MATERIALS AND METHODS

1. Animals

Male Swiss albino mice, 6-8 weeks old, weighing 25-30 g, were used in all the experiments. The animals were housed in polypropylene cages at 25 ± 2°C and allowed *ad libitum* access to tap water and standard mouse diet (Lipton India Ltd., India). The bedding material consisted of paddy husk which was changed regularly once in two days.

2. Irradiation

Irradiation was carried out in a gamma-chamber, ^{60}Co source, 204 TBq (5500 Ci), obtained from Bhabha Atomic Research Centre, India. The dose rate of the source was reduced by a factor of 19 using a 5 mm thick lead shielding. The dose rate (with the attenuator) decreased from 2.8 to 2.3 Gy/min during the period of study, as determined by Fricke's Ferrous sulphate dosimetry. Animals were restrained within individual compartments in a well-ventilated acrylic cylinder and placed in the gamma chamber for whole-body exposures.

3. Chemicals

All chemicals were of analytical grade and used without further purification. Ascorbic acid, α-tocopherol, β-carotene, caffeine, cysteamine and buthionine sulfoximine were purchased from Sigma (MO, USA). Foetal calf serum was from Gibco (Scotland). All other chemicals/reagents used in the present study were either from Sigma or from standard companies like Glaxo, SRL and BDH.

4. Chlorella vulgaris E-25

Dried samples of the algae E-25 strain were kindly provided by Prof. Masahiro Ogaki of the Pasteur Institute of Kyoto, Japan.

5. Sample preparation for bone marrow micronucleus test

Animals were killed by cervical dislocation and bone marrow from both femora was flushed out as a fine suspension into an eppendorf tube containing 1 ml of foetal calf serum. This suspension was centrifuged at 2000 rpm for 5 min and the pellet was resuspended in one drop of serum. A small drop of the cell suspension was placed on a clean slide and smeared with another slide. After air drying overnight, these were stained in May-Grunwald's and Giemsa stains. For each animal, 2500 polychromatic erythrocytes (PCEs) were scored for the presence of micronuclei (Mn). All slides were coded and scored blindly. The international guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes,as described in MacGregor *et al.* (1987), were rigidly followed.

6. Sample preparation for the analysis of chromosomal aberrations in metaphase cells of bone marrow

The recommended guidelines for conducting bone marrow chromosomal aberration analysis in mammals were followed (Preston *et al.* 1987). All animals were given colchicine (5mg/kg) by an intraperitoneal injection and were killed by cervical dislocation 2h later. The femurs were dissected out and bone marrow flushed into 1 ml of foetal calf serum using a 26 $^{1}/_{2}$ gauze needle. Following centrifugation at 2000 rpm for 5 min, the pellet was dissolved in 6 ml of [0.075] M KCl and kept at 37°C for 10 min. This hypotonic treatment continued as the sample was centrifuged

again at 2000 rpm for 10 min. The supernatant was discarded and the pellet was dissolved and fixed in ice-cold methnol acetic acid (3:1). After 1h, the cells were pelleted out and resuspended in 3 ml of fresh fixative and left overnight. Fixed cells were dropped onto clean, chilled slides and flame-dried. Later, slides were stained in 7% Giemsa and mounted in DPX. For each animal, 100 well-spread metaphase cells were scored under oil-immersion for determining the frequency of chromosomal aberrations. All aberrations were given equal weightage irrespective of the probable number of breakage events involved. For mitotic index, 1000 cells per animal were scanned.

7. Estimation of glutathione peroxidase activity in mouse liver

This selenoenzyme catalyzes the following reactions.

$$ROOH + GSH \longrightarrow GSSG + ROH + H_2O$$

$$H_2O_2 + GSH \longrightarrow GSSG + 2H_2O$$

Glutathione peroxidase (GPx) was assayed according to the method of Flohe and Guzler (1984).

Principle

Coupled enzyme assay with glutathione reductase (GR) is used. The glutathione disulphide (oxidized form) produced as a result of GPx activity is immediately reduced by an excess of GR thereby maintaining a constant level of glutathione reduced form in the reaction system. The assay takes advantage of concomittant oxidation of NADPH by GR which is measured at 340 nm.

$$H_2O_2 + 2GSH \xrightarrow{GPx} 2H_2O + GSSG$$

$$GSSG + 2NADPH \xrightarrow{GR} 2GSH + 2NADP^+$$

Sample preparation

Animals were killed by cervical dislocation and the liver was excised, weighed and homogenized in 0.25 M sodium phosphate buffer (pH = 7.4) using a Potter-Elvehjam type homogenizer fitted with teflon plunger. The ratio of tissue weight to solution volume was 1:10. The homogenate was centrifuged at 10,000 rpm for 30 min in a refrigerated centrifuge (Sorvall RC5C). The pellet, consisting of nuclear fraction and cell debris, was discarded. The supernatant was further spun at 45,000 rpm for 1h at 4°C using a Beckman ultracentrifuge. The supernatant, cytosol, was pipetted out into another tube taking care to avoid the lipid layer.

Assay

The final concentrations in 1 ml reaction volume were :

50 mM potassium phosphate buffer (pH 7.0) having 0.5 mM EDTA; 0.24 U/ml yeast glutathione reductase; 0.3 mM glutathione; 1.5 mM NADPH in 0.1% $NaHCO_3$; 1.5 mM H_2O_2 and an appropriate amount of sample.

The overall reaction was started by the addition of H_2O_2 and decrease in absorbance was monitored at 340 nm in Shimadzu UV-260 spectrophotometer. The nonenzymatic reaction rate was assessed by replacing the enzyme sample with buffer.

Specific activity, in µmoles of NADPH oxidized per min per mg protein, of the enzyme was expressed.

8. Estimation of glutathione-S-transferase activity in mouse liver

Glutathione-S -transferase catalyzes the following reaction-

$$RX + GSH \longrightarrow HX + R\text{-}SG$$

The enzyme was assayed according to the method of Habig *et al.* (1974).

Principle

The enzyme activity is measured by following the increase in absorbance at 340 nm of cDNB-GSH conjugate generated as a result of GST catalysis between glutathione and 1-chloro-2,4,-dinitrobenzene (cDNB).

$$cDNB + GSH \longrightarrow cDNB\text{-}GSH \text{ conjugate}$$

Sample preparation

same as that described for glutathione peroxidase.

Assay

The 3ml reaction volume contained final concentration of 0.1 M sodium phosphate buffer pH 6.5, 1mM cDNB in ethanol; appropriate amount of distilled water and enzyme sample. This mixture was preincubated at 37°C for 5 min. The reaction was initiated by the addition of 1mM GSH and the absorbance was recorded at 340 nm.

For calculation of enzyme activity, the millimolar extinction coefficient between cDNB-GSH conjugate and cDNB, which is 9.6, was used.

Specific activity of the enzyme was estimated as μmoles of GSH-cDNB conjugate formed per minute per mg protein.

9. Estimation of total glutathione (tGSH) in liver and bone marrow

The'total'glutathione includes reduced glutathione (GSH), oxidized glutathione(GSSG), and mixed disulphides (GSSR).

Principle

Catalytic amounts of GSH or GSSG and glutathione reductase bring about the continuous reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by NADPH according to the following reactions:

$$2\ GSH + DTNB \xrightarrow{\text{nonenzymic}} GSSG + 2\ TNB$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2\ GSH + NADP^+$$

$$NADPH + H^+ + DTNB \xrightarrow[\text{GSSG reductase}]{\text{GSH/GSSG}} 2\ TNB + NADP^+$$

The formation of 5-thio-2-nitrobenzoate (TNB) is followed spectrophotometrically at 412 nm.

Sample preparation

The total glutathione level (reduced + oxidized) was estimated by the method of Akerboom and Sies (1981). Bone marrow cells from each animal were flushed into 0.5 ml of double distilled water and homogenized by passing it repeatedly through a 26 $^1/_2$ G needle. Liver was excised, frozen in liquid nitrogen and pulverized. The powdered tissue (0.1 g) was dissolved rapidly in 0.5 ml of double-distilled water and homogenized. The bone marrow and liver samples were centrifuged at 5000 rpm for 10 min and an aliquot of the supernatant was used for determination of protein using bovine serum albumin as standard (Bradford, 1976). The rest of the supernatant was deproteinized by addition of an equal volume of 2 M perchloric acid containing 4mM EDTA and centrifuged at 3000 rpm for 5 min. To an aliquot of the acid extract, equal volume of a solution containing 2 M KOH and 0.3 M N-morpholinopropane sulfonic acid (MOPS) was added and neutralized. The resulting clear supernatant was used immediately for the determination of total glutathione.

Assay

The kinetic assay as described by Tietze (1969) was used. Briefly, an aliquot of the acid soluble extract was incubated with potassium phosphate buffer (0.1 M), DTNB (3.7 mM) and glutathione reductase (6 units/ml) at room temperature. The reaction was started by addition of NADPH (4.4 mM) and the linear increase in absorbance at 412 nm was followed for 6 min in a Shimadzu UV-160 spectrophotometer. The reaction was calibrated against a standard and tGSH concentration in the sample was estimated from the absorbance data using 14.3, the millimolar extinction coefficient of the nitrobenzoate ion.

Total glutathione levels were expressed in nmoles per mg of protein in the sample.

10. Estimation of ascorbic acid concentrations in liver and bone marrow

Ascorbic acids in biological samples include both the oxidized (AH^-, A^{2-}) and the reduced forms (AH_2). The most commonly used methods for ascorbic acid analysis involve oxidation of the reduced form and subsequent formation of a hydrazone or flourophor which may be estimated by spectroscopic or flourimetric analysis (Omaye *et al.* 1969).

Principle

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketogulonic acid. These products are treated with 2,4-dinitrophenylhydrazine to form the derivative bis-2,4-dinitrophenylhydrazone. This compound, in strong sulphuric acid, undergoes a rearrangement to form a product with an absorption band that is measured at 520 nm. The reaction is run in the presence of thiourea to provide a mildly reducing medium, which helps to prevent interference from nonascorbic acid chromogens.

Sample preparation

Bone marrow cells from each animal were flushed into 0.5 ml of double-distilled water and homogenized by passing it repeatedly through a 26 $^1/_2$ G needle. The samples were deproteinized by addition of an equal volume of ice-cold 5% TCA. Liver was excised, weighed and homogenized

in 9 ml. of ice-cold 5% TCA per gram tissue. The homogenates were centrifuged at 5000 rpm for 20 min. In order to form the bis-2,4-dinitrophenylhydrazone, 0.5 ml of supernatant was mixed with 0.1 ml of DTC reagent (2,4-dinitrophenylhydrazine/thiourea/copper sulphate) and incubated for 3h at 37°C. To convert this to the rearranged product, which is measured by spectroscopy, 0.75 ml of ice-cold 65% H_2SO_4 was added and mixed well, and the solution allowed to stand at room temperature for another 30 min. Absorbances were read at 520 nm in a Shimadzu UV-160 spectrophotometer. Standards were made in 5% TCA and ranged from 0 to 20 μg/ml.