Malignant transformation of Syrian hamster embryo (SHE) cells in culture by malachite green: An agent of environmental importance


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Malachite green (MG), consisting of green crystals with a metallic lustre, is very soluble in water and is highly cytotoxic to mammalian cells in culture and also acts as a liver tumour promoter. In view of its industrial importance and possible exposure to human beings, MG poses a potential environmental health hazard. Accordingly, we have studied the effect of MG on the formation of free radicals using ESR analysis with 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin trapping agent. ESR analysis showed formation of reactive free radicals during exposure of MG to Syrian hamster embryo (SHE) cells. As per mechanism-based toxicology in cancer risk assessment, the chemicals that have the potential to be metabolized to active free radical species could be human cancer hazards. So, we have investigated the effect of MG on the formation of Type II and Type III morphologically transformed foci using SHE cell transformation assay. MG induced dose related transformed foci. Some of these transformed foci were taken out using selective trypsinisation and established immortal cell lines. One of these immortal cell lines was characterized extensively. This immortal cell line showed enhanced DNA synthesis in the form of BrdU incorporation, increased presence of proliferating cell nuclear antigen (PCNA), bcl-2 and p53 proteins by immunohistochemistry. When these immortal cells were injected subcutaneously into nude mice, they developed tumors which were transplantable and histopathologically sarcomas. The present studies indicate that MG could be a potential candidate for two year chemical carcinogenesis rodent bioassays.

There is growing evidence that majority of human cancers are of environmental in origin. About 80-90% of human cancers are supposed to be caused by both environmental and lifestyle factors. One key feature of lifestyle is diet and results from epidemiological studies have suggested an important role of diet in the etiology of cancer. Some of these dietary factors include presence of several nonpermitted food colouring agents such as Malachite green, Orange II, Metanil yellow and Rhodamine B in colourful food stuffs available in roadside eateries. These additives as well as industrial colours are dyes containing amino or methyl groups which either form nitronium ions or carbonium ions which can yield DNA adducts thus increasing risk of cancer. One such compound is Malachite Green (MG), a triarylmethane dye. (Fig.1) It has been identified as harmful by WHO/FAO Expert Committee and is toxicologically classified under category CIII. It is also used extensively for dyeing cotton, wool, jute, leather, as a laboratory reagent and also as topical antiseptic. Despite the government ban, there is continuous occupational exposure of MG to workers in printing and textiles industries and it is also reported to be present in an unscrupulous way in juices and other eatables consumed by people from lower socio-economic strata especially school children. In view of its multipurpose utility, MG poses a great health hazard and is of considerable environmental concern.

Several reports are available on the cytotoxicity, carcinogenicity and mechanism of action of several
environmental carcinogens although they are believed to act through very different mechanisms. The nicotine-derived N-nitrosoamine NNK which is present in tobacco smoke induces lung tumours in rodents and is most likely involved in lung carcinogenesis in smokers. Metabolic activation of NNK by cytochrome P450 is required for carcinogenic activity. Benzo(a)pyrene must be bioactivated by enzymes such as cytochrome P450 in order to acquire its mutagenic and carcinogenic properties. The ultimate product of B(a)P diolepoxide (DE2) is extremely reactive and can bind to macromolecules such as DNA, RNA and proteins.

Cyclooxygenase, by way of its peroxidase activity catalyzes the oxidation of a wide range of xenobiotics including benzidine and 2-aminofluorene. Benzene induces increased levels of chromosome aberrations in circulating lymphocytes and chlorinated hydrocarbons enhance the activation of peroxisome proliferator activated receptor which could lead to hepatocarcinogenesis. Arsenic is known to target specific cellular proteins through sulphhydryl interactions and chromium induces oxidative-type DNA damage which is responsible for chromium carcinogenesis. All these studies clearly indicate wide variation in the mechanism of action of these agents. However, in the case of malachite green, despite it being a suspected environmental carcinogen, there is still a large vacuum in the information on cytotoxicity, carcinogenicity and the mechanism of action.

Earlier studies carried out in our laboratory have shown that MG is extremely cytotoxic to mammalian cells in culture. In addition, treatment of Syrian hamster embryo (SHE) cells with MG results in increased lipid peroxidation and catalase activity in a dose dependent way. Since the cytotoxicity of MG to the mammalian cells observed is unusually high which is associated with the increased lipid peroxidation, it is possible to assume that observed cytotoxicity could be due to the formation of free radicals. One of the recent criteria based on the mechanism-based toxicology in cancer risk assessment suggests that chemicals that have potential to be metabolised to active free radical species could be human cancer hazards. Moreover, substantial evidence is available which implicates the involvement of free radicals in causative role of many deleterious biological processes such as cytotoxicity, carcinogenicity, aging and numerous other diseases. In order to gain insight into potential mechanisms of MG based toxicology, in the present investigation we have studied the free radical formation by electron spin resonance (ESR) analysis, and detected single stranded breaks indicating the possible damage to the DNA. We have also tested the malignant transforming potential of MG on SHE cells and obtained a MG transformed cell line. These malignantly transformed cells are further characterised in relation to cell proliferation and antiapoptotic markers, chromosome pattern, their growth properties and tumour induction compared to that of control cells of SHE.

**Materials and Methods**

**Cells and culture conditions**—Primary SHE cultures were set up from 10-14 day gestation fetuses collected aseptically. Cells were grown in Dulbecco’s modification of Eagle’s medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) and Gentamycin (50 μg/ml) and maintained at 37°C in 5% CO2 atmosphere. For secondary cultures, cells were transferred by trypsinisation with 0.25% trypsin.

**MG treatment**—Stock MG solution was prepared fresh in sterile saline, passed through millipore membrane filter, diluted with saline and added to the culture dishes at appropriate concentrations. Control cultures received only sterile saline in place of MG. The duration of exposure to MG was 24 hr, after which time a media change was made without MG. Between days 8 and 10 when the colonies were discrete and well defined, the dishes were washed with saline, fixed with methanol, stained with Giemsa and the colonies were counted.

**ESR spectroscopy**—Cells were washed twice with PBS (pH 7.4) and again suspended in PBS and incubated at room temperature for 30 min in a total volume of 1 ml of PBS at a cell density of 1 million
cells /ml, DMPO (100 mM) and MG at different concentrations. ESR spectra were recorded at room temperature on a Varian E-112 Century series spectrometer with the following instrumental settings: microwave power, 2.0 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; field scan, 200 G; receiver gain, 5×10^6 mS; magnetic field, 3390 G. DMPO was used for trapping reactive free radicals.

**Evaluation of DNA damage in the form of strand breaks by alkaline elution technique**—Media of primary cultures was changed after 48 hr of setting up and the cells were labelled with tritiated thymidine (20 µCi/ml, Sp. Act. 6500 mCi/µM) for 24 hr. Cells were trypsinised and suspended in culture media with the appropriate concentration of MG and then processed for alkaline elution technique. Alkaline elution was performed essentially according to the method of Barbin et al with some modifications. Cells (1.5-2 million/ml) were utilized. The extent of DNA damage was estimated by comparing two fractions: the non-eluted DNA (i.e. DNA remaining on the filter + DNA in the second fraction) versus the fraction of DNA eluted. The difference between control and the treated was analysed by Wilcoxon's test.

**Morphological transformation**—Transformation assays were carried out as per the method essentially described by Reznikoff et al. Cells were seeded in 60 mm dishes (20 petridishes per group) at density of 5000. Five days after seeding, media change was given and after that the cells were treated with MG at concentrations 0.025 and 0.05 µg/ml based on cytotoxicity studies carried out. One week later, media change was given. Subsequently, media was changed at weekly intervals and at 6th week, dishes were fixed with methanol, stained with Giemsa and morphologically transformed foci were counted. In all the assays, the solvent in which MG was dissolved i.e. saline served as negative control. Transformed foci were classified as type II and type III using the morphological criteria described by Reznikoff et al. One of the MG transformed foci was used for establishing an immortal cell line and this cell line was utilised for characterization studies.

**Assay for growth in soft agar**—Assay for growth in soft agar was carried out by the method of Macpherson and Montagnier as adopted by Roberts et al. Agar plates (15 per group) were prepared in 55mm petridishes by first applying a 2 ml base layer of agar containing 1 ml of 1% agar (Difco) + 1 ml double DMEM containing 20% FBS and Gentamycin at 100 µg/ml concentration. Over this basal layer an additional 2 ml layer of agar (1ml of 0.6% agar +1ml same medium) containing 2500 cells in logarithmic phase (Passage No 14) were added. The petridishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and refed after 7 days with 2 ml of overlay agar medium mixture but without cells. At 2nd week, 0.5 ml of (1 mg/ml) sterile solution of Nitroblue Tetrazolium NBT (Merck) was layered over the agar and incubation was continued for another 48 hours. After removal of excess dye solution, photographic pictures were taken.

**Growth curve studies**—Control cells (Passage No 3-4) and transformed cells (Passage No 54) were plated in complete medium with 10% FCS in a 100 mm petridish at the cell density of 0.1 million cells per petridish. Analysis for the viable cell number was performed at time points of 24, 48, 72, 96, and 120 hr. For cell counts, cells after trypsinisation (0.02% EDTA-PBS and 0.25% Trypsin at 1:4 dilution) were pelleted by centrifugation, washed twice with PBS and the final pellet was suspended in 1 ml of PBS. Viable cell number was assayed by Trypan Blue exclusion by staining the cells with 0.4% Trypan Blue for 5 min and counting.

**Immunohistochemical analysis for PCNA, p53, and bcl-2**—Cells were grown until subconfluent stage on coverslips in 35mm petridishes, and fixed in 2% formaldehyde in acetone for 30 min at -20°C. After washing in PBS, the coverslips were immersed in 1% normal goat serum for half an hour for blocking, and subsequently incubated with the respective monoclonal primary antibodies (Dako:bcl-2 Oncoprot-124 at 1:40; p53 DO-7 at 1:40; PCNA PC-10 at 1:100 dilution) for 3 hr at room temperature, followed by overnight incubation at 4°C. Three washes with PBS, the coverslips were incubated with universal multilinked biotynilated secondary antibody (E-0453 DAKO KIT) for 1 hr at room temperature at 1:100 dilution. Coverslips were again washed thrice with PBS and subsequently incubated for 1 hr at room temperature with Streptavidin/ horse radish peroxidase (P-0397 DAKO KIT) at 1:500 dilution. The peroxidase reaction was performed using DAB-H_2O_2 as substrate to produce brown reaction product at the site of antigen expression. The cells were then counterstained with Hematoxylin, dehydrated with increasing grades of alcohol, treated
with xylene, and mounted in DPX. Negative controls were performed by omitting the primary antibodies.

**Immunohistochemical analysis of BrdU incorporation**—Cells were grown until subconfluent stage on coverslips in 35mm petridishes. Fresh medium was added along with BrdU (Sigma) at 10 µm concentration for 2 hr. The coverslips were washed thrice in PBS and fixed in Methanol: Acetic acid (3:1) at -20°C for 1.5 hr. After three washes with PBS, they were denatured with 2N HCl at room temperature for 20 min. After neutralizing the pH with several washes of PBS, the cells were incubated with BrdU Ab (20µl neat/cover slip, Becton and Dickenson) at 37°C for 1 hr followed by overnight incubation at 4°C. The remaining procedure was as described above.

**Immunohistological scoring**—Cells were scored with the grid using the light microscope. For scoring, each coverslip was divided into 4 parts. From each part, 5 areas were counted, each area consisting of 16 squares. Staining with p53, bcl-2, PCNA, and BrdU Abs were scored as positive if only cell nuclei were stained well above the background level. Positive scoring is further graded as moderate (+) or intense (++) . The labelling index is the number of cells with nuclear positive staining divided by the number of the cells counted.

**Flow cytometric analysis**—For flow cytometric analysis control cells (Passage No 3-4) and transformed cells (Passage No 50-55) were grown until subconfluent stage in 25 cm² flasks. The cells were harvested by EDTA-Trypsin-PBS solution, pellet washed twice with PBS, and the cells were fixed in 70% alcohol at 4°C for 2 hr. The fixed cells were stained with propidium iodide and RNase at the final concentration of 50 µg/ml and 0.4% Tween 20 . Normal hamster spleenocytes separated on Ficoll were used as a diploid reference standard in each assay batch. Cells were analysed using FACS Calibur Becton-Dickenson flow cytometer using Modfit LT software cell cycle analysis.

**Chromosomal analysis**—Control (Passage No 3-4) and transformed cells (Passage No 55-65) in the logarithmic stage were treated with trypsin-EDTA-PBS solution to obtain a single cell suspension. These cells were incubated with 0.01 µg of Colcemid/ml of complete medium for 1 hr. After centrifugation, hypotonic solution of 0.075 M KCl was added for 15 min at 37°C, fixed in fresh Carnoy’s fixative, dropped onto clean slides, baked at 60°C and stained with Giemsa.

**Tumorigenicity studies**—Tumorigenicity studies were done in Nude mice approximately 8-12 weeks old. Transformed cells at different passages (Passage No 25-30) were harvested, suspended in DMEM and about 2 million cells were injected (sc) into the dorsal side and the mice were monitored for the period of several months. Latency period was taken as the number of days from the injection of cells into mice until tumours first became visible. When the tumour of the size of about 2 cm was attained, the animal was sacrificed and tumour removed aseptically. One part of the tumour was again transplanted in nude mice for two generations and the second part was utilised for cell culture. Paraffin sections were cut for all these tumours and sections were stained with Hematoxylin and Eosin as well as with PCNA, p53, bcl-2 monoclonal antibodies.

**Results**

**Cytotoxicity assays**—Cytotoxicity studies of MG were carried out at different concentrations ranging from 0.00625 to 0.075 µg/ml by the colony survival assays. Fig. 2 shows the concentration dependent cytotoxic effect of MG on SHE cells. MG was found to be toxic to SHE cells at concentrations above 0.05 µg/ml. At 0.075 µg/ml concentration, the absolute plating efficiency decreased to 7%. (Fig. 3).

**ESR spectroscopy**—Since the cytotoxic effect of MG which was unusually high suggested the possible involvement of free radicals, we tested the free radical formation using ESR analysis. Figure 4A shows ESR spectra of free radicals generated by MG in SHE cells at different time periods. No signals were observed with only cells, MG, DMPO, cells+DMPO, MG+DMPO, and cells+MG. However, incubation of cells with DMPO as the spin trapping agent together with MG showed the formation of well defined ESR spectra, indicating the generation of free radicals. (Fig 4B). Fig 4C shows ESR spectra of free radicals generated at various concentrations of MG. A gradual increase was found in the signal height with increasing concentrations of MG up to 2 µg/ml, beyond which no further increase was observed. At 4 µg/ml concentration of MG, a decrease in signal intensity was observed, suggesting the possible cytotoxic effects.

**Evaluation of DNA damage in the form of strand breaks by alkaline elution assay**—Fig. 4D shows...
induction of strand breaks on exposure of cells to MG for 4 hr (optimum period selected after initial kinetic experiments) at different concentrations. A dose related increase in the DNA strand breaks was observed with increasing concentrations of MG. The increase was not significant at 0.1 µg/ml, however at 1 and 2 µg/ml significant elevation in strand scission was observed compared to the controls.

**Morphological transformation**—The transforming potential of MG was tested on SHE cells using foci formation as the parameter. Table 1 shows the data on the transforming ability of MG on SHE cells. MG was tested at concentrations of 0.025 µg/ml and 0.05 µg/ml and at both the concentrations, Type II and Type III foci were observed. Solvent controls did not show any foci formation. At 0.025 µg/ml concentration, MG showed a 6-fold increase in total foci formation compared to 0.05 µg/ml. Decrease in the number of foci at 0.05 µg/ml concentration of MG appeared to be due to toxic effects of the compound. Controls did not show any morphological changes. Extensive piling up and crisscrossing of the cells at
MAHUDAWALA - MALACHITE GREEN & MALIGNANT TRANSFORMATION OF SHE CELLS

Table 1—Induction of morphologically transformed foci by MG in SHE cells

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Treatment Group</th>
<th>Dishes with Type II Foci/Total dishes</th>
<th>Total No. of Type II Foci</th>
<th>Dishes with Type III Foci/Total dishes</th>
<th>Total No. of Type III Foci</th>
<th>Total Type II and Type III foci/Total dishes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Solvent Control</td>
<td>0/20</td>
<td>0</td>
<td>0/20</td>
<td>0</td>
<td>0/20</td>
</tr>
<tr>
<td>2</td>
<td>MG (0.025 µg/ml)</td>
<td>10/20</td>
<td>9</td>
<td>7/20</td>
<td>19/20</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MG (0.05 µg/ml)</td>
<td>2/20</td>
<td>1</td>
<td>1/20</td>
<td>3/20</td>
<td></td>
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</table>

The periphery of the colonies were observed at both the concentrations (Fig.5).

*Morphology*—The MG transformed foci from both the groups and the cells from the control group were further passaged regularly and morphology of these cells studied. Normal cells in vitro have a limited life span whereas tumour cells often have the ability to grow indefinitely. Our control cells in earlier passages showed contact inhibition pattern of cell growth with a thin flat monolayer of cells. However, as they were further passaged, they progressively showed distinct morphological changes like gross enlargement, flattening, increased nuclear to cytoplasmic ratio, degeneration, etc—indicating typical characteristics of senescence. In contrast, transformed cells were more healthy, and epithelioid in appearance. They showed progressively decreased contact inhibition and an ability to overgrow the monolayer with extensive pilling up. There was a total loss of orderly growth and extensive crisscrossing of cells. (Unpublished observations)

These transformed cells were found to be aneuploid in nature, as determined by flow cytometry and chromosomal pattern. Although the SHE cells in primary culture were morphologically homogenous diploid cell population, on transformation, they were clearly pleomorphic and about 52% of the cells in the transformed group had chromosome numbers in the aneuploid range. (Fig 6).

*Anchorage independent growth*—Growth of cells as colonies in soft agar is considered to be one of the best assays for cell transformation in vitro. Hence the MG transformed cells, which were maintained in culture by regular passaging were tested for anchorage independent growth in soft agar at Passage No 14. In our studies, control SHE cells remained as predominantly single cells in soft agar and failed to produce colonies indicating the anchorage dependent growth of these cells. On the other hand, transformed counterparts showed the formation of colonies in soft agar, (Fig 7) indicating that these transformed cells had acquired certain properties associated with their progression towards the malignant phenotype.

*Growth curve studies*—Fig.8 shows the growth curves for control and transformed cells. Transformed cells showed a higher growth rate compared to the control cells, with the doubling time of 16 hr as compared to 19 hr for the control cells.

*Characterisation studies*—The cells were characterised with respect to cell proliferation and antiapoptotic markers. Cell proliferation activity was studied immunohistochemically using PCNA expression and BrdU incorporation as the markers of

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Fig.3—Absolute plating efficiency of SHE cells at different concentrations of MG in primary cultures. Experimental details are given under Materials & Methods. [Each value represent mean ± SE of 10 values].

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cell proliferation. After 2 hr of BrdU labelling, about 6% of the control cells showed BrdU incorporation, as compared to 34% of the transformed cells. (Fig 9, A & B and Table 2). Higher incorporation of BrdU showed an increased fraction of S phase cells in transformed group, indicating that they were proliferating at faster rate. PCNA as a marker is expressed only in proliferating cells but not in resting cells. It is a nonhistone nuclear protein which enhances the activity of DNA polymerase δ, which is essential for DNA replication. PCNA expression was studied in transformed and control cells using PC-10 as monoclonal antibody. A labelling index of 0.88 was observed for PCNA staining in the case of transformed cells as compared to 0.54 of control group. Even though 54% of the control cells showed positive staining for PCNA, the intensity of staining varied between the two groups.

![Graphs and data](Fig.4—ESR spectra of DMPO adducts generated at various experimental conditions and formation of DNA strand breaks in SHE cells exposed to MG. A) With reference to time of exposure. B) Conditions optimal for the generation of reactive free radicals. C) With reference to various concentrations. D) Strand breaks at various concentrations. Experimental details are described under Materials and Methods.)
controls showing much lighter staining. (Fig.9, C & D and Table 2).

Antiapoptotic activity was studied using p53 and bcl-2 as antiapoptotic markers. Normal wildtype p53 is associated with suppression of cell proliferation or induction of apoptosis. Mutations in p53 blocks apoptosis and increases genetic instability due to continuous cell cycling. p53 expression was studied in control and transformed cells using DO-7 as primary Ab. Seventy percent of the cells in the transformed group showed p53 staining as compared to 10% in the control group. Also p53 positive staining was found to be more intense in transformed group as compared to the controls. (Fig 9, E & F and Table 2). Mutations in p53 gene in most cases can be detected by immunohistochemistry method because most p53 mutations lead to a significantly longer half life of p53 protein, causing an apparent overexpression of p53 protein. Thus it can be considered that the detection of p53 protein by Biotin-Strepavidin method using DO-7 Ab indicated the presence of mutant p53, although DO-7 Ab recognises both wildtype and mutant protein. It has been reported that bcl-2 is overexpressed in several normal and neoplastic tissues. Its overexpression allows increased survival of cells due to its ability to suppress cell death by apoptosis. Bcl-2 expression was studied in transformed and control cells using bcl-2 oncoprot-124 as primary Ab. Forty percent of the transformed cells showed an overexpression of bcl-2 with an exclusive nuclear localisation compared to control cells which did not show any expression. (Fig 9, G & H and Table 2).

Tumorigenicity studies and transplantation—These immortal cell lines were further tested for their ability to induce tumours in nude mice. Table 3 shows the data on tumour induction and transplantation in nude mice. All the 3 nude mice injected subcutaneously produced tumours with latency period of 2-3 months. (Fig 10A) Transplantation of the original tumour was carried out for two generations. All the 3 nude mice which were transplanted with the tumour showed the

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control Intensity</th>
<th>Transformed Intensity</th>
</tr>
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<tbody>
<tr>
<td>BrdU 2 h</td>
<td>0.07 +</td>
<td>0.34 +</td>
</tr>
<tr>
<td>PCNA</td>
<td>0.54 +</td>
<td>0.88 ++</td>
</tr>
<tr>
<td>p53</td>
<td>0.10 +</td>
<td>0.7 ++</td>
</tr>
<tr>
<td>bcl-2</td>
<td>0 -</td>
<td>0.4 +</td>
</tr>
</tbody>
</table>

Details are described under Materials and Methods.

Fig.5—Morphologically transformed foci induced in SHE cells following treatment with MG. A) Solvent control. B) MG 0.025 µg/ml. C) MG 0.05 µg/ml.
tumour growth in 7-10 days. (Fig. 10, B & C) These nude mice tumours were classified histopathologically as having resemblance to sarcomas. (Fig 10D) Tissue sections from the nude mice tumours showed positive staining for bcl-2, p53, and PCNA Abs. (Table 3).

Discussion
Nutritional and occupational exposures are some of the environmental factors implicated in the causation of human cancer. Identification of these factors will ultimately help in preventing their use in human environment and also provide people with better basis for making decisions about their individual habits and lifestyle. Malachite green occurs as a spurious food colouring agent and in textile industry. Even though literature data is not much on the mutagenicity and carcinogenicity of malachite green, extensive data is available on other compounds of environmental interest. Exposure to polycyclic aromatic hydrocarbons on an acute or chronic basis can be mutagenic and carcinogenic to various organs. In vitro and in vivo hepatocellular toxicity and carcinogenicity of carbon tetrachloride has been shown by different authors. In lymphoblastoid cell lines 1-chlorohexane and 2,3-dichlorobutane were found to be mutagenic. Mutagenic potential of 1,2-
Malachite green is one of the environmentally important agents which is a water soluble triarylmethane dye. MG is cheap, easily available and has potency to impart brighter shades for consumer appeal and satisfaction. Even though MG has been banned for human consumption due to its harmful effects it is being used unscrupulously for colouring several sweets and other edible materials. The damage done to the human system by this food adulterant is difficult to estimate instantly since poisoning is usually gradual and the cumulative effects could be disastrous. Moreover continuous occupational exposure to MG also creates an additional human hazard. Accordingly, detailed studies are required using in vivo and in vitro experimental model systems, in order to obtain the cytotoxic and carcinogenic potential of MG. This prompted us to test the cytotoxic properties of MG, its capacity to generate free radicals and associated strand breaks, malignant conversion and characterization of these cells using Syrian hamster embryo cells in primary culture. The findings in the present study demonstrate that free-radicals are generated during exposure of MG to SHE cells and seems to be associated with the observed DNA strand breaks and malignant conversion.

Earlier investigations and as well as the present study indicated that MG is extremely cytotoxic to mammalian cells in culture. The cytotoxicity observed is quite drastic and several fold higher compared to several standard carcinogens (unpublished observations). Moreover exposure of SHE cells to MG resulted in increased catalase activity suggesting the possible formation of hydrogen peroxide (H₂O₂) and associated hydroxymethyl and hydroxyperoxide radicals. Accordingly, the effect of various concentrations of MG on the generation of free radicals was investigated using ESR spectroscopy with DMPO as the spin trapping agent. Fig.4 shows the generation of

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Tumors induced</th>
<th>Latent period</th>
<th>p53*</th>
<th>PCNA*</th>
<th>bcl-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Transformed</td>
<td>3</td>
<td>3</td>
<td>2-3 months</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Transplanted</td>
<td>3</td>
<td>3</td>
<td>7-10 days</td>
<td>+</td>
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Experimenal details are described under Materials and Methods

* Determined in tumor sections

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Table 3—Tumorigenicity data in nude mice with MG transformed cells and expression of PCNA, BCL-2 & p53 mutations in primary and transplanted tumours

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Fig.8—Growth curves of normal and MG transformed SHE cells. Viable cell number was determined by trypan blue exclusion at days indicated. Experimental details are described under Materials and Methods. Bars denote SE.
Fig. 9—Immunohistochemical characterization of normal and MG transformed SHE cells. BrdU incorporation (A & B), PCNA (C & D), p53 (E & F) and bcl-2 (G & H) protein expression (X 400). Experimental details are described under Materials and Methods.
Fig. 10—Nude mice injected subcutaneously with MG transformed cells showing tumors. A) Original tumor. B) 1st generation transplanted tumor. C) 2nd generation transplanted tumor. D) Histopathology of original tumor showing resemblance to sarcomas.
free radicals under different experimental conditions. The kinetic experiments showed that ESR signals were significant after 30 min of incubation (Fig.4A). The free radical generation with MG was strictly dependent upon the presence of cells, MG and DMPO and no ESR signal was detected without cells, MG and DMPO either individually or in different combination (Fig.4B). Also, a gradual increase was observed in signal height with increasing concentrations of MG up to 2 μg/ml, beyond which no further increase was observed (Fig.4C). Free radicals are known to damage DNA, RNA, proteins and lipids. Accordingly, we tested the induction of single strand breaks in the DNA on exposure of SHE cells to MG. A dose dependent increase in the formation of strand breaks was observed with the increasing concentration of MG indicating the possible genotoxic effects (Fig.4D).

As per mechanism based toxicology in cancer risk assessment, the compounds that have potential to be metabolized to active free radical species will have better possibility to be carcinogenic agents. Since MG showed generation of free radicals and associated strand breaks, we continued our investigations to see whether MG could be a transforming agent. MG showed both type II and type III foci in transformation assays with extensive piling up and crisscrossing in the periphery at both the concentrations tested (Fig.5). Three of these foci were selectively trypsinized and have been regularly passaged in culture and immortal cell lines are obtained. One of these cell lines has been extensively characterized. This cell line showed anchorage independent growth in soft agar assays. Also these cells are tumorigenic as they could produce tumours when injected subcutaneously into nude mice (Fig.10A). These tumours were found to be transplantable (Fig.10B & C). Histologically, these tumours possessed the appearance of sarcomas (Fig.10D). Growth rate experiments carried out for control and transformed cells showed that transformed cells had a doubling time of 16 hours as compared to 19 hours of the control (Fig.8). Transformed cells incorporated BrdU at a faster rate (Fig.9A and B) and also showed enhanced expression of PCNA as compared to control (Fig.9C and D) indicating faster rate of cell proliferation in transformed cells. Also our studies with p53 and bcl-2 showed the presence of p53 mutations and an overexpression of bcl-2 in transformed cells (Fig.9E, F, G, H). The overexpression of bcl-2 and p53 mutations indicate a block in the process of apoptosis in transformed group which seems to be associated with immortalization and further progression to malignant phenotype.

There are considerable molecular and cellular similarities in carcinogenic processes among mammals, including rodents and humans. For those agents identified as carcinogenic to humans, experiments in animals have shown remarkable target organ concordance. The general observation and the knowledge that all chemicals known to induce cancer in humans which have also been shown to cause cancer in laboratory animals indicates that chemicals which unequivocally induce cancer in laboratory animals, especially in multiple species, must be considered capable of causing cancer in humans. Similarly, the agents that can malignantly transform normal mammalian cells in culture will have greater possibility to be carcinogenic to experimental animals. In the present study MG by virtue of its capacity to generate free radicals which seems to be associated with strand breaks and finally induction of malignant transformation in SHE cells indicates that it could be a potential candidate for long-term rodent bioassays. Two year chemical carcinogenesis rodent bioassays for identifying the environmental carcinogens are very well accepted and also form the most useful major bioassays. However, due to limited resources and unpredictable nature of the chemicals to be tested for carcinogenicity, it will always be useful to have other assays which will help the planning of more efficient experimental design for long-term two year bioassays. For this, use of short-term cell transformation assays that are highly predictive of a compounds’ likelihood of carcinogenicity will be of great support for two year rodent bioassays.

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