with nucleic acids and their components; comparisons have also been made with the reactions of $SO_4^{-\cdot}$. Initial studies show that $SO_4^{-\cdot}$ and $HO^{\cdot}$ (at low pH), generate radical-cations from the pyrimidine bases which then transfer the radical site to the sugar-phosphate backbone. Thus, uridine reacts with $HO^{\cdot}$ to give a base-derived radical at pH 7. At low pH however, this radical is largely replaced by another base-derived radical and a sugar derived radical, whose precursors are believed to be the base radical-cation.

Experiments with a range of model compounds show that the hydroxyl group at the C2 in the sugar is crucially important for the transfer of radical site. Possible mechanisms for this process will be discussed.

Session 20

Free Radicals in Medicine IV (Red Cells, Hearts)

20.1 THE ROLE OF FREE RADICALS AND VITAMIN E IN HUMAN REPERFUSION INJURY.

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Results from studies into free radical production during human myocardial reperfusion shall be presented. Patients undergoing either percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting, for the treatment of acute, chronic, stable angina were studied.

In the first group of 10 patients, sequential coronary sinus sampling was performed and free radical production was assessed using the spin trap PBN. Free radicals were only detected during the reperfusion phase of this technique and radical production reached a peak between two and five minutes after reperfusion. Free radical production was found to correlate, positively, with the degree of the ischaemic insult.

Paired arterial and coronary sinus blood samples were obtained from patients undergoing routine coronary artery bypass surgery. Free radical induced damage was assessed by measuring the levels of thiobarbituric acid reactive material (TBARs). The levels of TBARs rose significantly, in both arterial and coronary sinus blood five minutes after reperfusion.

There was a net myocardial loss of vitamin E 30 sec after reperfusion, however, changes in vitamin E levels did not reach significance in either arterial or coronary sinus samples.

These studies provide direct evidence of myocardial free radical generation in man during both PTCA angioplasty and coronary artery bypass grafting.

20.2 GLUTATHIONE AND REDOX REACTIONS AT THE ERYTHROCYTE MEMBRANE

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The ^{1}H-NMR spectra of intact erythrocytes can be greatly simplified by using a multiple-pulse spin-echo technique (1), which eliminates proton signals belonging to macromolecules or molecules bound to large structures (like membranes), because of the very short nuclear magnetic relaxation times produced by slow molecular tumbling. We have used an improved version of this technique to analyse the response of intact human erythrocytes to additions of glutathione, which is known to be unable to cross the red cell membrane (2). A significant increase of the NMR signal of internal glutathione was observed (around 80%) in glucose deprived cells, which corresponded to a 40% increase as measured by chemical assays. This indicates that the NMR measurement is a better probe for changes of redox equilibria in the internal glutathione pool. In fact, the effect was dependent on the integrity of the cell membrane and on the reactivity of its exofacial thiols, suggesting a transduction of the reducing power of external glutathione via thiol-disulphide exchange across the red cell membrane. In line with this suggestion, washing out external glutathione produced an immediate decrease of the NMR signal, which gradually returned to the original intensity after 19 hours incubation of the washed sample at 25°C. Moreover, addition of glucose led to 20% increase of the glutathione signal. Subsequent addition of glutathione led to the full extent of increase as observed with glucose-deprived cells, which in the presence of glucose alone, could be obtained only after 3 hours incubation. Mixed disulphides with internal proteins were shown to be a likely source for the extra thiol equivalents. Erythrocytes treated with diamide (a permeant agent forming mixed-disulphides) showed a smaller NMR signal of intracellular glutathione and a larger increase after addition of external glutathione than untreated cells. Comparable data were obtained with aged erythrocytes (kept at 4°C in autologous plasma for several days). Spectrin was shown to be involved in the effects, as the extent of signal increase was found to be linearly related to the spectrin content of spherocytic red blood cells. It can be concluded that external glutathione can release the internal one from mixed disulphides by transduction of reducing power through thiol-rich membrane proteins (most likely the glucose carrier and spectrin) via a thiol-disulphide interchange mechanism. In this way extracellular glutathione might influence the redox balance of the cytosol by redistributing or increasing the thiol content of the membrane.

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20.3 DETECTION OF GSH-LEVELS IN PERFUSED RAT HEARTS BY MEANS OF A NOVEL ESR-METHOD

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Glutathione is considered to be a sensitive indicator of oxidative stress in reperfused organs due to its scavenging activity towards free radicals. Thus, the continuous monitoring of GSH-levels was taken as a reliable approach to estimate the existence of an "oxidative stress" following reperfusion of the ischemic heart. For the first time we have studied the validity of a new and highly sensitive method to detect steady state concentrations of tissue GSH-levels non-invasively. The essential of this method is a GSH-induced disruption of a biradical to a stable monoradical. Both types of radicals exhibit different ESR signals allowing qualitative and quantitative estimation of GSH. Differences in the partition coefficient of the mono- and biradical favoured the accumulation of the biradical in the extravascular space and redistribution of the monoradical into the vascular system. Monitoring of the paramagnetic reaction product in the effluent perfusate elicited significantly lower GSH-levels following ischemia compared to the controls. This finding clearly indicates that reperfusion of the ischemic heart causes metabolic alterations in favour of increased generation of reactive prooxidants.

20.4 PEROXIDATION OF THE ANTIMALARIAL PRIMAQUINE:CHARACTERIZATION OF A BENZIDINE-LIKE METABOLITE WITH METHEMOGLOBIN- FORMING ACTIVITY

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Primaquine is one of the most effective exoerythrocytic antimalarial agents; its clinical utility, however, is compromised by hematotoxicity. The latter effect is usually ascribed to a metabolite rather than to the parent compound. The only identified metabolites known to be more effective than primaquine in oxidizing hemoglobin and depleting GSH from erythrocytes, are those expected from cytochrome P_{450}-mediated hydroxylation such as 5-hydroxyprimaquine. Although proteins with peroxidase activity such as horseradish peroxidase, methemoglobin and myeloperoxidase are known to oxidize primaquine, none of the resulting products has been unambiguously identified. Now we report the characterization of an organic extractable product formed in incubations of primaquine (70mM) with hydrogen peroxide (70 mM) and horseradish peroxidase (0.5 mg/ml) in acetate buffer pH 4.2. The metabolite was identified as 5,5-di-{8-[(4-amino-1-methylbutyl) amino]-6-methoxyquinoline} by ^{1}H-NMR, mass, UV-visible and infrared spectroscopy. The elucidated benzidine-like structure demonstrates that a carbon-carbon coupling between two primaquine molecules occurs during peroxidation. The identified metabolite (0.2-0.5mM) was able to promote oxidation of hemoglobin in a time- and concentration-dependent manner, when incubated with suspensions of rat erythrocytes (25% in phosphate buffer-saline) at 37°C. The benzidine-like metabolite was more effective than primaquine in forming methemoglobin, but less efficient than the previously described hydroxylated metabolites.

20.5 CARNITINE AND CARNITINE PALMITOYLTRANSFERASE, KEY MODULATORS OF THE ACYL-TRAFFICKING IN THE COURSE OF THE SECONDARY ANTIOXIDANT RESPONSE TO OXIDATIVE STRESS.

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The antioxidant biochemical network may be envisioned as a system operating at two different levels: a primary defense barrier which prevents oxidative injury by scavenging toxic oxidants, and a secondary defense system which eventually repairs the damage occurred after the oxidative attack. The deacylation-reacylation process of membrane phospholipids is considered a major component of the secondary antioxidant defense system. In fact, it is known that peroxidation of polyunsatured fatty acids esterified in membrane phospholipids is followed by an increased membrane phospholipid fatty acid turnover.

We have recently demonstrated that long chain acylcarnitines are metabolic intermediates of the reacylation pathway in erythrocyte membrane phospholipids. This process takes place in the absence of ATP, suggesting that long-chain acylcarnitines represent an important pool of intracellular fatty acids available for the reacylation of lysophospholipids, and that carnitine palmitoyltransferase (CPT) modulates the acyl flux. CPT catalyzes the reversible transfer of the fatty acid from CoA to carnitine, and because of its near equilibrium condition, the net flux of this reaction is highly sensitive to the mass action ratio of the reactants. In addition, the inhibition of CPT resulted in a marked reduction of the reacylation process in human erythrocytes exposed to ter-butyl hydroperoxide. Thus, CPT may affect the reacylation process of membrane phospholipids by modulating the size of the acyl-CoA pool between the activation step of the fatty acid and its transfer into lysophospholipids.

20.6 THE PROCESS OF HUMAN HEMOGLOBIN AUTOXIDATION AS SOURCE OF FREE RADICALS IN RED CELLS

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We studied the processes of reversible oxygen binding and nonreversible autoxidation of human hemoglobin. Superoxide anion was generated in the latter process. The activation energy of the oxygen binding, as determined by the temperature dependence of the P_{50} parameter, was 26 ± 4 kJ/mol, the activation energy of the autoxidation, as determined by the temperature dependence of the apparent rate constant of autoxidation, was 120 ± 15 kJ/mol. The rate of autoxidation appreciably increased as the pH value of the medium decreased, reflecting, probably, protonation of the distal histidine of the hemoglobin. The activation energy of the autoxidation was independent of pH. Aliphatic alcohols also increased the rate of the autoxidation process, probably, either by stabilization of the hemoglobin T-state, or by direct nucleophilic displacement of the oxygen molecule as superoxide anion.

20.7 SHAPE CHANGE AND SECRETION INDUCED BY N-ETHYL-MALEIMIDE IN HUMAN PLATELETS

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N-ethyl-maleimide (NEM) is a cell-penetrating thiol agent with several biochemical and physiological effects on blood platelets. NEM inhibits platelet aggregation, but not shape change induced by ADP. Stimulation of platelet adenyl-cyclase by prostaglandins (PGE1) is enhanced by NEM, and high concentration (2mM) of NEM cause irreversible depletion of platelet nucleotides. Further, NEM induces a dose-dependent increase in cytosolic Ca++ (as measured by fluorescent dyes) and production of thomboxane A2. NEM (2 mM) also causes a change in platelet shape, which in aggregometer tracings appears to be triphasic; the first phase causing a slow increase in optical density (OD) of platelet rich plasma (PRP), the second being responsible of a rapid decrease, the third, quite late, causing a wave of increase in OD. This work for the first time correlates biochemical and aggregometric changes with the ultrastructural modifications, as studied by scannig and transmission electron microscope and by morphometry.

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20.8 FREE RADICAL PRODUCTION FOLLOWING THROMBOLYTIC THERAPY IN ACUTE MYOCARDIAL INFARCTION

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In animal models myocardial ischemia and reperfusion are associated with free radical production, although the relevance of such studies to the human myocardium is unclear. We have therefore assessed free radical production following thrombolytic therapy for acute myocardial infarction. Sixty-six subjects were seen within 6 hr of an acute myocardial infarction and received 2 bolus injections of rt-PA (alteplase) separated by 30 min (total dose 70 or 100mg). Coronary patency was assessed by angiography 90 min after the first bolus injection (TIMI 0-3). Malondialdehyde (MDA, μmol/l), a marker of lipid peroxidation, and the major lipid-phase antioxidant vitamin E, (vit E, μmol/mmol cholesterol), were measured at 0, 30, 60 and 90 min, 6 hr and 24 hr using HPLC.

TIMI		0min	30min	60min	90min	6hr	24hr
2or3 (patent)	MDA	0.91	0.95	1.01*	1.02*	0.85	0.90
n=52	vit E	7.13	6.90	6.71	6.64*	6.96	6.96
0or1 (closed)	MDA	0.90	0.96	0.86	0.89	0.88	0.87
n=14	vit E	6.19	6.08	7.62	7.81	6.79	6.48

* = $p<0.05$ compared to 0 min

The rise in MDA and fall in vit E following successful thrombolysis are consistent with significant free radical production. The potential use of antioxidant supplementation with thrombolytic treatment to prevent these changes should be investigated.

20.9 OXYHEMOGLOBIN AND METHEMOGLOBIN AS ANTIOXIDANTS AGAINST HYDROXYL AND ALDEHYDE FREE RADICALS.

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We have shown that reduced gluthatione (GSH) and cysteine are oxidized to GSSG and cystine as well as to products of more profound oxidation, such as cysteic acid, GSO_2H and GSO_3H under the influence of hydroxyl radicals generated by ultrasound during oxyHb oxidation in Fenton's reactions.

Free radicals of aliphatic aldehyds accelerate the oxidation of oxyHb to metHb and also that of cysteine and GSH. Free radicals of alcohols and aldehydes reduce metHb to deoxyHb in a competitive reaction.

In the presence of oxyHb, a decrease of the oxidation rate of cysteine and GSH was observed. Thyil radicals of cysteine, glutathione and aldehyde free radicals reduce metHb to deoxyHb. The oxidation of aliphatic aldehydes free radicals to monocarbonic acids by metHb or their reduction to alcohols by HbO_2 exert a protective effect on red cells, because these processes diminish the level of aldehyde free radicals.

Detection of electron spin resonance signals in blood of HIV seropositive patients. 20.10

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Introduction :
An increased production of oxygen free-radicals (FOR) by unstimulated granulocytes has already been recorded in HIV seropositive patients. This oxidative stress is assessed in such patients by an increase of plasma MDA levels and a decrease of Vitamin E and Selenium in plasma. During endotoxic and haemorrhagic shock in rats, an unequivocal detection of ESR signal attributed to heme NO complexes has been described. Blood samples of 42 patients fullfilling the CDC criteria of AIDS or ARC and of 4 controls immediately after their collection were transferred into esr quartz tube. Spectra were recorded at 102 k on a ESP Bruker.

Results :
Signals were detected in three spectral areas :
-a : $1,500 < H < 1,700$ G ($g \# 4$)
-b : $2,600 < H < 3,600$ G ($g \# 2$)
-c : $5,000 < H < 6,500$ G ($g \# 1$)
Similar signals has been detected in several patients samples and only a very weak signal in area a, in controls. The signals were recorded only in erythrocytes.

Conclusion :
The observed spectra may be related to the presence of at least 3 or 4 different paramagnetic species in low concentration. A this moment there is no sufficient information to assign firmly the signals to any well-definited molecule of complex.

20.11 EFFECTS OF GM-CSF STIMULATION ON VARIOUS HUMAN GRANULOCYTE FUNCTIONS AND POSSIBLE TRANSMEMBRANE SIGNALLING WAYS WITH AGING

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The human hormone granulocyte-macrophage colony stimulating factor (GM-CSF) is an important in vivo regulator of granulopoiesis and neutrophil functions. The addition of GM-CSF to mature human neutrophils primes these cells to stimulation by the chemotactic factor FMLP. Thus, in addition to its role in stem cell proliferation GM-CSF plays a role in host defense. Our aim was to determine whether the GM-CSF has different effects on PMNLs of young and elderly subjects and whether the PT can modulate its action, meaning the participation of a PT sensitive G protein. We measured in case of 10 young (<25yrs) and 10 elderly (>65) healthy subjects the O_2^- production, the intracellular free calcium metabolism, the intracellular killing and ADCC activities of PMNLs. We found in case PMNLs of young subjects that 1. GM-CSF primed the FMLP induced superoxide anion production, while it was not the case for intracellular free calcium; 2. the PT could influence the stimulating activity of GM-CSF, as in case of FMLP; 3. GM-CSF primed also the O_2^- production of PMNLs of elderly, but to a much lesser degree, while it was not the case for intracellular free calcium; 4. the PT did not influence the GM-CSF or FMLP stimulations in case of PMNLs of elderly. The intracellular killing and ADCC activity were stimulated by GM-CSF in case of PMNLs of young and elderly, but to much lesser degree in the latter case. We can conclude that 1. GM-CSF modulate strongly the activity of PMNLs of young subjects via a PT sensitive G protein, 2. while in case of PMNLs of elderly its effect is much less and did not seem to act through the same transmembrane signalling pathway.

OXYGEN RADICAL PRODUCTION BY THE LEUKOCYTES OF FANCONI ANEMIA PATIENTS: THE USE OF ANTIOXIDANT THERAPY 20.12

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Fanconi anemia (FA) is an autosomal recessive disorder, which is supposedly induced by decreased SOD activity and enhanced superoxide production. We found that blood and bone marrow leukocytes of FA patients not only produce the enhanced amounts of oxygen radicals in comparison with normal leukocytes but these radicals contain higher levels of active hydroxyl or hydroxyl-like radicals. These data were obtained from the comparison of luminol- and lucigenin-dependent chemiluminescence intensities of FA and normal leukocytes. We concluded that elevated reactivity of oxygen radicals produced by FA leukocytes may be one of major factors of FA developing. Since bioflavonoid rutin (vitamin P) strongly inhibited luminol-dependent CL produced by FA leukocytes, we applied it for the treatment of FA patients. Long-term rutin treatment of 3 female children resulted in an essential decrease in CL of blood leucocytes, a decrease in the amount of chromosome aberrations, and improvement of hematological characteristics and health of FA patients. Therefore rutin can be recommended as and effective drug for the treatment of FA patients.

20.13 CHEMILUMINESCENCE DETECTED DURING THE FORMATION OF FERRYL HEME FROM PERFERRYL COMPOUNDS

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Recently we postulated the transient existence of a ferryl species in the reaction of hydroxylamines or butylated hydroxyanisole with oxyhemoglobin (1). More detailed studies revealed transient light emission in this reaction. Chemiluminescence was also observed when H_2O_2 was mixed with MetHb or MetMb, but not with hematin. This suggested the following set of reactions:

$$HX\text{-}Hb(III) + H2O2 \rightarrow {}^{\cdot}X\text{-}Hb(IV)\text{-}OH + H2O$$

$${}^{\cdot}X\text{-}Hb(IV)\text{-}OH + H \rightarrow HX\text{-}Hb(IV)=O + H^{+} + light$$

The identity of the light emitting species is unclear. Wavelength analyses of the emitted light as well as the influence of oxygen exclude $^1O_2$ as a source of chemiluminescence in these reaction sequences. The main subject of our investigation is to clarify whether the observed chemiluminescence is characteristic for the involvement of ferryl compounds so that it could be used for their detection.

(1) K. Stolze and H. Nohl, Methemoglobin Formation from Butylated Hydroxyanisole and Oxyhemoglobin. Comparison with Butylated Hydroxytoluene and p-Hydroxyanisole. Free Rad. Res. Comms. (1992), in press.

20.14 HYPOCHLOROUS ACID-INDUCED MOBILIZATION OF ZINC IN ISOLATED RAT HEART MYOCYTES.

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Hypochlorous acid (HOCl), a potent neutrophil oxidant, is suspected of causing injury in reperfused myocardium. We have shown recently that HOCl can cause oxidative damage by mobilizing zinc from isolated metalloproteins. The present study examined the ability of HOCl to cause intracellular zinc mobilization in rat cardiac cells. Isolated myocytes in calcium-free Tyrode solution were loaded with N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide (TSQ), a Zn^{2+}-specific fluorescent chelator. Changes in fluorescence were monitored in single cells with a spectrofluorometer. Superfusion with 1μM Zn^{2+} but not Cu^{2+} (200μM), Fe^{2+} (10μM), or Mn^{2+} (200μM) increased fluorescence, confirming the specificity of TSQ for Zn^{2+}. Superfusion with HOCl (50μM) caused a rapid increase in fluorescence indicative of zinc mobilization. TPEN (10μM), a membrane permeant heavy metal chelator, rapidly decreased fluorescence, but EGTA (100μM), a non permeant zinc chelator, did not, suggesting that Zn^{2+} mobilization occurred intracellularly. Dithiothreitol (DTT,0.5mM), a dithiol membrane-permeant zinc chelator which we have shown to be capable of reversing HOCl injury in heart, also decreased cellular fluorescence. The data suggest that HOCl may cause cardiac injury through the mobilization of cellular zinc, and that membrane-permeant zinc chelators (eg. DTT or TPEN) may be able to reverse this injury.

20.15 COMPARISON OF ANTIOXIDANTS IN HEARTS

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Hearts of various experimental animals /most frequently used in ischemic -reperfusion experiments/ have quite different antioxidant systems. Comparative studies were conducted to detect the relationship between the antioxidant enzymes and lipid peroxidation of different parts of rat, rabbit, cat and dog hearts. At the same time the amounts of the most important water soluble antioxidant, reduced glutathione /GSH/ were compared.

Experimental results showed, the antioxidant systems in dog heart are most similar to that of human heart.

20.16 INCREASE OF Fe^{3+}-INDUCED LIPID PEROXIDATION IN MITOCHONDRIA ISOLATED FROM SENESCENT RAT HEARTS.

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The interrelationship between respiratory function and lipoperoxidation of heart mitochondria, isolated from 3 and 24 month-old male Wistar rats, has been evaluated. The respiratory activity(RCI) did not change during lifetime. Tissue and mitochondrial malondialdehyde (MDA) levels were also unchanged, whereas an elevation of tissue content of lipofuscine was evident in aged rats. When a reaction of lipid peroxidation was induced by incubating the mitochondria with a mixture of ADP and Fe^{3+} for 10 min, an increase of MDA mitochondrial levels and a decrease of RCI values were observed particularly in the aged rats. The increased of Fe^{3+}-induced lipid peroxidation in the mitochondria of the oldest group could be correlated with a parallel increase of C20:4 and C22:6 mitochondrial polyunsatured fatty acid content observed in the same group. These results seem to suggest that heart mitochondria of 24 month-old rats, although do not show an impaired respiratory activity when compared to the young rats, are more exposed to damages induced by peroxidative stress.

Research supported by grants from M.U.R.S.T. and C.N.R. (Ageing project).

20.17 PRESENCE OF AN ACTIVITY THAT REDUCES NITRO BLUE TETRAZOLIUM IN HUMAN ERYTHROCYTE MEMBRANES

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During the study of lipid peroxidation of erythrocyte membranes catalyzed by iron, we have observed the reduction of nitro blue tetrazolium (NBT^{2+}). In our experimental conditions, the ghosts were incubated in the presence of $FeCl_2$ and NBT^{2+}. During this incubation no blue formazan was produced. The reduction of NBT^{2+} occurred only when a chloroform/ methanol mixture was added to the reaction. The reaction needs the presence of $FeCl_2$, is blocked by the iron chelator o-phenanthroline and the extent of colour development depends on $FeCl_2$ concentration. The amount of blue formazan formed depends also on ghost concentration. To assess the specificity of the chloroform/methanol mixture to induce the development of the colour we have tried other solvents in different ratios. Some compounds, with general anesthetic action, were effective whereas others, among which hexane, heptane, benzene, cyclohexane, were not. The activity was lost when the membranes were incubated with sodium dodecyl sulphate.

Selective solubilization of proteins from red blood cell membranes showed that the activity was mainly associated to the Triton X-100 extract.

We developed a method to specifically stain the activity on polyacrylamide slabs. Isoelectric focusing experiments conducted on the solubilized membranes showed that the activity banded approximately with a pI = 6. The activity could be, in fact, adsorbed on DEAE cellulose at pH 7.6 and eluted by increasing the ionic strength.

Studies are in progress to better characterize this activity and to ascertain its function.

This work was supported by grants from Italian M.U.R.S.T.

20.18 ACUTE IRON-LOAD INCREASES AGGREGATION, CALCIUM UPTAKE AND ARACHIDONATE METABOLISM IN RAT PLATELETS.

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We investigated the influence of an *in vivo* acute iron load on several platelet parameters. Iron load was achieved in rats by injecting iron dextran (0.1 mg Fe^{3+}/kg). Analysis were performed 18 h after injection. By comparison with controls, in iron-injected animals, we found significant increases of serum total iron (by 110%) and thrombin- and ADP-induced platelet aggregation (by 350 and 120%, respectively). The thrombin-stimulated release of [^{14}C]-arachidonate preincorporated into platelet phospholipids and metabolite formation (thromboxane B_2), were significantly increased. We also found in plasma an increase (by 67%) of malondialdehyde as well as a decrease of vitamin E (by 60%). When vitamin E was injected the day before iron injection, plasma MDA, platelet hyperactivity and thromboxane biosynthesis were reduced. Given the key role of calcium, we studied and also found that the thrombin-induced Ca^{2+} uptake was increased by 218% in platelets from iron-treated rats by comparison with controls. In contrast, the Ca^{2+} uptake of platelets from iron-loaded animals and pretreated with vitamin E, was greatly reduced. Our results suggest that modification of the calcium homeostasis due to an oxidative injury might explain platelet hyperactivity. In these conditions, vitamin E supplementation has a protective effect on acute iron load-induced platelet abnormalities.

20.19 TIRILAZAD MESYLATE PROTECTS ENDOTHELIUM FROM DAMAGE BY REACTIVE OXYGEN

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Tirilazad mesylate (U-74006F) is a 21-aminosteroid inhibitor of lipid peroxidation under development for the treatment of acute CNS trauma and ischemia. We examined the ability of tirilazad mesylate to protect endothelial function from acute damage by reactive oxygen. Endothelium dependent relaxation to acetylcholine was measured in rabbit aortic rings contracted with 0.25 μM phenylephrine. Acetylcholine produced a dose dependant relaxation of the rabbit aortic rings that was abolished by a 30 min treatment with the superoxide generating system xanthine, 0.4 mM, plus xanthine oxidase, 0.1 U/ml, (X/XO). Relaxation to the endothelium independent vasodilator, nitroglycerine, was not affected by X/XO treatment. Protection against X/XO mediated damage by various antioxidants was assessed by comparing the amount of relaxation produced by 1 μM acetylcholine before and after X/XO treatment. Inhibitors were added to the baths 25 min prior to X/XO. Catalase, 1000 U/ml, and oxypurinol, 10 μM, completely protected against X/XO damage; acetylcholine relaxations were 101±12 and 96±15% of pretreatment control values respectively. Superoxide dismutase, 150 U/ml, partially protected against X/XO (39±11% control) while vitamin E had no significant effect (13±9% control). Tirilazad mesylate, 0.05 μM, protected against X/XO mediated damage to endothelium dependant relaxation (81±9% control). These results demonstrate that tirilazad mesylate can protect endothelial function from damage by reactive oxygen species. Preservation of endothelial function might represent an important component of the activity of tirilazad mesylate *in vivo*.

20.20 THE APOENZYME OF GLUTATHIONE REDUCTASE

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The antioxidant flavoenzyme glutathione reductase (GR) is of interest as a target of drugs against parasitic infections. In regions with a high prevalence of infectious diseases, malnutrition going along with riboflavin deficiency is very common. This deficiency is reflected by a high erythrocyte glutathione reductase activation coefficient (EGRAC) which represents low intracellular FAD levels and a high proportion of inactive apoGR (1).

In vivo FAD, apoGR, and holoGR do coexist (K_d = 53 nM) and apoGR, in contrast to holoGR, cannot be modified by the GR-inhibitor BCNU. In vitro, however, holoGR does not dissociate even at concentrations lower than 1 pM; BCNU modifies isolated apoGR by more than 90%. As factors which might control the in vivo concentrations and protect apoGR from modification, glucose-6-phosphate dehydrogenase (G6PD) and ATP are prime candidates. G6PD interacts sterically with GR and in G6PD-deficiency low EGRAC-values are maintained even in severe malnutrition. MgATP (1 mM) slows down the complementation of apoGR (130 nM) with FAD (410 nM) by more than a factor of 10. A role of apoGR in the regulation of antioxidant mechanisms is discussed.

(1) Becker, Krebs & Schirmer. Internat J Vitam Nutr Res 1991; 61: 180-187.

20.21 OXIDATIVE STRESS RELATED BLOOD PARAMETERS IN AGED PATIENTS SUBMITTED TO ANTIOXIDANT THERAPY.

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It has been proposed that the aging process may result from deleterious and irreversible changes caused by free radicals on cell macromolecules. There is also large evidence indicating the participation of oxygen free radicals in several human pathologies. Despite of controversial data concerning that hypothesis, it is well accepted that the unbalance between the prooxidant and antioxidant activities favouring the first, can lead to oxidative stress condition which may result in cell death. Considering this possibility a group of aged patients, with or without apparent disease, was submitted to antioxidant therapy (metal chelation and daily oral antioxidant supplementation). Clinical behaviour of these patients was evaluated during the treatment. Blood samples of them were taken at the beginning and during the treatment. The total antioxidant capacity of patient's plasma was measured against two different systems: rat brain homogenates undergoing autoxidation and patient's red blood cells challenged with t-butylhydroperoxide. The last system mentioned also allows the evaluation of red blood cell endogenous anti-prooxidant status. Plasma levels of liposoluble antioxidant vitamins were also evaluated. The first results obtained show that the clinical improvement of some patients are accompanied by an increase in plasma and/or red blood cell antioxidant capacity.

20.22 $O_2^{\cdot-}$ GENERATED BY BENZO- AND NAPHTHOQUINONES INCREASES CARDIAC CONTRACTILITY "IN VITRO".

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To ascertain the contribution of quinone-generated active oxygen species in altering myocardial contractility "in vitro", two benzoquinones (2,5-dimethylbenzoquinone and duroquinone) and two naphthoquinones (menadione and its 2,3-dimethoxy analog) were tested for $O_2^{\cdot-}$ generation in guinea-pig and rat subcellular cardiac preparations and for their effect on the force of contraction of electrically-driven left atria.

Our results indicate that:

- in both guinea-pig and rat cardiac tissue benzoquinones generate $O_2^{\cdot-}$ mainly (90%) through mitochondrial one-electron reduction, naphthoquinones through both mitochondrial one-electron reduction (70%) and soluble two-electron reduction (DT-diaphorase) (30%);
- only quinones which generate $O_2^{\cdot-}$ and active oxygen species are able to increase cardiac contractility through a catecholamine-mediated mechanism, the effect being antagonized by scavengers of active oxygen species, SOD, catalase, mannitol and desferoxamine;
- a linear relationship (r=0.90) between the amount of $O_2^{\cdot-}$ generated by benzo- and naphthoquinones in guinea-pig and rat cardiac tissue and the extent of the catecholamine-mediated positive inotropic effect is evident.

20.23 IN VITRO STUDY OF DAMAGE INDUCED BY PRODUCTS OF LIPID PEROXIDATION ON CARDIAC TISSUE: COMPARISON OF THE EFFECTS OF AN ANTIOXIDANT AND AGENTS INTERFERING WITH CALCIUM REGULATION.

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In this work, we evaluate the effects of oxidative stress induced by products of lipid peroxidation using 3 "in vitro" models: (1) kinetics of lipid peroxidation, (2) necrosis of cardiac cells and (3) electromechanical coupling of papillary muscle. We compare the activity of a powerful antioxidant (BW755C) with that of 2 agents interfering with cellular calcium regulation (amlodipine, R56865). These 2 molecules are devoid of intrinsic reactive oxygen species (ROS) scavenging properties.

First model: Only BW755C inhibited various kinetic parameters of non-enzymatic peroxidation of arachidonic acid initiated by photochemically produced ROS. Second model: Amlodipine and R56865, but not BW755C, protected cardiomyocytes against necrosis induced by calcium paradox. In contrast, only BW755C protected cultured cardiac cells against oxidative necrosis initiated by peroxidized lipid. Third model: BW755C and R56865 completely inhibited the electromechanical disturbances induced by peroxidized lipid on guinea-pig papillary muscle.

These results suggest that products of lipid peroxidation may play an important rôle as a "relay" of the toxic effects of unstable and labile ROS. The electrophysiological results suggest that products of lipid peroxidation may induced ionic disturbances interfering with intracellular calcium homeostasis. These alterations were inhibited both by an antioxidant (BW755C) and by an agent protecting cardiac cells against calcium overload (R56865).

20.24 PURIFICATION OF RED CELL GLUTATHIONE PEROXIDASE ON A L-PENICILLAMINE-SEPHAROSE AFFINITY COLUMN

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Hamster liver glutathione peroxidase (GSH-Px, EC 1.11.1.9) is strongly inhibited by penicillamine (1). We have investigated the use of this inhibitor as an affinity ligand for the purification of red cell GSH-Px. L-penicillamine was coupled according to the manufacturer's instructions to Sepharose 4B, linked to an activated 6-aminohexanoic acid spacer arm. GSH-Px from whole blood haemolysate was first partially purified on a DEAE-Sepharose column by a modification (2) of the method of Corrocher *et al.* (3). This step typically gives a 50-fold purification. The partially purified enzyme was applied to the affinity column, which had been equilibrated with 0.1 M potassium phosphate buffer, pH 8.0. Unbound proteins were washed through with the equilibrating buffer, and GSH-Px was eluted with the same buffer, containing 50 mM glutathione and 0.5 M NaCl. Enzyme activity was measured by a modification of the method of Agergaard & Jensen (4) in a Thermomax microplate reader (Molecular Devices Corporation). The affinity step typically gives a 450-fold purification, and an overall purification of 22000 is thus obtained in two easy steps with good yield. However, D-penicillamine, which inhibits red cell GSH-Px to a similar degree as the L-isomer, is not a useful affinity ligand for the isolation of the enzyme. We conclude that L-penicillamine, coupled via a spacer arm to Sepharose 4B, is a useful affinity ligand for the purification of red cell glutathione peroxidase.

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20.25 METABOLISM OF QUINONES AND $O_2^{\cdot-}$ GENERATION IN GUINEA-PIG AND RAT CARDIAC PREPARATIONS.

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Two benzoquinones (2,5-dimethylbenzoquinone and duroquinone) and two naphthoquinones (menadione and its 2,3-dimethoxy analog) were tested for their metabolism through mitochondrial and microsomal NAD(P)H-dependent one-electron reductases and soluble two-electron DT-diaphorase, and for their ability to generate $O_2^{\cdot-}$ in subcellular cardiac preparations from guinea-pig and rat.

Our results show that:

- mitochondrial reductase and soluble DT-diaphorase account for about 90% and 10%, respectively, of benzoquinone metabolism, whereas mitochondrial reductase and soluble DT-diaphorase account for about 50% and 50% of naphthoquinone metabolism;
- the total metabolizing capacity of guinea-pig is higher than that of rat, the apparent V_{max} of DT-diaphorase being 2-4-fold higher in guinea-pig than rat, while the apparent K_ms are similar;
- both one- and two-electron reductions are responsible for quinone-induced $O_2^{\cdot-}$ generation.

The relationship between metabolism and $O_2^{\cdot-}$ generation is shown by the higher amount of $O_2^{\cdot-}$ generated in guinea-pig than in rat. This relationship is lacking for duroquinone; although metabolized to a great extent, it does not form $O_2^{\cdot-}$.

20.26 GENERATION OF OXYGEN SPECIES BY PHAGOCYTIC LEUCOCYTES IN PLATELET AND LEUCOCYTE-RICH PLASMA.

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Routine chemiluminescence (CL) technique to study generation of oxygen species by leucocytes involves several centrifugation steps to isolate neutrophils which are then suspended in a buffer for study, or the study is carried out in highly diluted whole blood. Both methods are unphysiological and may not reflect in vivo phagocyte oxidative function. In this study, we removed 99% of the red cells by mixing 9 vol. of heparinised human blood with 1 vol. of 6% dextran T-70 without centrifugation, and the platelet and leucocyte-rich plasma obtained was used for CL experiments without further separation; phagocyte count was adjusted to 10^6/ml with Hanks' buffer. In this medium, phagocytes responded to stimulation by opsonized zymosan or FMLP, producing strong luminescence intensity (LI) in the presence of a highly sensitive luminescence enhancer, MCLA. The LI was abolished by 0.5μM SOD, suggesting that the luminescence reaction is attributable to $O_2^{\cdot-}$, but the LI was also partially inhibited by 20μg/ml catalase or 15μM azide, indicating some contribution to the LI by H_2O_2, HClO, myeloperoxidase system. As this medium contains all the leucocyte populations, platelets, residual red cells and plasma proteins, it is a step towards a physiological condition for studying phagocyte-generated oxygen free radicals using the CL technique. The exhaustion of leucocytes by repeated centrifugation should be avoided.

20.27 ESR STUDIES ON NEUTROPHILS – CYTOCHROME *b*-558 AND PEROXIDASES

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Neutrophils have a unique membrane-bound electron transport chain named the NADPH oxidase system, which consists of a membrane-associated cytochrome b_{558} (cyt b-558) and cytosolic components. Cyt b-558 has been proposed to be the terminal site of the electron transport chain, i.e., the $O_2^{\cdot-}$-forming site, but its functional role has not been determined. We examined ESR spectra of cyt b-558 in concentrated cellular and subcellular fractions of pig blood granulocytes. A thick cell suspension containing mostly neutrophils showed typical high spin ESR signals due to myeloperoxidase (MPO) and a low spin signal at g value of 3.20. A similar thick granulocyte suspension containing eosinophils showed not only these signals but also another low spin heme signals at g values of 2.86, 2.13 and 1.66, which has been previously reported to be cyt b-558. MPO and eosinophil peroxidase (EPO) were released from the membrane fractions, and then were highly concentrated, in which no cyt b-558 was detected by their absorption spectra. The signal at g_z value of 2.86 was found only in the EPO fraction, suggesting that this signal is derived from a low spin form of an EPO-complex, but neither from MPO nor cyt b-558. The $O_2^{\cdot-}$-forming NADPH oxidase was solubilized from the peroxidase-depleted membranes and concentrated up to 45 μM cyt b-558. Cyt b-558 showed an anisotropic ESR signal (g=3.20), which was cyanide-insensitive and reducible with reductants. These results suggest that the g=3.20 signal is characteristic of the low spin heme iron in cyt b-558.

20.28 GLOBIN-HEME INTERACTION IN FREE RADICAL-INDUCED HEMOGLOBIN OXIDATION

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Oxidative injury to Hemoglobin (Hb) is associated with formation of metHb and degradation to a group of pigments collectively called hemichromes. In intact red blood cells, this denaturated Hb precipitates in the form of Heinz bodies that are attached to the red cell membrane. We have investigated the role of carbon and oxygen-centered radicals in the formation of Hb irreversible cross-linking and in the oxidation of aromatic aminoacids during the denaturation process. As radical source we used an azocompound (AAP) that, by thermal decomposition at 37°C, produces a carbon radical and, in the presence of oxygen, peroxyl/alkoxyl radicals. The electrophoretic analysis of AAP-oxidated Hb showed a time-dependent loss of Hb monomer and the formation of Hb-dimer and oligomers. In agreement with a low heme oxidation observed at high pO_2, the increase of oxygen concentration reduced by 24% Hb aggregation. The protective effect of oxygen was more evident on Hb oligomers. At high pO_2 a protective effect was also observed on oxidation of aromatic aminoacid while at low pO_2 this process was accelerated. We hypothesize that AAP carbon radicals can interact with some globin aromatic aminoacid working as electron-transfer centers for the heme iron

20.29 ANTIOXIDANT DEFENSES, LIPID PEROXIDATION AND PROLIFERATIVE RESPONSE IN SPORTSMAN LYMPHOCYTES

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Physical exercise results in an increased production of oxygen free radicals and it produces changes in immune cell function, concretely on lymphocytes, the principal cells implicated in the immune response.The present work represents the first time that antioxidant levels in blood lymphocytes from sportsmen are measured and correlated with the most representative property of lymphocytes, its proliferative response. Fifty elite sportsmen of different modalities were distributed in 2 groups: A) the aerobic group (bicyclists) and B) aerobic-anaerobic group (athletes and canoeists). In addition, 25 subjects with moderate training exercise (C group) and 25 sedentary controls were studied. Superoxide dismutase (SOD) activitiy was measured by spectrophotometric method, and malonaldehyde, vitamin E and vitamin C concentrations by HPLC. The proliferative response was estimated as ^{3}H-thymidine incorporation in cultures of 72 hours in the presence of the mitogen phytohemagglutinin (PHA). The results indicate that in relation with the control group SOD activities increased in B and C groups and they were not modified in A group. The vitamin C concentration increased in A and C groups, but decreased in B. The vitamin E concentration increased in A and C groups and was unchanged in group B. Malonaldehyde was increased in B and C groups and decreased in group A. The lymphoproliferative response was increased in group C and decreased in the other groups respect to the controls. Therefore, no relation was found between the levels of antioxidants and lipoperoxidation, and the proliferative function of lymphocytes.

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20.30 THE MAIN MOLECULAR SPECIES OF PHOSPHOLIPID HYDROPEROXIDES IN ERYTHROCYTE MEMBRANES OF CHILDREN.

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We report the concentrations of four individual molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and their corresponding hydroperoxides after lipoxygenase incubation in erythrocyte membranes of control children. After separation of PC and PE with Silica Sep-Pack column, we used a single RP 18 column to separate the molecular species and their hydroperoxides. The molecular species were detected at 205 nm and the eluate was then mixed with a chemiluminecence reagent to detect the corresponding hydroperoxides. Furthermore we determined α-tocopherol in plasma and erythrocyte membranes The hydroperoxide concentrations of PC 16:0/20:4, 16:0/22:6, 18:0/22:6 and PE 16:0/18:2 were positively related with membrane α-tocopherol concentrations. These last results suggest that when α-tocopherol level was low, an observed lower concentration of hydroperoxides was probably due to an overproduction of secondary products which were not detected in our experimental conditions On the other hand, the α-tocopherol membrane concentration was not correlated with the plasma level usually performed. In conclusion our work underlines the key role of membrane α-tocopherol upon the hydroperoxides formation in presence of lipoxygenase.

20.31 THE CLINICAL EFFECT OF SUPEROXIDE DISMUTASE ON REPERFUSION INJURY IN ACUTE MYOCARDIAL INFARCTION

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Human recombinant superoxide dismutase (h r SOD) was administered before reperfusion to patients with acute myocardial infarction undergoing successful percutaneous transluminal coronary recanalization (PTCR) within 6 hours, and the effects on cardiac function, reperfusion arrhythmias, and the infarct size investigated. The subjects comprised the control group (n 6, 60.5 ± 4 years) and SOD administered group (n 6, 54 ± 10), all of whom had a first myocardial infarction with blood flow successfully reestablished by PTCR within 6 hours of onset, infarct site proximal to the first diagonal branch of the LAD, and no collaterals. 3,500U/kg SOD was administered by iv bolus injection just prior to PTCR followed by 31,500U/kg by continuous drip infusion over a 120 min period. The degree of infarction was judged by the extent and severity scores determined by ^{201}Tl SPECT in the acute and chronic phases, and cardiac function evaluated by RIEF performed in the acute and chronic phases. Also, the degree of ventricular arrhythmias development was determind from 24-hour Holter ECGs. 【Results】 SOD administration showed no significant cardiac function ameliorating effect or infarct size reducing effect, although it significantly suppressed the development of ventricular arrhythmias within 24 hours after reperfusion.

20.32 EFFICIENCY OF GLUTATHIONE RECYCLING DURING HYDROGEN PEROXIDE STRESS IN ERYTHROCYTES OF NEWBORN BABIES AND ADULTS.

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Hydrogen peroxide catabolism by erythrocytes plays an important role in protecting the lung against oxygen toxicity. This mechanism may be particularly important in preterm babies, whose pulmonary antioxidant enzyme systems are immature. We compared, in vitro, the glutathione recycling system in erythrocytes of babies (cord blood, n=8 preterm and n=9 term) and of adults (n=10). The concentration of glutathione peroxidase was lower ($p<0.05$) and glutathione reductase higher ($p<0.05$) in the babies. In all subjects incubation of erythrocytes with H_2O_2, with or without inactivation of catalase, caused a rapid depletion of reduced glutathione (GSH) and concomitant accumulation of oxidized glutathione (GSSG) followed by recovery of GSH and fall of GSSG to initial values. Inactivation of catalase resulted in a 50% loss of intracellular glutathione ($p<0.005$), a larger maximum GSH-depletion ($p<0.05$) and a longer GSH-recovery time ($p<0.005$). Erythrocytes from newborn babies showed a smaller maximum GSH-depletion ($p<0.05$) and a shorter GSH-recovery time ($p<0.005$) compared with those from adults. These differences between the newborn and adult group persisted after inactivation of catalase. The efficient glutathione recycling was maintained in the first postnatal week in babies who were healthy or suffering from acute respiratory distress. Erythrocytes of newborn babies have a more efficient glutathione recycling compared with those of adults and this may help prevent the development of chronic lung disease in the preterm baby.

20.33 EFFECT OF NITRONE SPIN TRAPS AND NITROXYL RADICALS ON ISCHEMIC HEART INJURY

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Nitrone spin trap compounds are frequently used to detect free radicals by means of the ESR spin trapping technique in the ischemic heart. Via the spin trapping, nitroxyl radicals are formed. However, little is known about direct interactions of the nitrones and nitroxyl radicals with the heart. The nitrone spin trap dimethylpyrroline oxide (DMPO, 100 μM) was perfused into the isolated rat heart during postischemic reperfusion (30 min total and global ischemia, 30 min reperfusion): irreversible ventricular fibrillation (VF) was completely abolished; the incidence and duration of VF, ventricular tachycardia (VT) and the number of ventricular ectopic beats were reduced. The postischemic recovery of left ventricular developed pressure (LVDP), contractility ($LVdp/dt_{max}$), aortic and coronary flow were significantly improved compared to the untreated control ($p<0.05$, $n=10$). The nitroxyl radical pentamethylimidazoline oxyl (PMIO·, 2000 μM), which is considered as a nitroxyl derivative of DMPO, significantly reduced the incidence of VF and the duration of VT. The functional recovery also was better in comparison to the control ($p<0.05$, $n=12$). Comparing the effective concentration range of two compounds studied, it is concluded that the cardiopretective effect of DMPO is rather related to the radical trapping activity of the molecule than to the nitroxyl radical formed after the trapping procedure.

20.34

Reduced glutathione peroxidase activities as potential ischaemic heart disease risk factors.

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Plasma, platelet and erythrocyte glutathione peroxidase activities were measured in ischaemic heart disease patients and comparison subjects matched for age, sex, race, smoking habit and month of vene puncture. Both mean platelet and plasma peroxidase activities were significantly lower in IHD patients compared to controls ($p=0.0001$). Mean erythrocyte peroxidase and plasma lipid levels were similar in the two groups.
No correlation was observed between plasma, platelet and erythrocyte glutathione peroxidase levels. Examination of plasma and platelet enzyme values in a two dimensional manner showed the IHD group to be heterogenous and the combination of the two variables produced an 86% discrimination between IHD and non-IHD subjects. Our data suggest that plasma glutathione perosidase amy be relevant to the control of intravascular atherogenic lipid products and be a previously unrecognised disease risk factor.

20.35 EVIDENCE OF OXIDATIVE DAMAGE IN ALCOHOLIC PATIENTS: RELATIONSHIP WITH ALCOHOL INTAKE.

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The involvement of free radical mediated damage in the pathogenesis of alcoholic liver diseases is still matter of debat mainly because the lack of convincing evidence supporting the presence of oxidative injury in humans. To elucidate this point we have exhamined 26 alcoholics that were grouped according to the estimated daily alcohol consumption as ALC1 (100-200 g ethanol/day) and ALC2 (> 200 g ethanol/day) and 20 controls all drinking less than 100 g ethanol/day. In ALC1 and ALC2 groups the level of malonildialdehyde (MDA) in the erythrocytes and plasma lipid hydroperoxides (LOOH) were found to be, respectively, 2-3 and 5-6 time higher than in controls. Significant differences in MDA and LOOH values were also appreciable between ALC1 and ALC2. The detection of lipid peroxidation was associated with a 50% decrease in the erythrocyte GSH content of both ALC1 and ALC2 groups. Similarly, plasma vitamin E levels in ALC1 and ALC2 were also significantly different from controls, but not among the alcoholics groups. These results demonstrates that signs of oxidative damage are actualy present in alcoholics and correlates with the alcohol consumption, but not with changes in the antioxidant content of blood.

AFANAS'EV	IGOR	12.1
ALCARAZ	MARIA JOSE	8.5
ALLEN	D. RANDAL	6.5
ALMEIDA	LEONOR MARTINS	2.10
ALOJ TOTARO	EUGENIA	1.8
ANDERSSON	CARL-MAGNUS	6.11
ANNONI	GIORGIO	C.5
ARAGNO	MANUELA	6.31
ARAHUETES	ROSA MARIA	15.10
ARDUINI	ARDUINO	20.5
ARRIO	BARNARD	2.16
ARUOMA	OKEZIE I.	11.11, 14.3
ASADA	KOZI	8.1
ASMUS	KLAUS-DIETER	6.2
ASPBERG	ANDERS	10.10
AUGUSTO	OHARA	14.14, 20.4
AZZI	ANGELO	7.5
BACON	BRUCE	C.4
BAGNASCO	M.	A.5
BAGNATO	GIANFILIPPO	5.8
BALANSKY	R.M.	A.5
BALDI	ELISABETTA	9.20
BANNI	SEBASTIANO	11.2
BARENGHI	LIVIA	18.9
BARNARD	HENDRIK C.	9.10
BARROS	S.B. DE MOREAS	11.9
BARTOLI	GIANNA MARIA	14.8
BASAGA	HUVEYDA	6.13
BATTELLI	MARIA GIULIA	17.16
BAUD	LAURENT	5.4
BECHARA	ETELVINO	18.13
BECK SPEIER	INGRID	5.22
BECKER	KATJA	20.20
BECKMAN	JOSEPH S.	13.4
BELLOMO	GIORGIO	9.24, 16.26
BENOV	LUDMIL C.	9.5
BENNICELLI	C.	A.5
BERGER	MAURICE	19.10

BIASI	FIORELLA	4.23, 9.29
BINDOLI	ALBERTO	9.11
BISBY	ROGER HUGH	6.3
BLA'ZOVICS	ANNA	15.8
BLACHE	DENIS	12.5, 20.18
BLASIG	INGOLF	12.30, 20.33
BORNMAN	LIZA	10.12
BORRELLO	SILVIA	7.4
BOTTI	BARBARA	4.25
BOYER	SCOTT	3.4
BOVERIS	ALBERTO	18.2
BRADAMANTE	SILVIA	9.3
BRUCHELT	GERNOT	10.4
BUC CALDERON	PEDRO	9.13
BUDINI	ROLANDO ANTONIO	8.7
BUKO	VYACHESLAV	4.11
BULKLEY	GREGORY B.	D.3
BURDON	ROY HUNTER	14.6
BURTON	GRAHAM	16.1
BUSTOS	GLORIA	17.30
BUTLER	JOHN	4.5
BUZADZIC	BILJANA	16.16
CABELLI	DIANE ESTHER	1.3, 1.10
CADENAS	ENRIQUE	6.1
CADET	JEAN	19.1
CAJONE	FRANCESCO	12.13
CAMARERO	VALERIA	17.20
CAMOIRANO	A.	A.5
CANTONI	ORAZIO	16.22
CANUTO	ROSA ANGELA	5.15
CAPOLONGO	FRANCESCA	15.11
CARGNONI	ANNA	9.14
CARINI	RITA	8.6
CARPENEDO	FRANCESCA	20.22
CASINI	ALESSANDRO	16.6
CATTERALL	HELEN	19.15
CAVALIERI	ERCOLE L.	4.4
CERRUTI	PETER	B.1
CHARVERON	MARIE	15.12

CHEESEMAN	KEVIN H.	B.4
CHEVALIER	GASTON	12.36
CHING	TJONG-LIE	17.22
CHIRICO	SUSANNA	10.15, 12.24
CHOPRA	MRIDULA	6.21
CHRISTIE	NEIL A.	5.27
CINI	MASSIMO	12.14
CIZ	MILAN	6.19
CLOT	PAOLO	20.35
COMOGLIO	ADRIANA	4.31, 8.13
COMPAN	INES	7.7
CONTE	ENRICO	6.14
CONTI	MARC	12.16
COTTALASSO	DAMIANO	4.12
CROSS	CARROL E.	5.1
CURZIO	MARINA	17.26
CUTLER	RICHARD	A.1
D'AGOSTINI	F.	A.5
DARGEL	ROLF	4.19
DARLEY-USMAR	VICTOR	13.1
DATTILO	M.	D.4
DAVIES	JOANNA M.S.	17.6
DAVIES	MICHAEL J.	2.12
DAVIES	KELVIN	7.1
DE FLORA	S.	A.5
DE LAMIRANDE	EVE	5.12
DE MATTEIS	FRANCESCO	18.17
DE PASCALE	ANTONELLA	5.26
DEAN	ROGER	A.2
DEL RIO	LUIS A.	16.23
DELLA ROVERE	FILIPPO	14.9
DI MASCIO	PAOLO	12.7
DI MAURO	CLELIA	17.27
DI SIMPLICIO	PAOLO	4.15
DINIS	TERESA	17.18
DIZDAROGLU	MURAT MIRAL	19.2
DJUJIC	IVANA	9.16
DROY-LEFAIX	MARIE-THERESE	15.9
DUTHIE	GARY	10.2

EGNER	PATRICIA	14.19
EK	BENGT	12.26
EMERIT	JACQUES	16.5, 20.10
ESTERBAUER	H.	PM2
EVERETT	STEVEN	6.18
FARR	SPENCER	7.3
FARRUGGIA	GIOVANNA	12.17
FEHER	JANOS	15.1
FEIG	DANIEL	19.6
FELDMAN	DAVID L.	2.19
FERRALI	MARCO	18.6
FERRANDEZ	DOLORES	20.29
FERRANDIZ	MARIA LUISA	17.29
FERRARI	ROSA PIA	4.32
FERRO	MARGHERITA	4.13
FIORUCCI	PATRIZIA	12.32
FLISS	HENRY	20.14
FLITTER	WILLIAM D.	20.1
FLOREANI	MAURA	20.25
FOURNIER	JEANINE	14.7
FRAGA	CESAR	19.3
FRANCONI	FLAVIA	16.10
FRANZINI	ELIZABETH	1.11
FREDERIKS	WILMA MARIA	9.9
FREISLEBEN	HANS J.	10.11
FUBINI	BICE	14.4
FUJII	HIROTADA	20.27
FULOP	TAMAS	17.9
GADALETA	MARIA NICOLA	10.14
GAGNON	CLAUDE	5.13
GASBARRINI	ANTONIO	4.22, 9.25
GEBICKI	JANUSZ M.	2.13, 2.14
GELVAN	DAN	6.22
GERBER	GERHARD	9.2
GERBER	MARIETTE	14.5
GHEZZI	PIETRO	17.32
GHIGO	DARIO	13.11
GIDROL	XAVIER	7.11
GIESEG	STEVEN P.	6.20

GOLDSTEIN	BERNARD	B.2
GONENNE	AMNON	5.19
GONZALEZ FLECHA	BEATRIZ S.	9.27
GOSS SAMPSON	MARK	15.4
GOTOH	NAOHIRO	2.8
GREEN	EMMA	12.31
GREENLEY	TINA	14.12
GRUNE	TILMAN	12.22
GUARNIERI	CARLO	9.6, 20.16
GUICHARDANT	MICHEL	11.7
GUIXIAN	GAO	12.37
GUTIERREZ	PETER L.	4.7
GUYTON	KATHRYN Z.	14.18
HAKIM	GABRIELE	20.17
HASEGAWA	TOHRU	10.5, 10.9
HASELOFF	REINER	8.4
HAUCK	MATYAS	9.8
HENNEKENS	CHARLES H.	B.3
HENROTIN	YVES	9.15
HEWGILL	FRANK	11.3
HICKS	MARK	16.7
HIRSCHELMANN	ROLF	17.11
HOGG	NEIL	2.6
HOLLEY	ANNE	12.12
HONN	KENNETH V.	3.3
HORVATH	MONIKA	15.7
HU	MIAO LIN	17.5
HUNT	NICHOLAS	17.2
IINUMA	SHOJI	15.18
ILIOU	JEAN PIERRE	20.23
INGELMAN-SUNDBERG	MAGNUS	C.3
IULIANO	LUIGI	3.7
IZZOTTI	A.	A.5
JAMIESON	DANA	12.29
JANSSEN	YVONNE	14.11
JOCHMANN	CLAUDIA	4.9
JOVANOVIC	SLOBODAN	6.16
JUNQUEIRA	VIRGINIA	20.21
KAGAN	VALERIAN	15.2

KALYANARAMAN	BALARAMAN	2.2, 9.28
KANBAYASHI	YASUHIRO	12.27
KANEDA	HAJIME	6.30
KANEKO	TOSHIRO	17.14
KANTENGWA	SALOMÉ	16.11
KASAI	HIROSHI	19.9
KAUR	HARPARKASH	5.25
KAWAMURA	NAOHISA	5.23
KELLY	FRANK J.	5.2
KETTLE	ANTHONY	4.3
KOKURA	SATOSHI	17.13
KOLTOVER	VITALI KIMOVICH	10.8, 13.6, 18.10
KOOIJ	ARNOLD	9.21
KORAC	BATO	4.20
KORKINA	LUDMILA	5.31, 20.12
KORPELA	HEIKKI	11.6
KORWIN-ZMIJOWSKI	CARLA	16.17
KOSTIC	MILOSAV	4.6
KOSTYUK	VLADIMIR A.	6.10
KRAMSCH	DIETER M.	2.17
KRAUTH-SIEGEL	LUISE	14.15
KUBISH	HANS-MICHAEL	19.11
LAMBELET	PIERRE	11.4
LANDI	LAURA	6.4
LANGLEY	SIMON	5.9, 5.10, 5.11
LECCIA	MARIE THERESE	1.6
LEITE	LUCIANA	4.17
LEMASTERS	JOHN J.	9.1
LENZ	ANKE-GABRIELE	5.24
LEONCINI	GIULIANA	18.11
LEVINE	RODNEY L.	7.8
LILES	MARK	15.5
LIN	YI GUANG	12.34
LINDAHL	RONALD	14.2
LINDLEY	KEITH	12.18
LINK	EVA MARIA	3.5, 16.14
LISSI	EDUARDO	12.6
LIU	GENG TAO	8.8
LIVREA	MARIA A.	12.28

LLESUY	SUSANA F.	10.19
LOJEK	ANTONIN	1.9
LOPEZ-TORRES	MONICA	5.3, 12.25
LUCCHI	L.	5.34
LUX	ORA	15.13, 18.16
MADRA	SUKHDEEP	14.13
MAIORINO	MATILDE	16.3
MALLOZZI	CINZIA	20.28
MARESCA KOSCHATZKY	MARIA	17.17
MASINI	ALBERTO	6.12
MASON	RONALD P.	4.1
MASOTTI	LANFRANCO	12.3
MASUDA	SADAO	9.4
MASUMOTO	NOBUYUKI	18.14
MATSUMOTO	SHIGENOBU	6.27
MATSUO	MITSUYOSHI	6.26
MATSUYAMA	KIICHI	17.15
MAVELLI	IRENE	6.32
McARTHUR	KATHRYN	6.25
MEDEIROS	MARISA HELENA	19.13
MEISTER	ALTON	D.1
MENEGHINI	ROGERIO	19.8
METSA-KETELA	TIMO	2.15
MIGLIETTA	ANTONELLA	12.19
MIKI	MASAYUKI	16.4
MILLER	NICHOLAS	5.30
MINAMIYAMA	YUKIKO	13.8
MINISCI	F.	D.4
MINO	MAKOTO	D.2
MINOTTI	GIORGIO	18.4
MIRABELLA	FRANCESCA	4.29
MITSUI	AKIRA	9.22
MIYATA	NAOKI	4.30
MKRTCHIAN	SOUREN	4.10
MOHACSI	ATTILA	18.8
MOISON	RALF	5.29, 20.32
MONNIER	VINCENT M.	A.3
MONTESINOS	MARIA D. CARMEN	8.10
MORGANTE	EMANUELA	15.19

MOUTET	MARC	16.18
MULLER	DAVID	10.16
MURACA	ROBERTO	7.12
MURPHY	MICHAEL	13.2
MURPHY	JOHN A.	19.7
MURRELL	GEORGE ANTHONY C.	10.6, 10.7
NAGANO	TETSUO	18.15
NAGATA	YUICHIRO	2.9
NAITO	YUJI	13.5
NAKAHASHI	YAYOI	13.7
NAKAMURA	SATOSHI	16.12
NAKAMURA	MASAO	6.23
NALVARTE	ELISABET	19.4
NAVAB	MAHAMAD	C.1
NAVIDAD-NOVALVOS	RAFAEL	9.19
NERIISHI	KAZUO	1.2
NIEMINEN	ANNA LIISA	15.6
NISSINEN	EAKKI AARNE	16.13
NOGUCHI	NORIKO	2.4
NOHL	HANS	20.3, 20.13
ODETTI	PATRIZIO	15.3
OHKUBO	SHINJI	20.31
OKADA	SHIGERU	5.17
OLDFIELD	FIFI F.	10.3
ORAK	JOHN	5.6
OSIPOV	ANATOLY	6.24
OTEIZA	PATRICIA	12.38
PACE-ASCIAK	CECIL ROBERT	3.1
PAGANGA	GEORGE	2.5
PALASCIANO	A.	D.4
PALOZZA	PAOLA	16.20
PAOLETTI	FRANCESCO MAURO	14.10
PAROLA	MAURIZIO	7.6, 16.25
PARSONS	BARRY J.	17.31
PASQUIER	CATHERINE	1.12
PEA	FEDERICO	5.18
PEARSON	DAVID	20.34
PEREZ-CAMPO	ROSA MARIA	15.15
PERIN	MARIA	19.12

PHILLIPS	GARY	5.7
PIAZZA	GIUSEPPE	5.33, 14.22
PIETRAFORTE	DONATELLA	13.10
PINAMONTI	SILVANO	5.14
PINCEMAIL	JOEL	5.5
POCH	BERTRAN	9.7
POGIRNITSKAYA	ANGELIKA	4.14, 14.20
POMPELLA	ALFONSO	12.15
POTAPOVICH	ALLA IVANOVA	4.27
POVOA	HELION	12.33
PRONAI	LASZLO	17.8
PUHL	HERBERT	2.1
PULCINELLI	FABIO	20.7
PUNTARULO	SUSANA	4.16
PUPPO	ALAIN	8.3
PUTVINSKY	ALEXEY	17.10
QUARESIMA	VALENTINA	4.8
RABL	HANS	10.13
RAMDATH	DAN D.	18.12
RAO	GOVIND	19.14
RAVINDRANATH	VIJAYALAKSHMI	9.26
REMACLE	JOSE	16.8
REZNICK	ABRAHAM	10.1
RIGOBELLO	MARIA PIA	9.12
RILEY	PATRICK A.	14.1
ROCH-ARVEILLER	MONIQUE	17.21
RODNEY	MATHEWS	20.19
ROMEO	MARIA RAFFAELLA	6.15
ROSSI	TIZIANA	4.28
ROTILIO	GIUSEPPE	20.2
ROYALL	JAMES A.	5.20
RUULS	SIGRID R.	10.18
RYTER	STEFAN	7.9
SAGAI	MASARU	14.24
SAICIC	ZORICA S.	16.15
SANDSTROM	JAN	17.3
SANIABADI	ABBY	20.26
SANZ	MARIA JESUS	8.11
SARAN	MANFRED	13.3

SCHAUR	RUDOLF	12.21
SCHOENBERG	MICHAEL H.	17.28
SCHOENHEIT	KATRIN	9.23
SCHONEICH	CHRISTIAN	6.8
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