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Evaluation of Carcinogenic Potential of Benzo(α)pyrene and Dimethylbenzantracene in Mouse leukemic cells

A thesis submitted to the Faculty of Science of the United Arab Emirates University in partial fulfillment of the requirements for the Degree of Master of Science in Environmental Science

by

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ABSTRACT

The carcinogenic potential of benzo[α]pyrene (B(α)P) and dimethylbenzanthracene (DMBA) was tested using mouse leukemic cell lines L-5178 R and S types in tissue culture condition. It was found that B(α)P and DMBA inhibited the synthesis of DNA of R and S types of cells to about 34%, at 10 mM concentration. While B(α)P and DMBA did not show any acute toxic effect on mouse cell line R and S types at 1 mM concentration separately. Both R and S types of cells were allowed to grow for several days in the presence of 1 mM DMBA and B(α)P respectively. Several independent clones were isolated from both DMBA and B(α)P treated cells and cultured separately. The effect of DMBA and B(α)P on changing the structure of DNA (in relation to their growth and oncogenicity) was then checked in these clones. It was found that several clones have changed their growth pattern showing a shorter generation time. By soft agarose colony formation assay, the oncogenic behavior of B(α)P and DMBA treated clones was determined. It was found that 25% of the clones have changed (15% increase and 10% decrease) their oncogenic behavior. This suggests that B(α)P and DMBA caused some permanent changes (most likely DNA) in the cell. Attempts were made to characterize these changes at the molecular level by analyzing expression of glutathione-S-transferase (GST) and Ras GTPase by immunoblotting. There was no significant change in the expression level of GST and Ras protein. Mutation in p53 gene exons 6, 8, and 9 was also checked by polymerase chain reaction (PCR). No large scale mutation was found in exons 6, 8, and 9 of p53 gene. This investigation suggests that both B(α)P and DMBA have the ability to change structure of DNA leading to increased carcinogenicity in tissue culture condition. This study has provided an additional support, using in vitro cell culture techniques to show the increased risk of cancer due to the exposure to B(α)P and/or DMBA present as environmental pollutant.
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<tr>
<td>B[α]P, Benzo[a]pyrene</td>
<td>R, Mouse leukemic cell line L-5178 of R (resistant) type</td>
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<td>DMBA, 7, 12 dimethylbenzanthracene</td>
<td>S, Mouse leukemic cell line L-5178 of S (sensitive) type</td>
</tr>
<tr>
<td>RNA, Ribonucleic acid</td>
<td>DNA, Deoxyribonucleic acid</td>
</tr>
<tr>
<td>AGE, Agarose gel electrophoresis</td>
<td>PAGE, Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR, Polymerase chain reaction</td>
<td>TBE, Tris borate EDTA buffer</td>
</tr>
<tr>
<td>TBS, Tris buffer saline</td>
<td>TBST, Tris buffer saline with tween-20</td>
</tr>
<tr>
<td>SDS, Sodium dodecyl sulphate</td>
<td>TE, Tris EDTA</td>
</tr>
<tr>
<td>FCS, Fetal calf serum</td>
<td>GST, Glutathione S-transferase</td>
</tr>
<tr>
<td>PBS, Phosphate buffer saline</td>
<td>IAA, Isoamylalcohol</td>
</tr>
<tr>
<td>Krpm, Kilo rotation per minute</td>
<td>Kbp, Kilo base pair</td>
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<tr>
<td>Kda, Kilo dalton</td>
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INTRODUCTION
Cancer is undoubtedly a multistage genetic disease (Foulds, 1969, 1975). Multiple genetic changes occur during the transformation of normal cells into cancer cells. A cancer cell is characterised by the uncontrolled growth and spread of abnormal cells. Cancer cells proliferate without regard for the regulatory mechanisms that restrain cell reproduction, crowding out their normal counterparts. Scientists have always worked at the cells replicative machinery for a hint about the common basis of all cancers. This line of reasoning led to the conclusion that errors in replication ultimately cause abnormality in cell growth (Siminovitch, 1976). Therefore, all cancers can be considered as genetic diseases, in the sense that they originate with changes in the genetic material DNA. There is increasing evidence that a number of cancers are also genetic diseases in the sense that a predisposition to the conditions are inheritable. To understand the origin of malignancy, however, one must know the molecular basis of the development of tissues and organs from single cells. Most cancers are thought to arise when DNA changes within a single somatic cell that lead to the loss of growth control and rapid proliferation. Thus, the cells that make up a tumor (solid or liquid) are progeny, or clones, derived from a single aberrant ancestor.

**Characteristics of Transformed (Cancer) Cells:**

Most primary cells (those taken directly from a living organism) are difficult to maintain in culture. They have a limited life span, typically undergoing on the order of 50 divisions before dying. However, a number of “immortal” cell types have been discovered, that live and reproduce essentially forever in culture. Some cell lines are derived from tumor (cancer) cells. Cancer cells differ from normal cells in many important characteristics when they are grown in tissue culture conditions. This includes:
1). Loss of Response to Growth Controls: Fundamental to all neoplastic cells, benign and malignant, is uncontrolled proliferation. Unfortunately the nature of these controlling mechanisms, even for normal cells, is mysterious. We only know that a number of factors are involved in wound regeneration by stimulating and controlling normal cell proliferation. Some of these are long-range, such as somatotropin and steroid hormones, whereas others are short range, including serum macromolecules. Cell-to-cell signals and undoubtedly other mechanisms are yet undiscovered. Whatever the controlling mechanisms may be, neoplastic cells appear to have escaped their effects, and there is a strong suspicion that such escape may be related to membrane changes.

2). Morphological Changes: Cancer cells range from those that are well differentiated and remarkably similar to their normal counterparts to those that are totally undifferentiated, making it difficult or impossible to determine from microscopic examination the cells of origin of the anaplastic tumor. However, in tissue culture condition, cancer cells have distinct morphology.

3). Transplantability: Cancer cells can be readily explanted into appropriate culture media or syngeneic hosts. In contrast, except for the fibroblast, it is very difficult and sometimes impossible to establish in vitro cultures of normal cells.

4). Cancer Cells are Immortal: Cancer cells can, with appropriate care, be subcultured in vitro indefinitely or be surgically transplanted from one appropriate host to another without time limit. Many standard tumor cell lines (Ehrlich ascites cells) have been maintained for decades.

5). Loss of Anchorage Dependence for Growth: Most malignant neoplastic cells will grow readily in semisolid or even fluid media while normal cells can only grow in fluid media.
6). **Loss of Contact Inhibition:** When growing in culture media, normal cells form a monolayer on the surface of the culture flask. When they come into contact with each other, further cell division and mobility ceases in the direction of the cell's contact. Cancer cells fail to respond to such controls and grow in a disorderly fashion, piled up on each other to create multilayered, tangled masses (Harris, 1973).

7). **Diminished Density-Dependent Inhibition of Cell Growth:** The phenomenon of contact inhibition was thought to arrest cell replication in culture media because of the increasing density of the population of cells (Holley, 1975). However, it is now appreciated that contact inhibition and density-dependent inhibitions of growth are separate phenomena. Cells grown in culture are dependent on many factors present in the serum in medium, such as proteins, vitamins, hormones and minerals as well as other constituents. When the medium is depleted of essential components required for growth, the culture becomes "density inhibited". One of the dramatic change in characteristic of neoplastic transformation is a lowered requirement for constituents of serum, thus representing diminished density-dependent inhibition of cell growth (Holley, 1975). The basis of this diminished dependence is unclear but may relate to increased rates of transmembrane transport of vital cell substrates.

8). **Progressive Acquisition of the Cancerous Phenotype:** There is substantial evidence that neoplastic transformation does not occur in "one swell foop". Rather, it represents a sequential process involving successive generations of cells, with each generation becoming more deviant. This phenomenon has been termed by Foulds "tumor progression" (Foulds, 1975). Neoplastic transformation requires cell replication. Tumors are composed of many subpopulations of cells that have karyotypic, antigenic and other aggressive attributes that are different from those of normal cells. In experimental models, benign tumors may over the span of time evolve into malignant neoplasms (Nowell, 1976). Cloning experiments have
demonstrated that within a single neoplasm some cells have a greater ability to metastasize than others. This observation has given rise to the concept referred to as “independent assortment” of cancerous attributes, thus, as the basal cell carcinoma of the epidermis may invade but not metastasize. Analogously, ovarian carcinomas may metastasize by seeding the peritoneal cavity but may not have significant capacity to invade. Thus, neoplastic transformation is a dynamic process.

9). Irreversibility of the Cancerous Phenotype: It is generally held that once a cell has undergone neoplastic transformation, the process is irreversible. Tumor progression is almost always in the direction of greater aggressiveness. There are, however, rare instances, both in clinical practice and in experimental systems, of transformation that appears to be reversible (Editorial, 1978). In cell culture, depending on dose-time factors, some cells may acquire some of the morphologic and functional attributes of transformation yet later revert to apparently normal cells. There are a handful of remarkable case reports where patients have experienced the miracle of spontaneous regression of cancer (Everson, 1966). Neuroblastomas, which are highly malignant cancers, have in some instances reverted to relatively benign ganglioneuromas. There is no adequate explanation for such blessed behavior, but it does indicate that neoplastic transformation is not always irreversible, raising some intriguing speculations about the ultimate nature of cancer.

10). The ability to Form Cancers When Implanted in Appropriate Hosts: In the last analysis, the phenotypic attribute of a cell marking it most clearly as a transformed cell is its ability to indeed give rise to a cancer when implanted into an appropriate host. Often in culture media many of the other attributes mentioned above may be present, suggesting that the cell has undergone cancerous transformation, but will it grow in a host, invade,
possibly metastasize, and ultimately produce a malignant neoplasm, this is the most critical test of neoplastic transformation.

**Chemical Carcinogens**

Many chemicals have been judged by International Agency for Research on cancer to have caused cancer in human (Miller 1970). Chemical carcinogens elicit their specific defining adverse effects in the production of cancer in animals or humans. In many respects, carcinogenic chemicals are similar to other toxic agents or drugs. They undergo biotransformation, as would any similarly structured xenobiotics. Carcinogens interact with other environmental agents. Their effect is sometimes enhanced and sometimes decreased as occurs with drugs (Williams and Weisburger, 1991). Several types of chemicals were discovered to be carcinogenic in experimental animals after having first been suspected of causing cancer in humans. The association between exposure to soot and coal tar and cancer was identified in the late 18th century by the English physician Percival Pott, who observed that many of his patients who had cancer of scrotum were chimney sweeps (Williams and Weisburger, 1991, Robins, 1981, Lieberman and Lebovitz, 1990). That coal tar could cause cancer at the point of application in rabbits was reported independently from Japan (Yamagiwa & Ichikawa, 1916). Investigators in the United Kingdom directed by Kennaway & Cook extracted 50 grams of a chemically pure carcinogen, benzo[α]pyrene & dibenz[a,h]anthracene from two tons of crude coal tars (Williams and Weisburger, 1991). These pioneering observations documented the carcinogenicity of polycyclic aromatic hydrocarbons. Since then, hundreds of chemicals have been shown to be carcinogenic in animals (Miller, 1970, Weisburger, _et al_, 1975).
The Following Pertinent Observations Have Emerged From the Study of Chemical Carcinogens:

1). They are of extremely diverse structure and include both natural and synthetic products.

2). Some are directly reacting and require no chemical transformation to induce carcinogenicity, but others only become active after metabolic conversion. Such agents are referred to as procarcinogens and the active end-products as ultimate carcinogens.

3). All chemical carcinogens, both the direct reacting and the ultimate carcinogens, are highly reactive electrophiles (possess electron-deficient atoms) that react with nucleophilic residues (atoms rich in electrons) in DNA, RNA and cellular proteins (Miller and Miller, 1971).

4). The binding to DNA has no particular gene specificity but does have in some instances chemical specificity.

5). The carcinogenicity of many chemicals, particularly weak carcinogens, is augmented by agents which by themselves have little, if any cancerous activity. Such augmenting agents are referred to as cocarcinogens or promoters. However, strong carcinogens have no requirement for promoting agents.

6). Several chemical carcinogens may act in concert or with other types of carcinogenic influences, e.g., viruses or radiation, to induce neoplasm.

Polycyclic hydrocarbons are among the most potent of the carcinogens. They will induce in vitro neoplastic transformation of a variety of cell types derived from many animal species as well as cancers in vivo in most laboratory animals (Lieberman and Lebovitz,
The strongest carcinogens in this category are 3-methylcholanthrene, 7,12-dimethylbenzanthracene, dibenz[a,h]anthracene and 3,4-benzopyrene (Lieberman and Lebovitz, 1990). These agents are present in coal and coal products and mineral oils, and they are produced in the pyrolysis of many organic substances. Aromatic hydrocarbons are present in car exhaust fumes and notably in tobacco tar (Sinclair, 1978). However, the apparent carcinogenicity of cigarette smoke cannot be attributed solely to polycyclic hydrocarbons, since the smoke also contains a number of other potential carcinogens, such as radioactive compounds, as well as other cancer suspected agents (Schmahl, 1977).

Critical to the carcinogenicity of the polycyclic hydrocarbons are double bonds located in a particular region of the molecule. Once absorbed into cells, oxygen is added to the double bond to form an epoxide, the ultimate carcinogens. A microsomal enzyme, arylhydrocarbon hydroxylase, catalyzes this reaction. The epoxide may then bind to DNA, RNA or protein. The carcinogenicity is most likely linked to the binding to DNA (Brookes, et al, 1964).

The aromatic amines are another type of chemical carcinogens whose study also stems from the discovery of cancer in human exposed to them. In the late 1800 the German physician Rehm noted a number of cases of cancer of urinary bladder among workers in the dye industry. The experimental evidence for the carcinogenicity of amines to which these workers were exposed did not appear until 1937 when Hueper and associates in the U.S. found that 2-aminonaphthalene could cause bladder cancer in dogs reproducing the lesion seen in human (Williams and Weisburger, 1991).

Aromatic amines and azo dyes are also powerful carcinogens in animals as well as in human. The major agents in this category are β-naphthylamine, benzidine, 2-
acetylaminofluorine, originally synthesized as an insecticide and the azo dyes scarlet red and butter yellow (dimethylaminoazobenzene). Both of these dyes were developed to color food, the latter to give margarine the natural color of butter. This category of agents was the first to illustrate the requirement for metabolic conversion of procarcinogens (Miller et al 1966).

β-naphthylamine, principally encountered in aniline dye and rubber industries, induces bladder cancer in animals and in industrial workers, having produced in the past a fifty fold increased incidence in those chronically exposed (Kleinfeld, et al 1965). Yet when directly introduced into the bladder in an animal, it is non-carcinogenic. After absorption, β-naphthylamine is converted in the liver into a carcinogenic aminophenol. The liver then conjugates the aminophenol to glucuronic acid, detoxifying it. But when extracted in the urine, the nontoxic conjugate is split by the urinary enzyme glucuronidase to release the carcinogenic aminophenol. Analogously, other aromatic amines and azo dyes require conversion into ultimate carcinogens, which can interact with proteins and particularly with nucleic acids. Ehrlich in Germany discovered that exposure to a bis azo dye, scarlet red or C.I. solvent Red 24, led to a reversible proliferation of liver cells. It was not until 25 years later between 1932 and 1934 that in a pioneering study in Japan, Kinosita and Yoshida independently discovered the carcinogenic effect of some azo dyes in rodents (Williams and Weisburger, 1991). Thereafter, many other classes and types of chemicals were found to be carcinogenic in animals. Some of these chemicals such as vinyl chloride, were discovered after they were suspected of being involved in the development of cancer in humans. Some chemicals were found to be carcinogenic in the course of bioassay for the detection of a diverse effect in a chronic toxicity study. This was the case with 2-acetylaminofluorene and dimethylnitrosamine. Some chemicals were also discovered to be carcinogenic during investigations that attempted to reproduce in laboratory animals adverse effects that had been
observed in humans or domestic animals. A study dealing with the possible causative factors of anyotrophic lateral sclerosis prevalent on pacific islands led to the finding that the plant product Cycasin was a potent carcinogen. 1,2-dimethylhydrazine was found to be a carcinogen as a result of its structural similarity to a glycone of cycasin, methylazoxymethanol (Williams and Weisburger, 1991).

Nitrosamines are direct reacting carcinogens that are not normally found in human foods. Of concern is the possibility that nitrates, used as meat preservatives, may be converted into carcinogenic nitrosamines in the gastrointestinal tract. Artificial sweeteners, particularly saccharin, are suspected of causing bladder cancer. The most recent studies do not indicate significant carcinogenicity, yet in those with a low background risk, such as nonsmoking women, heavy users of saccharin have a slightly increased incidence of this neoplasm. Also at risk are infants born of mothers consuming relatively large quantity during pregnancy, who are then weaned to a diet rich in saccharin (Hoover, et al, 1980, Clive, et al, 1979).

Asbestos predisposes to a variety of cancers in humans, particularly mesotheliomas and bronchogenic carcinomas (Lieberman and Lebovitz, 1990, Robins, 1981). Vinyl chloride has induced hemangiosarcoma of the liver in heavily exposed industrial workers. Chromium and nickel compounds, when volatilized and inhaled, have caused cancer of the lungs. Alkylating agents, used in the chemotherapy of neoplasms, have themselves induced a significantly increased incidence of acute leukemia (Bergsagel, et al, 1979, Reimer, et al, 1977). The polyhalogenated biphenyls and many of the insecticides, such as aldrin, dieldrin and chlordane, are also suspect (Heidelberger, 1975).
Chemical carcinogens may be present in human diets or be produced from dietary substances (Sinclair, 1978). The fungus Aspergillus flavus which grows on improperly stored grains and nuts, produces aflatoxins, that are potent hepatocarcinogen for animals and humans (Miller, et al 1976). Aflatoxins have been found in human foods in many regions of the world and are thought to underlie the extremely high incidence of liver cell carcinoma in regions of Africa & Indochina (Peers, et al 1976).

Other studies in 1950's investigating the case of Turkey X disease, which was responsible for extensive losses of livestock, pinpointed aflatoxin B1 and later other mycotoxins as hepatotoxins and a potent carcinogens. The powerful carcinogenicity of bis-chloremethyl ether was observed first in the laboratory and few years later lung cancer was noted in individuals exposed occupationally (Williams and Weisburger, 1991).

**Mechanism of Cell Transformation:**

The chemical carcinogens have opened many windows into the mechanisms of neoplastic transformation (Ryser, 1971, Weinstein, 1976).

First, chemical carcinogenesis is dose-dependent. The larger the dose, the greater the incidence of tumors in a series of animals and the more widespread the neoplastic transformation of cells *in vitro*. To a large extent, the sequential administration of small doses has the same effect as a comparable total dose given at once, depending on the potency of the carcinogen, the length of the time interval between doses and the effectiveness of repair mechanisms. Thus, it appears that cancers may emerge from multiple small hits and moreover, the effects of a carcinogen are irreversible.
Second, there is a latent period between the administration of a chemical carcinogen and neoplastic transformation. The duration of the lag period varies with the strength of the agent, the susceptibility of the host and probably other factors as well. In humans, this lag phase ranges from 5 to 30 years, in lower animals the lag is proportional to the lifespan of the species, but there appears to be an absolute minimum period of latency of the order of 50 or more days. During this period, generations of cells have spawned. Thus neoplastic transformation is a multistep process involving successive clones.

Third, the evidence is strong that carcinogenic chemicals ultimately exert their effect by binding to DNA, thus, induce a mutation. Although interaction with RNA and cellular proteins cannot be excluded, the weight of evidence speaks in favor of chemical alteration of the nucleotides in DNA. Although many lines of evidence support this conclusion, perhaps most convincing is that the overwhelming majority of chemical carcinogens can be shown to be mutagenic in the Ames test (Ames, 1979). If the agent is mutagenic, some bacteria will back-mutate from a defective histidine gene to a functional gene, thus allowing them to synthesize histidine required for their growth. The number of revertants is a rough measure of the mutagenicity of the agent, which correlates to a large extent with its carcinogenicity.

Other studies indicate that for most carcinogens, guanine is the preferred locus of binding. This, in essence, creates a point mutation, which may lead to base-pair substitution or frame-shift mutations. The altered guanine may pair incorrectly with thymine or adenine instead of its correct partner, cytosine. Such mispairing may lead to substitution of one amino acid for another when translated into protein. Other DNA alterations may also occur, but it should be emphasized that binding to RNA and proteins has not been excluded as the carcinogenic event. There is some evidence that cancer is not always irreversible. Indeed, in
cell cultures with low dosage of carcinogenic agents and under other appropriate conditions, cells that display some of the attributes of neoplastic transformation may occasionally revert (Stanbridge, 1976, Sager and Kovac, 1978, Ringertz and Savage, 1976). Thus, the concept that carcinogenesis is a mutational process is not irrefutable, the possibility that it is epigenetic and a disorder of differentiation has not been excluded. However, it should be pointed out that reversion of transformed cells to normal may not of necessity point to epigenetic mechanisms, since there is good evidence that mutations can be repaired.

Fourth, cell replication favors the carcinogenicity of chemicals. Indeed, carcinogenic transformation probably would not occur in the absence of cell proliferation (Cayama, et al 1978). Promoters themselves may act as cocarcinogens (Berenblum, 1941). It was demonstrated that nontumorogenic doses of a carcinogen would evoke tumors when followed by the application of croton oil (active principle, phorbol ester) or phenol, themselves virtually noncancerous. The carcinogen is now referred to as the initiator and the croton oil as the promoter. When given in reverse order, that is, promoter first, no tumors appear. Moreover, as has been said, the initiator induces an apparently irreversible effect, the action of the promoter is transient. It is now apparent that the principal action of the promoter is stimulation of cell proliferation. Analogously, tissue injury and repair, non-neoplastic hyperplasia and other cell replicative processes all provide a soil for the action of chemical carcinogens (Hennings, et al, 1970). Cell replication may be required to permanently fix the mutation or to amplify it. With cell replication, unrepaired mutations may be compounded. Chemical carcinogenesis has taught us that the emergence of the neoplastic cell is in all likelihood a multi-hit, multistep process involving successive generations of cells. Berenblum's model has been referred to as the two-stage induction of cancer (Berenblum,
13.

1949). It is more likely that many stages and indeed, more than a single carcinogenic agent, are involved. There is ample documentation that cells previously exposed to radiant energy are more vulnerable to chemical carcinogens and that viral oncogenesis may be favored by chemical agents. Thus, in humans, the emergence of a cancer may be the unfortunate consequence of many carcinogenic influences acting in concert or sequentially over a long time span. It is for this reason that many experts contend that there is no safe minimum level of exposure to potential carcinogens, a view that can now be seen to have sound foundation.

**Control of Cell Growth:**

Glutathione-S-transferase (GST) is a family of enzymes that act as catalysts between reduced glutathione and a variety of electrophiles (Jakoby, 1977; Mannervik, *et al.*, 1985). There are theoretical reasons why patients with reduced GST expression have an increased risk of developing cancer. Harada, *et al.*, (1992) showed that patients with gastric carcinomas had a higher frequency of GST gene deletion than healthy controls.

The ras oncogene family has three members Ha-ras, Ki-ras and N-ras. They are thought to play a role in signal transduction, for cell proliferation, transformation and differentiation depending on tissue. Many types of human tumors (Barbacid, 1987) have either mutations or high expression of ras oncogene. Most common mutations in Ras genes have been reported to change amino acid residues at position 12, 13, 59 and 63 (Bos, 1987, 1989). Ki-ras mutations have been described in well-differentiated gastric carcinomas, but not in all cases, and usually at a low frequency. A recent publication on this subject showed ras mutation in only 3% of patients with gastric carcinomas (Koshiba, *et al.*, 1993). Investigations of ras over-expression using immunocytochemical technique have also yielded
inconsistent results. Mutation in ras genes has been found to be 90% in human pancreatic cancer (Maruta and Burgess 1992). It is believed that Ras plays a critical role in the developing of 30% of total human cancer (Bollag and McCormick, 1991).

The P53 gene is a tumor suppressor gene located on chromosome 17p13. Inactivation of the p53 tumor suppressor is a common event in the development of diverse types of human cancers. About half of all cancer cases involve missense mutations of one p53 allele coupled to the deletion of the second allele (Hollstein et al, 1991), and many of the remaining cases involve cellular or viral oncogenes that inactivate p53 (Oliner, et al, 1992). Different carcinogens seem to cause different characteristic mutations (Harris, 1993). Exposure to one common carcinogen, ultraviolet light, is correlated with transition mutations at dipyrimidine sites (Brash, et al, 1991); dietary aflatoxin B-1 exposure is correlated with G:C to T:A transversion in hepatocellular carcinoma (Hsu, et al, 1991) and exposure to cigarette smoke is correlated with G:C to T:A transversion in lung and neck carcinoma (Takeshima, et al, 1993). The majority of mutations in p53 are in the hydrophobic mid-region of the protein (Harris, 1993) spanning exons II to IX of the gene.
Mouse Leukemic Cell Line L-5178 Y:

The L-5178Y cell line was originally isolated by Dr. Lloyd W. Law in the laboratory of Cell Biology at the National Cancer Institute in the early 1950's. Dr. Law was attempting to induce tumors in female DBA/2 mice by painting them with various doses of 3-methylcholanthrene. He isolated several leukemic cell lines (including L-1578) that were carried as intraperitoneal ascites (D. Clive, et al 1983). It was not possible to propagate leukemic cells continuously in the absence of a feeder layer of nonleukemic cells (DeBruyn, et al 1949). In 1938 Bichel reported that leukemic cells from mice would not reproduce in the culture media used at that time, but required a feeder layer of chick fibroblasts (Fischer, 1958). These studies were confirmed and extended by DeBruyn who grew leukemic lymphoblasts on a supporting layer of mouse fibroblasts (DeBruyn, et al 1949).

Fischer described the successful culture of several strains of L-5178, a lymphocytic mouse leukemia. The medium he developed, which contains peptone and a very high level of folic acid, permits the continuous reproduction, \textit{in vitro} and in the absence of feeder layers, of the leukemic cells. Several lines of evidence have been obtained in support of the statement that leukemic cells representative of L-5178 reproduce in the medium devised. Titration studies on susceptible strains of mice with cells grown in culture have demonstrated that there is no loss of virulence on long-continued propagation \textit{in vitro}. The cells in culture retain their morphologic characteristics unaltered, continue to grow in the supernatant fluid of the medium, and retain their round-cell character and staining properties. Cultures obtained from the ascitic cavity of mice during the logarithmic phase of their growth, resume logarithmic rates of reproduction with an intervening lag period of no more than 5 hours. In addition Fischer observed that, cytological studies have demonstrated...
that the diploid character of the cells is retained after 60 cell generations in culture (Fischer, 1958). Finally, single cells, isolated from populations grown in culture, have given rise to genetically homogeneous populations of cells with the same general properties as those exhibited by parent strains (Fischer, 1958).

The medium that he developed at Yale University in 1958 and which bears his name, therefore one clone of L-5178 cells, which he and his colleagues used for most of their works (Fischer, 1971) was termed L-5178Y (the ‘Y’ stands for Yale), (D. Clive, et al, 1983). The L-5178 cell line possessed many Characteristics that made it an excellent choice for biochemical and genetic studies: adaptability to protocol changes and various genetic end points, rapid growth in suspension culture (generation time reported from 7-14 and 12-20 hours), high plating efficiency, and stable, near diploid chromosome number (40+/−1) (Fischer, 1971, Fischer, et al, 1974, Amacher, et al, 1982, Goldenberg et al, 1965, Clive, et al 1979, 1983). Derivatives of the L-5178Y were used in the studies presented in this paper which are L-5178YS (‘S’ stands for sensitive) and L-5178YR (‘R’ stands for resistance), for simplicity therefore it will be referred as ‘S’ and ‘R’.

B(α)P and DMBA are Potential Carcinogens:

Polycyclic aromatic hydrocarbons (PAH) are a ubiquitous class of chemicals produced during the combustion of fossil fuels. It is estimated in the United States that nearly 900 tons of benzo(α)pyrene, is emitted into the air each year (Williams and Weisburger, 1991) Benzo(α)pyrene occurs in many environments such as forest soils, river water, marine flora and fauna, and has been found in industrial effluents, atmospheric particulate matter, car exhaust fumes and food stuffs (Sinclair and Frost, 1978). In 1978
Swanson reported the concentration of B(α)P in the atmospheric air at the environmental level of 10 picograms/m³ air in rural area, to 460 nanograms/m³ air in industrial area, (Swanson, et al, 1978). Gelboin reported B(α)P as an arylating agent which causes mutations, chromosome breaks, and cytogenetic abnormalities in sea urchin gastrulae (Gelboin, 1980). Hose, et al reported that the exposure to B(α)P resulted in depressed mitotic rates in sea urchin embryos (Hose, et al, 1983). Santodonato suggested that the mitotic inhibition may be caused by toxic metabolites of PAH which appear to selectively attack DNA in the S phase of the mitotic cycle (Santodonato, 1981). Other studies showed representatives of this chemical compound were physically complexed with DNA in vitro (Boyland and Green, 1962. Lesko, et al, 1968, Liquori, et al, 1962) as well as becoming covalently bound to cellular macromolecules in vivo (Brooks and Heidelberger, 1969. Brooks and Lawley, 1964, Heidelberger and Moldenhauer, 1956, Miller, 1951). Strong evidence has recently been obtained that the binding of hydrocarbons to the macromolecular constituents of cultured cells, including DNA, was of the firm covalent variety and might accordingly be expected to be more important for carcinogenesis and mutagenesis (Kuroki and Heidelberger, 1971). However, the exact molecular mechanism involved in this chemical binding, the extent of their attack on the various cell components (DNA, RNA and proteins) and the role of these different reactions to the process of malignant transformation still remains to be reported (Huberman, et al, 1971., Kuroki and Heidelberger, 1971., Miller and Miller 1971). Fahmy studied the mutability at the rRNA genes in Drosophila by DMBA and B(α)P (Fahmy and Fahmy, 1970, Fahmy and Fahmy 1973). These known potent carcinogens and their derivatives have been extensively tested in different mutation assays. As mentioned, the strongest carcinogens in this category are DMBA, and B(α)P and they are present in coal and coal products and mineral oils, and they are produced in the
pyrolysis of many organic substances. The important part of these polycyclic hydrocarbons are the double bonds located in a particular region of the molecule termed as K-region. Once absorbed into cells, oxygen is added to the K-region double bond to form an epoxide, the ultimate carcinogen. A microsomal enzyme, aryl hydrocarbon hydroxylase, catalyzes this reaction. The epoxide may bind to DNA, RNA or proteins, but the carcinogenicity is most closely linked to the binding to DNA (Bradley, et al, 1981, Clatt et al, 1980, Fahmy, et al, 1973, Clive, et al, 1979, Conney, et al, 1994, Amacher, et al, 1982). The structure of benzo(α)pyrene and dimethylbenzanthracene are shown in figure-1.
Figure-1: Structure of Benzo(\(\alpha\))Pyrene and Dimethylbenzanthracene

Benzo[\(a\)]pyrene

7,12-Dimethylbenz[\(a\)]anthracene
Test for Carcinogenicity of Chemical Pollutants:

Scientists believe that alterations in the structure and function of DNA play a crucial role in the production of cancer by chemicals. The animal bioassay for detecting carcinogenic chemicals is a large, complex and very expensive scientific study using hundreds of rodents to which the suspect chemical is administered for most of their life. Also, the specific locus test in mice (Searle, 1975), which is one of the assays for detecting heritable gene mutation in mammals, requires the examination of many thousands of offspring and is equally expensive and time consuming.

Therefore, with the multitude of chemicals introduced into the environment this century, and the hundred of new compounds being synthesized each year, only a small fraction can be tested in conventional animal studies, for these reasons, the last two decades have seen the introduction of a number of relatively rapid tests for detecting mutagenic and carcinogenic chemicals. Such tests are economical in resources and produce results in a matter of weeks. All of these short-term procedures are based on the demonstration of chromosomal damage, gene mutation, or DNA damage and many of them are in vitro assays, and the test organisms ranging from bacteria and yeast to insects, plants and cultured mammalian cells.

Ames (Ames, 1977, Ames, et al, 1973) reported that the results of the salmonella typhimurium microsome test for the mutagenic potential of chemicals corresponded with observations of carcinogenicity in laboratory animals in 80-90% of the chemicals examined. More recently a lower level of correlation was indicated (Kretzer, et al, 1979), the failure to detect all animal carcinogens by this test could be due to one of three factors: a) the animal studies led to erroneous conclusions, b) the Ames test is not appropriate for use with
specific classes of chemicals, or c) the chemical induced cancer by a mechanism other than induction of genotoxicity. The major difficulty is that current definitions of the term carcinogen do not consider the mechanism by which cancer is induced in specific cases. Thus, it was suggested (Clayson, 1962, 1981); a carcinogen is an agent or process which significantly increases the yield of malignant neoplasms in a population.
AIM OF PRESENT INVESTIGATION

Benzo(a)pyrene (B(α)P) and DMBA are known as potent carcinogens. These compounds change the nucleotide sequence of DNA. These changes may occur in genes responsible for control of cell growth and behavior. In that conditions cell morphology and growth patterns are affected. Techniques are now available to look at the changes in cell behavior upon exposure to chemicals. This investigation was designed to evaluate the potency of B(α)P and DMBA to change DNA molecules in the cell in relation to its oncogenic (cancerous) behavior as well as expression of early marker in tissue culture condition (p53, ras, GST) of cancer. In this investigation it was found that both B(α)P and DMBA could change the oncogenic behavior of the cell, most possibly by affecting DNA structure.
MATERIALS AND METHODS
1. Cell culture:

a) Reagents: Fischer’s medium (Sigma Chemicals Co.), agarose (Sigma Chemicals Co.), fetal calf serum (Life Technologies), penicillin/streptomycin (Sigma Chemicals Co.), sodium pyruvate (Sigma Chemical Co.).

b) Preparation of Media: About 10.6 g Fischer’s medium and 1.125 g sodium carbonate and 0.1 g sodium pyruvate were taken in a conical flask and were dissolved in 850 ml of distilled water. The medium was sterilized by filtering through 0.22 µm membrane (Nalgen filters) and 80 ml of fetal calf serum was added. Then 1 ml of penicillin/streptomycin was added per 100 ml of medium and stored at 4°C (Freshney, 1987).

c) Culture of mouse R and S cells: Mouse leukemic cell line L-5178, R and S were cultured in the Fischer’s medium containing 10% fetal calf serum at 37°C in 95% atmospheric air and 5% CO₂. The cells were allowed to grow until it reached log phase. Cells were collected by centrifugation at 4000 rpm for 10 minutes and suspended in fresh medium so that final concentration of cells was about 5 x 10⁴ cells per ml. Fresh medium was exchanged every week and cells were used for various experiments.

The cells were cultured in the Fischer’s medium with 10% fetal calf serum in 30 ml culture flask containing 10 ml of medium, and it was subcultured every 3 days by transferring aliquots to fresh medium. Cells for the inoculum were taken from stock cultures in the logarithmic phase of growth and diluted to get a concentration of 2 to 3 x 10⁵ cells per milliliter. The cultures were incubated in a humidified incubator supplied with a constant amount of 5% CO₂ in air at 37°C. Every three days the fresh populations of cells
were centrifuged and the supernatant was aspirated and the pellet was suspended in 10 ml of fresh medium and then 1 ml of the suspension was added to 10 ml fresh medium in culture flask. The media were kept for 30 minutes at 37°C in a water bath before use.

2. Treatment of Mouse R and S Cells with Carcinogens:

a) Preparation of carcinogen (benzo(a)pyrene and dimethylbenzanthracene) solutions:

2.5 mg of Benzo[α]pyrene (m.w. 252) and dimethylbenzanthracene (m.w. 256) were dissolved in 1 ml of acetone in an eppendorff tube to get a 10 mM concentration. 100 μl of this solution was diluted to 1 ml to get a concentration of 1 mM. The solutions were aliquoted and stored at -70°C for long time storage and at -20°C for regular use.

b) Carcinogen treatment of R and S cells:

i) Treatment of R and S cells with B(α)P and DMBA: The 20 ml R and S type cells were transferred from culture flasks to 50 ml falcon tubes and centrifuged at 4000 rpm for 10 min. The supernatant was aspirated and 3 ml of fresh medium was added to the pellet and suspended. Each one ml of this suspension was transferred to new sets of tubes, total of 6 tubes and that were labeled according to the cell type and carcinogen, RC (control of R), RBP (B(α)P treatment of R), RD (DMBA treatment of R), SC (control of S), SBP (BP treatment of S), SD (DMBA treatment of S). Ten μl of acetone was added to the control, 10 μl of 10 mM DMBA to each of R and S, and 10 μl of 10 mM B(α)P to each R and S type cell. The cells were incubated for 1 hour at 37°C in the incubator. After one hour of incubation 9 ml of fresh medium was added to each and incubated for seven days. Later the cells were collected and centrifuged. The supernatant was discarded and the cells were lysed for DNA analysis.
ii) Treatment of S cells with different concentration of B(α)P and DMBA: The S cells (5x10⁴) were taken from flasks and transferred to falcon tubes and centrifuged at 4000 rpm for 10 min. The supernatant was discarded and the pellet was suspended in 3 ml of fresh medium. One ml of this suspension was transferred to a new set of tubes marked SC (control), SB1 (B(α)P treatment of 1 mM), SB10 (B(α)P treatment of 10 mM), SD1 (DMBA treatment of 1 mM), and SD10 (DMBA treatment of 10 mM). Ten μl of acetone was added to the control and 10 μl of either 1 mM or 10 mM carcinogen was added to the specific tubes. It was mixed and left for 5 minutes, 9 ml of fresh medium was added and incubated for seven days at 37°C in 95% atmospheric air and 5% CO₂. Cells were collected and DNA was isolated.

iii) Treatment of R and S cell line with repeated doses of B(α)P and DMBA: A set of R and S cells were taken (5x10⁴) and centrifuged. The supernatant was discarded and the pellet was suspended in 3 ml fresh medium. One ml of this suspension was transferred to 9 ml fresh medium and 10 μl of acetone, 10 μl of 10 mM B(α)P or 10 mM DMBA were added to the appropriate flasks. The treatment was carried out for three consecutive days and then cells were collected and DNA was isolated.

3. Isolation of Carcinogen Treated Clones:

Mouse leukemic S cells were cultured at a density of 5 x 10⁴ cells per ml in 150 mm dishes. The S cells were then treated with 10 mM B(α)P or 10 mM DMBA for three consecutive days. The medium from the plate was removed (with suspending cells) and fresh medium was added to it. The cells were allowed to grow for two weeks. Independent colonies of various sizes appeared.
Sixteen colonies (clones) were isolated under the microscope and transferred to 96 well plate containing 300 μl of medium in each well and incubated for 3 days.

4. Determination of Generation (Doubling) Time:

Each of the clones was transferred to 6 well plates containing 3 ml of medium, cultured separately and labeled as SBI to SB16. Part of individual clones was transferred to a new set of 6 well plates (each well containing $5 \times 10^4$ cells per ml) and incubated for one week. Aliquots from each culture was withdrawn and the density of cell was calculated everyday. Growth curve (Fig.3) was plotted for each of the clones. Generation (doubling) time was determined from the log phase of the curve.

The same procedure was carried out for S cells which was treated with 10 mM DMBA, in a group of 8, and two sets of controls, one for B(α)P 10 mM in a group of 10, and the other for 10 mM DMBA, in a group of 5 (total number of 39).
Each clone was plated at $5 \times 10^4$ cells per ml and incubated for one week. Aliquots from each culture were withdrawn and the cell density was counted every day. The growth curve was made by plotting the log value of the cell number versus the time. The figure shown above represents a growth curve for B(α)P treated clone.
5. Soft Agarose Colony Formation Assay:

a) Soft Agarose Preparation:

i) Preparation of base agarose (0.5%): One gram of agarose was taken in 100 ml of distilled water and heated until it completely dissolved. Agarose solution was kept to cool down to 50°C. Fischer's medium (2 x) containing 20% fetal calf serum was warmed up to 40°C. Equal volumes of the two solutions were mixed. About 1.5 ml of the mixture was added to 35 mm petri dishes. The gel was allowed to solidify and then stored at 4°C.

ii) Preparation of top agarose: About 0.7 grams of agarose was taken in 100 ml of distilled water and heated until it was dissolved completely. The solution was cooled to 50°C and Fischer’s medium (2 x) containing 20% fetal calf serum was warmed up to 40°C. Cells number from different clones were counted and adjusted to get around 4 x 10⁴ cells per ml. The 35 mm petri dishes with base agar were removed from the refrigerator 30 minutes before adding the top agar. In a 15 ml corning tube, 2.5 ml of 2x Fischer’s medium with 20% fetal calf serum and 2.5 ml of 0.7% agar was taken and was mixed gently. To this solution, 100 μl of cell suspension was taken and mixed. About 1.5 ml of this suspension was poured to plates (triplicate for each clone). The agarose suspension was kept at room temperature until it was solidified. The plates were incubated in a humidified incubator at 37°C with 95% atmospheric air and 5% CO₂ for 14 days (Nur-E-Kamal, et al., 1992, Nur-E-Kamal, et al., 1993). The number of colonies were counted under the microscope.

6. Isolation of DNA from B[α]P and DMBA Treated Clones:

For each clone 25 ml of culture was made to get a cell density of 5x10⁵ cells per ml. Culture was taken in a corning tube (50 ml) and centrifuged at 4000 rpm for 10 min. The
pellet was washed three times with PBS. Then the cells were suspended in 1 ml PBS and transferred to 15 ml corning tubes and centrifuged at 4000 rpm for 10 minutes, supernatant was discarded.

The pellet was suspended in 0.5 ml of TE and vortexed. Then the suspension transferred to eppendorff tube and centrifuged at 5000 rpm for 3 minutes, 0.5 ml of WBC lysis buffer (NaCl, 0.3M; Tris HCl pH 7.4, 10 mM:EDTA, 10 mM; Urea, 7 M; SDS 2%) was added and mixed by vortex and heated at 65°C for 10 minutes. After heating, 0.6 ml of Phenol/ chloroform/isoamylalcohol (25:24:1) was added to each tube and mixed by vortex. The mixture was centrifuged at 12000 rpm for 7 minutes and the upper phase was transferred to new tubes. About 0.6 ml of chloroform/isoamylalcohol (24:1) was added to each tube and mixed by vortex, later centrifuged for 5 minutes at 12000 rpm. The upper phase was transferred to new sets of tubes and 0.4 ml of isopropyl alcohol was added to each tube, mixed and left overnight at room temperature.

Next day it was centrifuged at 12000 rpm for 10 minutes and supernatant was discarded, and the precipitate were dried under vacuum for about 15 minutes. DNA pellet was dissolved in 0.1 ml of TE buffer. The solution was then analyzed by spectrophotometer and agarose gel electrophoresis.

7. Analysis of DNA by agarose gel electrophoresis:

a) Preparation of TBE (Tris-borate EDTA buffer):

To prepare a 5X TBE the following chemicals were used:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>54 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>27.5 g</td>
</tr>
<tr>
<td>0.25 M EDTA(pH 8)</td>
<td>40 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>960 ml</td>
</tr>
</tbody>
</table>
Total volume 1000 ml was prepared and it was diluted 5 times before use (Maniatis, 1982).

b) Preparation of agarose gel:

Different concentration (0.7% to 2%) of agarose were used. Required amount of agarose were taken in 100 ml of 1 x TBE, and were melted in a microwave oven. Five μl of ethidium bromide (10 mg/ml) were added to the solution and mixed well. The gel solution was kept at room temperature to cool down to 65°C and then poured on the DNA-subcell electrophoresis plate and was allowed to solidify (Maniatis 1982).

c) Agarose gel electrophoresis:

The gel was transferred to the electrophoresis tank containing 1 X TBE and ethidium bromide (0.5 mg/ml). Ten μl of each DNA sample was mixed with 1 μl of loading dye solution and loaded on to the gel. Five μl of lambda Hind III (size marker) was added to one well on each gel. The electrophoresis tank was connected to the power source and set at 80 volts (constant) for 90 minutes.

d) Visualization of DNA in agarose gel:

The gel was kept under ultraviolet light (transilluminator, Flowgen-TFX-20 M, San Gabriel, CA.) and picture of the gel was taken by using a polaroid camera (DS 34 with EP H6 Hood).

8. Analysis of protein from B[α]P and DMBA treated cells

a) Preparation of sample buffer:

Sample buffer was prepared by using and mixing the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>4 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>1 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>Sucrose 10%</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>2-β-mercaptoethanol</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>0.05% (w/v) bromophenol blue</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>
b) Extraction of protein:

Cells were cultured to get about $1.3 \times 10^6$ cells and collected from the culture flasks by centrifugation at 4000 rpm for 10 min. The supernatant was discarded and the pellet was suspended in 0.3 ml of sample buffer. The suspension was heated upto 95°C in a water bath for 15 minutes. Then the solution was transferred to eppendorff tubes, and stored at -20°C.

c) Estimation of Protein Concentration by Bradford Method:

A standard protein solution (1.4 mg per 1 ml) was prepared by using Bovine plasma gamma globulin (Bio-Rad protein assay kit, Standard 1) which was dissolved in 20 ml of distilled water.

Three different concentrations (14, 70 and 140 μg) of bovine plasma gamma globulin were diluted to 1 ml with distilled water and 4 ml of Bradford solution (Bradford, 1976) was added to these solutions and mixed well. The solution was kept at room temperature for 10 minutes. The absorbance of each sample was determined at 595 nm using DU-70 (Beckman) spectrophotometer. A standard curve (Fig.3) was made by plotting O.D. (Y axis) versus the concentration of protein (X axis). Protein solution from each of the clones was treated with Bradford reagent (Bio Rad) in a similar way. The absorbance of the solution was determined at 595 nm and then the concentration of protein was determined by using the standard curve.
Three different concentrations (14, 70 and 140 μg) of bovine plasma globulin solution (1.4 mg/ml) were diluted to 1 ml with distilled water. To these samples 4 ml of Bradford solution was added and mixed. The absorbance of each sample was determined at 595 nm. The standard curve was made by plotting O.D. against concentration of protein.
9. Polyacrylamide Gel Electrophoresis (PAGE):

a) Preparation of 5X running buffer:

The following amount of chemicals were taken:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trizma base</td>
<td>9 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>43.2 g</td>
</tr>
<tr>
<td>SDS</td>
<td>3 g</td>
</tr>
<tr>
<td>Distilled water to</td>
<td>600 ml</td>
</tr>
</tbody>
</table>

The chemicals were dissolved completely and stored. 1 X solution was made by diluting 60 ml to 300 ml with water before use in electrophoresis.

a) Preparation of Gel:

i) Preparation of Separating gel (12%):

The following chemicals were taken in a conical flask:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water:</td>
<td>3.35 ml</td>
</tr>
<tr>
<td>Tris-HCl buffer pH 8.8:</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS:</td>
<td>100 μl</td>
</tr>
<tr>
<td>Bis/Acrylamide 30%:</td>
<td>4 ml</td>
</tr>
</tbody>
</table>

The chemicals were mixed properly. Then 50 μl of ammonium persulfate (10%) and 5 μl TEMED were added to the mixture. The solution was poured into the plate immediately. Distilled water was overlayered gently on top of the solution and kept for 30 minutes for polymerization.

ii) Preparation of Stacking gel (4%):

The following chemicals were mixed:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water:</td>
<td>6.1 ml</td>
</tr>
<tr>
<td>Tris-HCl pH 6.8:</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS:</td>
<td>100 μl</td>
</tr>
<tr>
<td>Bis/Acrylamide 30%:</td>
<td>1.3 ml</td>
</tr>
</tbody>
</table>
The chemicals were mixed properly. Then 50 μl of ammonium persulfate (10%) and 10 μl of TEMED were added and mixed. Water overlayer of separating gel was discarded and stacking gel solution was poured into the plate. A comb was inserted into the stacking gel solution so that the comb remain 0.5 inch above the separating gel. The gel was left at room temperature for half an hour for polymerization. After polymerization the comb was removed and the gel was ready to use.

c) Polyacrylamide Gel Electrophoresis:

The gel was transferred into the electrophoretic tank and assembled properly. About 300 ml of 1 X running buffer was poured on top and bottom chamber of electrophoresis system. The samples as well as proteins low range molecular weight size marker (Bio-Rad) were heated at 95°C for 5 minutes and then cooled down to room temperature and loaded onto the gel. The gel was connected to the power source set at 200 volts for 45 minutes. The gel was then taken out of the plate and used either for staining or immunoblot.

d) Staining of Gel with Coomassie Blue:

The gels were stained in a solution containing 0.1% Coomassie blue in fixative (40% methanol/10% acetic acid) for 15 minutes and then it was destained in a solution (40% methanol/10% acetic acid) for several times (15 minutes for each) to remove the background stain. The gels were transferred to a filter paper and dried.

e) Silver Nitrate Staining:

The gel was kept in 10% ethanol for 5 minutes then transferred to 1% nitric acid for 5 minutes and rinsed with fresh 1% nitric acid. Then rinsed with distilled water and was
transferred to 0.5% silver nitrate solution (two changes of 15 minutes each) and rinsed twice with distilled water (10 minutes each).

Colour development solution (3 g sodium carbonate, 500 μl formaldehyde and 0.2 mg sodium thiosulphate were dissolved in distilled water to make 100 ml solution) was added for two changes of 2-3 minutes each till the protein bands were clearly visible and then transferred to 10% acetic acid to stop the colour development. The gel was transferred to filter paper and dried.

10. Protein Immunoblotting:

a) Reagents:

i) Preparation of Towbin’s buffer: 6.06 g of Tris and 28.8 g of glycine were dissolved in 800 ml of distilled water and 400 ml of methanol was added to the solution. Then the volume was made to 2 liters by adding distilled water.

ii) Preparation of TBS (Tris Buffer Saline): The following chemicals were measured: 0.8 g of Tris base, 6.85 g of Tris HCl and 8.9 g of Sodium chloride into a conical flask. 800 ml of distilled water was added and the chemicals were dissolved. The volume of solution was then made to one litre by adding distilled water.

iii) Preparation of TBST (Tris Buffer Saline with Tween-Twenty): One milliliter of Tween-Twenty was added to 1000 ml of TBS solution to get TBST.

b) Polyacrylamide Gel Electrophoresis:

The polyacrylamide gel was prepared as described in previous section. Ten μl of the samples boiled at 95°C for 5 minutes were loaded to the gel and it was kept in the mini-protein cell and connected to the power source set at 200 volts for 45 minutes.
c) Transfer of Protein from Gel to Nitrocellulose Membrane:

After electrophoresis the separated proteins in the gel were transferred onto nitrocellulose membrane according to the following protocol. The nitrocellulose membrane and filter paper were cut to the size of gel. In a tray, the gel holding cassette was kept open. The fibers pad, nitrocellulose membrane and filter paper were soaked in Towbin’s electrolyte buffer. On the grey panel of the cassette a fiber pad was placed, then a filter paper, gel was carefully placed on the filter paper. The nitrocellulose membrane was placed on top of gel. Another piece of filter paper was then placed on the nitrocellulose membrane. A fiber pad was placed next to filter paper and finally the white panel of cassette. The cassette was closed and it was transferred to the transfer cell. The transfer cell was filled with Towbin’s buffer and was connected to the power source at 15 volts for overnight. The protein transfer was carried out at 4°C (Towbin et al, 1979).

d) Antibody Treatment:

The membrane was taken out of the transfer apparatus. The membrane was then soaked in 5% low fat milk (dissolved in TBS) for 2 hours to block the nonspecific binding of proteins.

The membrane was washed with TBS for 15 minutes, and treated with primary antibody (either anti-GST or anti-RAS) for overnight. The membrane was then washed with TBST, four changes of 10 minutes each, and rinsed with TBS for 10 minutes, and then treated with secondary antibody coupled with alkaline phosphatase (GAM-AP) for 2 hours.
e) Colour Development:

After secondary antibody reaction, the membrane was washed with TBST, four changes of 10 minutes each, rinsed with TBS for 10 minutes. Colour development was carried out in the dark, by taking 20 ml of Alkaline phosphatase colour development buffer (AP-colour development buffer, Bio-Rad Immune-Blot Assay Kit). 0.2 ml of each of the two reagents (reagent A NBT and reagent B BCIP) was added to the buffer and immediately poured on membrane. When the bands appeared the reaction was stopped by transferring the membrane in distilled water. The membrane was photographed.

11. Polymerase Chain Reaction (PCR):

Exons of P\(^{53}\) gene were amplified by PCR (Saiki, et al., 1988; Mullis and Faloona, 1987). For each PCR reaction, the following chemicals (purchased from Gibco-BRL) were taken in a PCR tube:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>81.5 μl</td>
</tr>
<tr>
<td>10 x PCR buffer</td>
<td>10 μl</td>
</tr>
<tr>
<td>25 mM dNTP</td>
<td>1 μl</td>
</tr>
<tr>
<td>50 mM MgCl(_2)</td>
<td>3 μl</td>
</tr>
<tr>
<td>Template</td>
<td>2 μl</td>
</tr>
<tr>
<td>Primer(sense)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Primer(antisense)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5 μl</td>
</tr>
</tbody>
</table>

The following primers were used for amplification of P\(^{53}\) exons:

- Exon 6: Sense (5'-CTGATTGCTCTTAGGTCTGG-3');
- Exon 6: Anti-sense (5'-AGTTGCAAACCCAGACCTCAG-3');
- Exon 8,9: Sense (5'AGTGGTAATCTACTGGGACG-3');
- Exon 8,9: Anti-sense (5'-ATTCTCCATCCAGTGTTTC-3').
The mixture was centrifuged and three drops of mineral oil was added to each tube. PCR was carried out according to the following programme: 95°C for 1.5 min, 50°C for 1.5 min, and 72°C for 2 min. The reaction was repeated for 30 cycles. Five μl of each PCR product was mixed with 1 μl of loading dye and was loaded to 2% agarose gel (preparation of gel was described in previous section). The gel then transferred to an electrophoresis chamber and a voltage of 80 (constant) for 90 minutes was applied. The gel was treated with ethidium bromide (0.5 microgram/ml). DNA was visualized under ultraviolet light and the bands were recorded photographically.
RESULTS
Culture of R and S Type of Mouse L-5178 Cells:

Mouse cells L-5178 of R and S type were cultured in Fischer's medium containing 10% fetal calf serum in a mixture of 95% atmosphere air and 5% CO₂ at 37° C. As reported earlier, S type of cells were found to be more uniform in their shape and the colony formation was more rounded and more likely to be suspended in the medium (Fig. 4) while R type were not uniform in their size and shape and tend to be attached to the glass surface (Fig. 5). Both of these cell lines were growing happily with typical pattern of cell division (Fig. 6). Cells were stained with Papanicolaou stain, microscopical examination showed that cells were round with large nuclei which contain one or more macronucleoloe and having a thin rim of cytoplasm. The cells were showing a high mitotic rate under high power field of microscope.

Effects of DMBA and B[α]P on DNA Synthesis of Mouse L-5178 Cells of R and S Type:

Both S and R types of mouse leukemic cells were cultured in the presence of DMBA or B(α)P at 10 mM concentration. Total DNA was extracted and estimated. It was found that DNA synthesis in R cells was reduced to 68% after treatment with B[α]P, while the same cells (R) did not show any response after DMBA treatment (Table-1). Interestingly, when S type cells were treated with B[α]P and DMBA, an inhibitory role of DMBA was observed. It was found that the synthesis of DNA was inhibited to 66%. But there was no significant effect of B[α]P on the DNA synthesis of S cell type (Table-1). DNA was further characterised for their intactness by 0.7% agarose gel electrophoresis. It was found that the quality (molecular size) of DNA in DMBA and B[α]P treated R and S cells were same as untreated cells (Fig.7). There was no significant level of DNA degradation.
Figure 4. Morphology of Mouse Leukemic Cell L 5178 of S Type

Mouse leukemic cells were allowed to grow in Fischer’s medium. Photographs were taken under microscope.
Figure 5. Morphology of Mouse Leukemic Cell L5178 of R Type

Mouse leukemic cells were allowed to grow in Fischer's medium. Photographs were taken under microscope.
Mouse leukemic cells were allowed to grow in Fischer's medium. Cells were fixed with 95% ethanol followed by Papanicolaou staining. Photographs were taken under microscope.
Table I: Effect of B[α]P and DMBA on DNA content of mouse leukemic cells L-5178 of R and S type

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>DNA (µg/ml)</th>
<th>DNA (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC</td>
<td>128</td>
<td>100</td>
</tr>
<tr>
<td>RB</td>
<td>86.4</td>
<td>68</td>
</tr>
<tr>
<td>RD</td>
<td>137.2</td>
<td>107</td>
</tr>
<tr>
<td>SC</td>
<td>159.6</td>
<td>100</td>
</tr>
<tr>
<td>SB</td>
<td>137.6</td>
<td>86</td>
</tr>
<tr>
<td>SD</td>
<td>105.2</td>
<td>66</td>
</tr>
</tbody>
</table>

Mouse cells L-5178 of R and S type were allowed to grow in the absence or presence of either B[α]P or DMBA as described in materials and methods. DNA was extracted from those cells and estimated spectrophotometrically.
Mouse leukemic cells of R and S type were treated with B(α)P and DMBA. DNA was extracted and analyzed by 0.7% agarose gel electrophoresis. DNA was then stained with ethidium bromide and visualized under u.v. light. 1, RC (control of R); 2, RB (B(α)P treatment of R); 3, RD (DMBA treatment of R); 4, SB (B(α)P treatment of S); 5, SC (control of S); 6, SD (DMBA treatment of S) and M is the λ Hind III marker.
2). Effects of different concentrations of B[α]P and DMBA on DNA synthesis of mouse L-5178 cells of S type:

Mouse L-5178 cells of S type were cultured to a cell density of $5 \times 10^4$ cells/ml. B(α)P was added to the culture to a final concentration of 1 mM or 10 mM. Cells were allowed to grow in this condition for seven days. Cells were collected and genomic DNA was extracted. Amount of DNA was measured spectrophotometrically at 260 nm. It was found that when S cells were treated with 1 mM B(α)P, there was no significant effect on DNA synthesis while 10 mM B(α)P inhibited DNA synthesis to about 24% as compared to the untreated cells (Table 2). On the other hand 1 mM DMBA decreased DNA synthesis by 21%, while 10 mM DMBA inhibited the DNA synthesis by 39%.

3). Isolation of B[α]P and DMBA treated cells:

Mouse L-5178 cells of S type were treated with 10 mM B[α]P or DMBA for three consecutive days. Cells were then diluted and cultured at lower number for two weeks to allow single cells to form colonies. A parallel experiment was performed without any treatment. Colonies were picked up from all B[α]P (B series) DMBA treated (D series) and untreated (C series) plates randomly under microscope in a sterile condition and cultured separately. Cells derived from each colony were named and characterised.
Table-II: Concentration dependent effect of B[α]P and DMBA on DNA content of S cells

<table>
<thead>
<tr>
<th>Clone name</th>
<th>DNA content Amount (μg/ml)</th>
<th>DNA (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td>18.7</td>
<td>100</td>
</tr>
<tr>
<td>SBP1</td>
<td>18.5</td>
<td>99</td>
</tr>
<tr>
<td>SBP10</td>
<td>14.4</td>
<td>76</td>
</tr>
<tr>
<td>SD1</td>
<td>14.4</td>
<td>79</td>
</tr>
<tr>
<td>SD10</td>
<td>11.4</td>
<td>61</td>
</tr>
</tbody>
</table>

Mouse cells L-5178 of S types were allowed to grow in the presence or absence of different concentration of B(α)P & DMBA for seven days. DNA was extracted and estimated spectrophotometrically. Values are an average of three independent experiments.
4) Growth pattern of B[α]P and DMBA treated cells:

Each of the clones was cultured separately and the growth rate was monitored by counting cell numbers at certain time interval. Cells from each clone were cultured separately. The density of cells were determined every day and growth curves were plotted. From the log phase, doubling time (hr) was determined. Growth curve for each clone was plotted. A typical growth curve is shown in Fig 2. From the log phase of growth curve, the doubling time of each clone was determined. It was found that some B[α]P treated cells (SB5, SB7, SB14) were growing faster while some cells (SB1, SB16, SD8) were growing slower than the parental (control) cells (Table-3). It suggests that B[α]P treatment has changed the behavior of cell growth.

**Oncogenic Behavior of B[α]P and DMBA Treated Mouse L-5178 Cells:** B[α]P and DMBA treated cells were tested for their ability to grow in soft agarose. Same number of cells from each clone were plated. The number of colonies which appeared after 2 weeks were then counted. It was found that the clones had different ability to grow. Some of the clones (SB6, SB11, SB14) had more ability to grow on soft agarose compared to the parental cells, while some (SB1, SD2, SD5, and SD8) could not grow at the level of parental cells (Table 4). This suggests that B[α]P and DMBA treatment has changed the oncogenic behavior of mouse L-5178 cells. It is important to note here that fast growing cells are more cancerous than slow growing one. (Fig. 8)
Table-III: Generation time (hrs) of B[α]P and DMBA treated cells

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Doubling Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1</td>
<td>12.5</td>
</tr>
<tr>
<td>SB2</td>
<td>7</td>
</tr>
<tr>
<td>SB5</td>
<td>2</td>
</tr>
<tr>
<td>SB6</td>
<td>7.5</td>
</tr>
<tr>
<td>SB7</td>
<td>2</td>
</tr>
<tr>
<td>SB8</td>
<td>3</td>
</tr>
<tr>
<td>SB11</td>
<td>3</td>
</tr>
<tr>
<td>SB12</td>
<td>2</td>
</tr>
<tr>
<td>SB14</td>
<td>2</td>
</tr>
<tr>
<td>SB15</td>
<td>3</td>
</tr>
<tr>
<td>SB16</td>
<td>8</td>
</tr>
<tr>
<td>SC5</td>
<td>5.5</td>
</tr>
<tr>
<td>SD4</td>
<td>6</td>
</tr>
<tr>
<td>SD5</td>
<td>9.5</td>
</tr>
<tr>
<td>SD8</td>
<td>12</td>
</tr>
</tbody>
</table>

Cells from each clone of B(α)P and DMBA treated clones were cultured separately. The growth of each was monitored by counting the cell number at every 24 hrs. Doubling time was determined from the log phase of growth of each clone. Values are an average of three determinations.
Table-IV: Transforming ability of B[α]P and DMBA treated mouse L-5178 cells

<table>
<thead>
<tr>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone</td>
<td>% Oncogenic</td>
<td>Clone</td>
</tr>
<tr>
<td>name</td>
<td></td>
<td>name</td>
</tr>
<tr>
<td>SB1</td>
<td>61</td>
<td>SB2</td>
</tr>
<tr>
<td>SB16</td>
<td>78</td>
<td>SB7</td>
</tr>
<tr>
<td>SD2</td>
<td>56</td>
<td>SB8</td>
</tr>
<tr>
<td>SD5</td>
<td>39</td>
<td>SB12</td>
</tr>
<tr>
<td>SD8</td>
<td>33</td>
<td>SD4</td>
</tr>
<tr>
<td>SD7</td>
<td></td>
<td>SD5</td>
</tr>
<tr>
<td>SC6</td>
<td></td>
<td>SC6</td>
</tr>
</tbody>
</table>

Cells from each clone were plated in soft agarose containing Fischer’s medium (10% FCS). The cells were allowed to grow in 5% CO₂ at 37°C for 14 days. Colonies that appeared in each plate were counted under microscope, and categorized based on their efficiency to grow on soft agarose. Group I, growing less than parental cells; Group II, grows same as parental cells, Group III; growing more than control.
Figure-8: Soft agarose colony formation assay of mouse leukemic cell

L-5178 of S type

Transforming ability of mouse leukemic cells were examined by allowing them to grow in soft agarose as described in “Materials and Methods”. A representative size of colony appeared from transformed (A) and normal (B) cell is shown above.
5). Total protein analysis of clones treated with B[α]P and DMBA:

Total protein was extracted from similar number of cells of each clone. It was found that total amount of protein obtained from each clone did not show significant difference (Table 5). The protein from each clone was extracted and then characterized by SDS-polyacrylamide gel electrophoresis. The proteins were visualized either by Coomassie blue (Fig. 9) or silver staining (Fig. 10). In both cases, there was no qualitative or quantitative significant difference in the pattern of protein both in intensity and number of bands.

Analysis of Expression of Glutathione S-transferase π (GST) and H-Ras by Immunoblotting:

Total protein from the B[α]P and DMBA treated cells were separated by SDS-PAGE and immunoblotted using specific antibodies. It was found that there was no significant change in the expression of GST π protein in B[α]P and DMBA treated cells (Fig. 11). In a similar experiment, the expression of H-Ras protein was analysed by using a primary antibody against human H-Ras protein. It was found that the expression level of H-Ras protein in B[α]P and DMBA treated cells as well as parental cells of L-5178 was very low (data not shown).

Analysis of P53 gene exons by polymerase chain reaction (PCR):

To check whether there is any mutation in P53 exons in B[α]P and DMBA treated cells, PCR analysis was performed. Using specific primers, exon 6, 8 and 9 was amplified and the size of PCR product was analysed by agarose gel electrophoresis. The size of exons in both B[α]P and DMBA treated cells were same as control (Fig. 12 and 13).
Table-V: Determination of total protein extracted from B[α]P and DMBA treated cells

<table>
<thead>
<tr>
<th>Clone name</th>
<th>Protein (mg)</th>
<th>Clone name</th>
<th>Protein (mg)</th>
<th>Clone name</th>
<th>Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1</td>
<td>1.5</td>
<td>SB13</td>
<td>1.8</td>
<td>R</td>
<td>2.2</td>
</tr>
<tr>
<td>SB2</td>
<td>1.7</td>
<td>SB14</td>
<td>2.4</td>
<td>S</td>
<td>2.5</td>
</tr>
<tr>
<td>SC1</td>
<td>2.5</td>
<td>SB3</td>
<td>1.6</td>
<td>SB15</td>
<td>1.7</td>
</tr>
<tr>
<td>SC10</td>
<td>1.7</td>
<td>SB12</td>
<td>1.6</td>
<td>SD8</td>
<td>1.4</td>
</tr>
<tr>
<td>SC2</td>
<td>1.9</td>
<td>SB4</td>
<td>1.7</td>
<td>SB16</td>
<td>1.9</td>
</tr>
<tr>
<td>SC3</td>
<td>1.6</td>
<td>SB5</td>
<td>1.4</td>
<td>SD1</td>
<td>1.8</td>
</tr>
<tr>
<td>SC4</td>
<td>1.6</td>
<td>SB6</td>
<td>1.3</td>
<td>SD2</td>
<td>1.2</td>
</tr>
<tr>
<td>SC5</td>
<td>2.5</td>
<td>SB7</td>
<td>1.7</td>
<td>SD3</td>
<td>1.6</td>
</tr>
<tr>
<td>SC6</td>
<td>1.9</td>
<td>SB8</td>
<td>1.4</td>
<td>SD4</td>
<td>2.4</td>
</tr>
<tr>
<td>SC7</td>
<td>1.5</td>
<td>SB9</td>
<td>1.2</td>
<td>SD5</td>
<td>2.2</td>
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<tr>
<td>SC8</td>
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<td>SB10</td>
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<td>1.8</td>
</tr>
<tr>
<td>SC9</td>
<td>1.9</td>
<td>SB11</td>
<td>2.4</td>
<td>SD7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Total protein was extracted from similar number of cells of each clone and protein concentration was determined by Bradford Method. Values are an average of three determinations which varies less than 10% from each other.
Figure-9: Coomassie blue staining of SDS-polyacrylamide gel of total protein extracted from mouse leukemic cell L-5178

Total protein extracted from different clones was separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue as described in Materials and Methods. Above figure shows the expression level of total cellular proteins. 1, SC (control of S); 2, SB1, 3, SB2, 4, SB7, 5, SB8, 6, SB11, 7, SB15 (2-7 are clones of the B(α)P treated S cells); 8, SD4, 9, SD8 (8 and 9 are clones of DMBA treated S cells), and M is molecular weight marker.
Figure-10: Silver staining of SDS-polyacrylamide gel of total protein extracted from mouse leukemic cell L-5178 of S type

Total protein was extracted from each clone. Proteins in each extract were separated by SDS-polyacrylamide gel electrophoresis. Gel was stained by silver nitrate. Above figure shows the expression level of total cellular proteins. 1, SC (control of S); 2, SB1, 3, SB2, 4, SB7, 5, SB8, 6, SB11, 7, SB15 (2-7 are clones of B(α)P treated S cells); 8, SD4, 9, SD8 (8 and 9 are clones of DMBA treated S cells), and M is molecular weight marker.
Total proteins extracted from each clone were separated by SDS-polyacrylamide gel electrophoresis and proteins were transferred from gel to nitrocellulose membrane as described in Materials and Methods. Membrane was treated with primary antibody (anti-GST Pi) as it was described in “Material and Methods”. Arrow shows the position of GST protein (22.5 KDa). 1, SC (control of S cells); 2, SB1, 3, SB2, 4, SB7, 5, SB8, 6, SB11, 7, SB15 (2-7 are clones of the B(α)P treated S cells); 8, SD4, 9, SD8 (8 and 9 are clones of DMBA treated S cells).
Figure 12: Characterization of exon 6 of P53 gene by polymerase chain reaction (PCR)

Exon 6 of P53 gene was amplified using total genomic DNA. The PCR product was characterized by 2% Agarose gel electrophoresis. The position of the PCR product of exon 6 of P53 gene is shown by arrow (95 bp). 1, SC (control of S cells); 2, SB1, 3, SB2, 4, SB7, 5, SB11 (2-5 are clones from B(α)P treated S cells); 6, SD4, 7, SD8 (6 and 7 are clones from DMBA treated S cells).
Figure -13: Characterization of exons 8 and 9 of P\textsuperscript{53} gene by polymerase chain reaction (PCR)

Exons 8 and 9 of P\textsuperscript{53} gene was amplified using total genomic DNA. The PCR product was characterized by 2% agarose gel electrophoresis. The position of PCR product of exons 8 and 9 of P\textsuperscript{53} gene is shown by arrow (320 bp). 1, SC (control of S cells); 2, SB1, 3, SB2, 4, SB7, 5, SB11 (2-5 are clones of the B(α)P treated S cells); 6, SD4, 7, SD8 (6 and 7 are clones of the DMBA treated S cells).
DISCUSSION
Polycyclic aromatic hydrocarbons are known to have the ability to bind to DNA double helices in their native structure or oxidized form. Such binding eventually leads to change in nucleotide structure (mutation) in DNA (Brookes, et al, 1964, Brookes and Heidelberger, 1969, Brookes and Lawley, 1964, Boyland and Green, 1962, Fahmy and Fahmy 1970, 1973, Heidelberger and Moldenhauer, 1956, Huberman, et al, 1971, Kuroki and Heidelberger, 1971, Lesko et al, 1968, Liquori, et al, 1962, Miller, 1951, Miller and Miller, 1971, Ames 1979). Mutation in a gene could change the structure and property of the cell such as growth rate, oncogenicity (cancer causing ability). B[α]P and DMBA are polycyclic hydrocarbons and widely distributed as the environmental pollutant such as in petroleum products. The ability of B[α]P and DMBA to cause changes in DNA was evaluated in tissue culture condition by exposing mouse L-5178 cells to these chemicals.

A condition was set up to culture mouse L-5178 cell lines of both R and S type. The effect of B[α]P and DMBA on mouse cell line L-5178 on either R or S type was tested at 1 mM and 10 mM concentration. It was found that 10 mM B[α]P has inhibited the growth of R cells significantly, while a significant inhibition of S cell growth was found after 10 mM DMBA treatment. The growth of S cells were found to be affected by DMBA and B[α]P treatment at 1 mM concentration. Because of less toxicity on growth, DMBA and B(α)P of 1 mM were chosen for S cells. After exposing with B[α]P and DMBA, colonies derived from each cell (clone) were picked up and cultured separately.

The effects of B[α]P and DMBA on mouse cells were analysed by determining the time for cell division (generation or doubling time) of each clone. Some B[α]P and DMBA treated cells were found to change growth behavior. Therefore, some clones growing either
faster or slower than the parental cell line. Recent finding in the study of cell growth control shows that there are mainly two groups of genes (proto oncogene and tumor suppressor genes) that control cell growth (Cooper, 1995). Activating mutation of protooncogenes or inactivating mutation of tumor suppressor genes cause an increase in cell growth while inactivation of proto oncogene or activation of tumor suppressor genes results in the inhibition of cell growth. Most probably the treatment of B(α)P and DMBA resulted in random mutations of either activating or inactivating type in protooncogenes and/or tumor suppressor genes. Thus, some cells have shown increase (SB5, SB7, SB14) or decrease (SB1, SB16, SB8) in growth rate than their parental cells. Interestingly, mutation in protooncogenes and tumor suppressor genes not only change the growth rate of cells but in some cases changes the oncogenic (cancer causing) behavior of the cells. One of the commonly used methods to evaluate oncogenicity is to check the ability of the cells to grow on soft agarose (known as soft agarose colony formation assay). The ability of B(α)P and DMBA treated cells to grow on soft agarose was tested and compared with parental cells. It was found that the oncogenic behavior of B(α)P treated clones SB5, SB6, SB11, SB14 and SB15 has increased. About 20% of total clones were found to show increase in oncogenic behavior while 15% showed a decrease. Changes in oncogenic behavior are most likely because of the mutation in either protooncogenes or tumor suppressor genes or both. It is likely that exposure of B(α)P and DMBA will cause similar effects once exposed to human cells. This finding suggests a possibility of increased cancer incidence if there is a pollution of B(α)P and DMBA.

We have tried to characterize molecular change(s) in the cell that resulted from B(α)P and DMBA treatment. Glutathione-S-transferase (GST) is considered to be a potential
marker for cancerous growth. The level of this protein expression changes in some cancer. Total protein from B[α]P and DMBA treated clones which showed increased oncogenicity were used to check expression level of GST protein by immunoblotting. But in this case, there was no change in GST expression in any of B[α]P and DMBA treated cells. It may be possible that expression of GST changes in specific types of cancerous situation as others believe (Harada, et al 1992; Hayes, et al 1987). Ras family of G proteins plays a key role in the development of cancer. The expression of Ras G protein was also analysed by immunoblotting using alkaline phosphatase conjugated second antibody. There was no detectable protein band in either parental cells or in carcinogen treated cells, while in control (mouse liver extract) a protein band of expected size (data not shown) appeared. There is evidence indicating that leukemic cells express very low amount of Ras protein (Maruta and Burgess 1992). Therefore, a more sensitive method such as hydroxy peroxidase conjugated system or radiolabeled antibody might be useful in detecting Ras proteins in these cell lines.

\( p^{53} \) is known as a tumor suppressor gene which regulates cell division. Mutation in \( p^{53} \) gene has been reported in its different exons (Hollstein, et al 1991; Harris 1993). Total genomic DNA of B[α]P and DMBA treated cells were analysed by PCR amplification of exons 6, 8 and 9. Analysis of these exons by agarose gel electrophoresis (AGE) did not show any difference with control (parental) DNA. This suggests that there was no significant structural change (deletion or insertion) in these clones. It might be worth to mention here that identification of a single base change is not possible by AGE. A more sensitive method like single stranded conformation polymorphism (SSCP) or nucleotide sequencing might be useful to detect small change in DNA. Moreover, analysis of all the exons or whole genes might clearly show the exact mutational status of this gene. Work is
now in progress to look on mutation of $p^53$, Ras gene and other genes that control cell growth. Identification of changes in these genes might be useful in understanding molecular mechanisms involved in altering oncogenic behavior of B[αP and DMBA treated cells.
1. Benzo(α)pyrene [B(α)P] and dimethylbenzanthracene (DMBA) were found to inhibit the synthesis of DNA.

2. DMBA and B(α)P were found to affect the generation time of mouse leukemic cells.

3. B(α)P and DMBA treated mouse cells were found to affect oncogenic (cancerous) property of 25% of total number of cell analysed suggesting that B(α)P and DMBA treatment resulted some permanent changes (most likely DNA) in the cell.

4. Expression of glutathione-S-transferase (GST) and Ras GTPase did not change significantly after B(α)P and DMBA treatment. There were no major change in size of the exons 6,8 and 9 of p53 gene.

5. B(α)P and DMBA were found to be potential carcinogens (cancer causing agent) in cell culture condition. It suggests that B(α)P and DMBA are important compounds that could be used to study the stages of carcinogenesis.

6. Pollution of B(α)P and DMBA might play important role in increasing the incidence of cancer.
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تم اختبار المكون السرطاني الكامن لكلا من البنزوبيرين وثنائي ميثيل بنزانتراتسين باستخدام خطوط خلايا اللوكيميا للفأر 15178 أنواع S و R في حالة زرع الأنسجة.

وقد وجد أن البنزوبيرين وثنائي ميثيل بنزانتراتسين يثبط منتكوين الحامض النووي في الخلايا إلى حوالي 32% بينما لم يظهر أي تأثير سمي من R في أنواع (DNA) البنزوبيرين وثنائي ميثيل بنزانتراتسين عند تركيز ميل مولار على خلايا الفأر أنواع S على التوالي. عدد من المستعمرات المستقلة عزلت من الخلايا المعالجة بالبنزوبيرين وثنائي ميثيل بنزانتراتسين ووزعت منفصلة.

وقد تم التحقق من تأثير ثانوي ميثيل بنزانتراتسين والبنزوبيرين في تغيير تركيب الحامض النووي في العلاقة إلى النمو وتكوينه الأورام) في هذه المستعمرات. وقد وجد أن العديد من المستعمرات تغيرت في نمط نموها وتكونت في وقت قصير. وتعادل تكوين المستعمرة بالأغادر الطري تم تعين سلوك تكون الأورام لكلا من البنزوبيرين وثنائي ميثيل بنزانتراتسين في المستعمرات المعالجة. وقد وجد أن 25٪ من المستعمرات تغيرت (15٪ ازدياد و 10٪ تناقص) في سلكها لتكوين الأورام. هذا يوحي بأن كلا من البنزوبيرين وثنائي ميثيل بنزانتراتسين صنعت بعض التغيرات الدائمة (غالبا في الحامض النووي DNA) في الخلايا.

لقد أجرينا عدة محاولات لوصف هذه التغيرات عند مستوى الجزيئي بالتحليل العثماني لجليتاثيون - اس - كاربات رابير (GST) والرأسم دي تي بي أي بواسطة التحليل البروتيني المناعي. ولم نجد حدوث أي تغيير. 

وبوساطة التحليل الفلازيمسي البوليميراز (PCR) وسمند جنح حدوث أي طفرة في هذه الأكسونات.

هؤلاء الاختبارات أوجت بأن كلا من البنزوبيرين وثنائي ميثيل بنزانتراتسين لهم القدرة على تغيير التركيب الحامض النووي (DNA) مما أدى إلى زيادة السرطانية في حالة زرع الأنسجة. وبذلك تتواصل إلى زيادة التأكيق بأن التلوث البيئي بالبنزوبيرين وثنائي ميثيل بنزانتراتسين قد يزيد من حدوث السرطان.
الإشراف

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