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Correlation between O^6-Methylguanine-DNA Methyltransferase Activity and Resistance of Human Cells to the Cytotoxic and Mutagenic Effect of N-Methyl-N'-Nitro-N-Nitrosoguanidine

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Veronica M. Maher
Major professor

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CORRELATION BETWEEN O^6-METHYLGUANINE-DNA METHYLTRANSFERASE ACTIVITY AND RESISTANCE OF HUMAN CELLS TO THE CYTOTOXIC AND MUTAGENIC EFFECT OF N-METHYL-N'-NITRO-N-NITROSOGUANIDINE

By

Jeanne Domoradzki

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ABSTRACT

CORRELATION BETWEEN O^6-METHYLGUANINE-DNA METHYLTRANSFERASE ACTIVITY AND RESISTANCE OF HUMAN CELLS TO THE CYTOTOXIC AND MUTAGENIC EFFECT OF N-METHYL-N'-NITRO-N-NITROSOGUANIDINE

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Cells from Gardner's syndrome (GS) and familial polyposis (FP) patients, persons with a hereditary predisposition to colon cancer, were compared to those of normal persons for sensitivity to the cytotoxic and mutagenic action of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). MNNG, a model compound, was chosen because methylating agents have been implicated in colon carcinogenesis. A positive result would support the hypothesis that mutations occurring in the colon epithelial cells of these patients at an abnormally high frequency are a contributing factor in the disease. The levels of O^6-methylguanine-DNA methyltransferase (MT) activity in FP and GS cells were also compared with that of other human fibroblasts. MT, a

protein acceptor molecule that removes alkyl groups from the O^6 position of guanine and transfers them to cysteine to form S-alkylcysteine and to regenerate guanine, has been shown to be present constitutively in human fibroblasts. Since O^6-methylguanine is considered the major lesion responsible for mutations induced by methylating agents in mammalian cells including human cells, there was a possibility that GS and FP cells would be deficient in such a DNA repair system.

FP cell line GM2355, GS cell lines 2938 and GM3948, and xeroderma pigmentosum (XP) cell line XP12BE exhibited normal sensitivity to the cytotoxic and mutagenic effects of MNNG and had normal levels of MT activity when compared to normal human fibroblasts. In contrast, GS cell line GM3314, cells from an apparently normal fetus GM0011, and fibroblasts from an SV40 virus-transformed XP cell line (XP12ROSV) showed extreme sensitivity to the killing and mutagenic effects of MNNG and had non-detectable levels of MT activity. An SV40 virus-transformed normal cell line, GM637, showed an intermediate level of sensitivity to MNNG and a reduced level of MT activity.

These results suggest that O^6-methylguanine, and/or any other adduct repaired by MT, is a potentially cytotoxic and mutagenic lesion. They also indicate that the predisposition to colon cancer of FP and GS patients is not necessarily correlated with an increased sensitivity of their fibroblasts to mutations induced by methylating carcinogens.

To

my parents, Stanley and Olympia Domoradzki;
my husband, Edward Valenzuela;
and my dear friend, Jinx Steiner

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ABBREVIATIONS

CHO,	Chinese hamster ovary (cells)
$D_{\emptyset}$,	dose required to reduce the survival 1/e along the exponential portion of the curve
$D_{1\emptyset}$,	dose that lowers the cell survival to 1Ø% of the untreated control
DEN,	diethylnitrosamine
DMH,	1,2-dimethylhydrazine
DMN,	dimethylnitrosamine
DMS,	dimethylsulfate
DMSO,	dimethylsulfoxide
DT,	diphtheria toxin
EDTA,	ethylenediaminetetraacetic acid
EMS,	ethyl methanesulfonate
ENU,	N-ethyl-N-nitrosourea
FP,	familial polyposis coli
G_1,	period in cell cycle between mitosis and S phase
G_1/S,	border of the G_1 and S phases of the cell cycle
GS,	Gardner's syndrome
HEPES,	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT,	hypoxanthine(guanine)phosphoribosyltransferase
^{3}H-TdR,	tritiated thymidine
3-MeA,	3-methyladenine
7-MeG,	7-methylguanine

MMS,	methyl methanesulfonate
MNNG,	N-methyl-N'-nitro-N-nitrosoguanidine
MNU,	N-methyl-N-nitrosourea
MT,	O^6-methylguanine-DNA methyltransferase; methyltransferase
O^6-MeG,	O^6-methylguanine
$\underline{s}$,	Swain-Scott constant
S phase,	semi-conservative DNA synthesis period in the cell cycle
SCE,	sister chromatid exchange
SV40,	simian virus 40
TG,	6-thioguanine
XP,	xeroderma pigmentosum

I. INTRODUCTION

Bauer (1928), Boveri (1929) and Sutton (1938) suggested that carcinogens could bring about the transformation of a normal cell into a tumorigenic one by introducing a mutational event, an alteration in the hereditary material. Introduction of this hypothesis, known as the somatic cell mutation theory of carcinogenesis, preceded knowledge of the molecular structure of genetic material. It is accepted today, but has had fluctuating levels of support in the past (Burdette, 1955; Kaplan, 1959). This theory accounts for the monoclonal character of cancer and its irreversibility. DNA alterations, as an efficient means for preserving and perpetuating new information, remain the most logical mechanism for the initiation of cancer.

Epidemiological evidence suggests that the majority of human cancers are caused by environmental factors. An early step in the carcinogenic process, initiation, is thought to involve the interaction of an electrophilic chemical or of radiation with cellular DNA (Miller, 1970). The resulting alterations in the DNA structure may interfere with the vital functions of the DNA molecules; replication and transcription into RNA molecules with subsequent translation into functional proteins. According to Strauss (1977), such

DNA damage, if unrepaired, could: 1) lead to cell death if a critical protein is no longer active, 2) have no effect whatsoever, if the change is in a noncoding (nonessential) region of DNA (silent mutation), or 3) lead to an altered expression of crucial proteins.

There exist cellular repair pathways for coping with a wide variety of types of DNA damage (Hanawalt et al., 1978, 1979; Lehmann and Karran, 1981). These pathways may involve simple reversal of the damaged structure to an undamaged one, removal of the damaged section of DNA and its replacement by resynthesis (excision repair), or tolerance mechanisms which enable daughter DNA strands to be synthesized despite the presence of persisting DNA damage in the parental strands. The rate, extent and accuracy of these DNA repair processes will determine the fate of the cell, whether it will die, survive unchanged, or survive with some permanently altered genotype (mutation).

Information on the series of events leading from DNA damage to tumor formation has come from the study of persons with a predisposition to cancer such as xeroderma pigmentosum (XP). XP is a recessively inherited disease in which sunlight-induced skin cancer occurs at a much higher frequency than in the general population. Fibroblast cells from xeroderma pigmentosum patients show greatly increased sensitivity to DNA damage caused by chemical and physical agents compared to cells from normal individuals. For example, XP cells have higher frequencies of mutants induced

by ultraviolet light (254 nm), simulated sunlight, and chemicals such as the reactive metabolites of benzo[a]pyrene, than normal human cells (Maher and McCormick, 1976; Patton et al., 1984; Yang et al., 1982). This implicates a molecular defect in their response to DNA damage and this defect has been found to be in DNA excision repair. The studies with xeroderma pigmentosum cells support the somatic cell mutation theory, that is, carcinogenesis is the result of DNA damage.

Other examples of cancer prone syndromes include Gardner's syndrome (GS) and familial polyposis coli (FP). Patients with these diseases are extremely prone to intestinal polyposis. Methylating agents such as 1,2-dimethylhydrazine have been implicated in colon carcinogenesis in experimental animals (IARC, 1974) and it has been reported that skin fibroblasts from familial polyposis coli and Gardner's syndrome patients are hypersensitive to killing, chromosomal aberrations, and transformation by DNA-damaging agents, including alkylating agents (Paterson et al., 1981; Little et al., 1980; Barfknecht and Little, 1982; Miyaki et al., 1982; Miyaki et al., 1980; Hori et al., 1980; Kopelovich et al., 1979).

The present study was undertaken to determine: 1) if skin fibroblasts from familial polyposis coli and Gardner's syndrome patients are abnormally sensitive to cell killing and mutations induced by the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 2) if familial polyposis

and Gardner's syndrome cells are deficient in the repair of MNNG-induced damage, and 3) the biologically important lesions induced by MNNG.

II. LITERATURE REVIEW

A. Mutagenesis in Mammalian Cells

A tumor cell has altered growth properties compared to normal cells. During the steps leading from a normal cell to a tumor cell, permanent changes occur that can be transmitted from cell to cell for many generations. These changes allow the tumor cell to grow autonomously instead of responding to the growth control mechanisms of normal cells (Braun, 1974). The theory of Boveri (1929) and Bauer (1928) that a single chromosomal or mutational event in somatic cells is the specific change responsible for tumorigenesis is known as the somatic cell mutation theory. This theory attempts to explain the irreversible nature of neoplastic transformation, the large variety of tumor types, and the clonal and hereditary nature of cancer.

Alterations in DNA provide a logical mechanism for the initiation of cancer because the neoplastic state is heritable. Other considerations indicating that DNA is the target for the initiation of carcinogenesis are: 1) many carcinogens are or can be metabolized to electrophiles that react covalently with DNA (Miller and Miller, 1971), 2) many

carcinogens are also mutagens (McCann et al., 1975), 3) defects in DNA repair such as in xeroderma pigmentosum (XP) predispose those individuals to cancer development (Cleaver and Bootsma, 1975) and 4) cancers display chromosomal abnormalities (German, 1972).

Epigenetic mechanisms such as interactions of carcinogens with proteins and RNA may also initiate carcinogenesis. The concept that chemical carcinogenesis might result from a primary alteration of cellular RNA developed from the fact that RNA can be transcribed as DNA, and the resulting DNA can be integrated into the host genome (Temin, 1974). A mechanism in which RNA could play a role in change of states of differentiation has been proposed by Dickson and Robertson (1976). Alteration of certain protein molecules could have the potential of permitting the development of cells with altered genomes. For example, β-propiolactone-treated DNA polymerase has an increased rate of error in the repair process (Loeb et al., 1974; Sirover and Loeb, 1974).

1. Types of DNA Damage

The types of DNA damage caused by chemical and physical agents and errors introduced during the course of replication have been described by Drake and Baltz (1976), Nagao et al. (1978), Hanawalt et al. (1978), and Roberts (1978). The most common DNA lesions are a missing base, an altered base, an incorrect base, deletion or insertion of a

base, a cyclobutane dimer, strand breaks and crosslinking.

Missing bases, such as the loss of purines, can occur as the result of acid and heat treatment. At physiological temperatures, 10,000 purines are lost per mammalian genome per day (Lindahl, 1979). Some alterations in DNA have a negligible effect on the functioning of DNA. An example of such an alteration is alkylation at the N-7 position of guanine (Strauss, 1976). However, it should be noted that N-7 alkylguanine can be slowly lost from DNA by spontaneous depurination.

Other alterations in DNA can cause small distortions in the double helix and interfere with the base pairing properties. Alkylation of the O-6 atom of guanine will convert the atoms around the 1-6 bond from the normal keto form into the enol form. Thus, this will cause the substituted guanine to be recognized as adenine (Loveless, 1969) such that during replication, a thymine will pair opposite the modified guanine (miscoding).

Improper base pairing may result from an incorporation error during replication and subsequent deficient proofreading. Deaminations (cytosine to uracil, adenine to hypoxanthine) can occur spontaneously because of inherent chemical instability or be induced, for example, by nitrous acid. Mutations due to deamination are transitions, that is, a pyrimidine replacing the other, or a purine replacing the other. For example, TA becomes CG.

Deletion or insertion of a base can be produced by

intercalating agents. These agents insert between the planar rings of the bases and induce replication or recombination errors by omission or addition of an extra base. These errors produce frameshift mutations which put the reading frame for translation of mRNA out of phase from the point that the nucleotide was inserted or deleted. Distortions in the helix covering several nucleotides can be produced by more bulky types of damage, for example, the cyclobutane pyrimidine dimer, the principal lesion produced in DNA by irradiation with ultraviolet light. Various chemical agents, such as bleomycin, and ionizing radiation can disrupt the physical structure of DNA by breaking the phosphodiester bonds of one strand of the double helix (single-strand break) or of both strands at neighboring sites (double-strand break).

Crosslinks between two strands can be produced by chemicals such as bifunctional alkylating agents and mitomycin C.

2. Consequences of DNA Damage

An alteration in DNA is a mutation and mutations may result from a number of factors (spontaneous, induced, oncogenic viruses) which change the base sequence in DNA and thus result in a change in genetic information. Mutations may have several effects upon a cell. Mutations may cause no noticeable change in the cell function, modify cell function but still be compatible with cell growth and

reproduction, or lead to death of a cell. Spontaneous mutations are the result of normal cellular operations and occur at a characteristic rate in any given organism. The occurence of mutations can be increased by treatment of cells with chemicals (induced mutations).

3. Assays for Measuring Carcinogen-Induced Increases in Mutant Frequencies

Assays for measuring mutation frequencies induced by carcinogens have been reviewed by Chu and Powell (1976) and Arlett (1977). In mammalian cell mutagenesis, four common assays to measure the mutational response are resistance to: 1) purine analogs, 2) bromodeoxyuridine 3) ouabain, and 4) diphtheria toxin. The genotypic change is identified through a phenotypic change such as loss of an enzyme (forward mutation).

The most widely used assay, developed by Szybalski (1964), measures the presence or absence of the activity of the x-linked enzyme hypoxanthine(guanine)phosphoribosyl-transferase (HPRT). Mutants lacking the purine salvage enzyme are identified by their resistance to purine analogs, such as 8-azaguanine and 6-thioguanine (TG), whereas normal cells die because they incorporate these analogs into DNA in place of guanine.

Another system selects for cells resistant to bromodeoxyuridine caused by loss of the activity of thymidine kinase. Bromodeoxyuridine is toxic to cells with

normal thymidine kinase activity because it is incorporated into DNA in place of thymidine. Therefore growth in bromodeoxyuridine allows only cells lacking thymidine kinase to survive. Another selective agent, ouabain, inhibits plasma membrane Na^+/K^+ - ATPase in normal cells, however, the basis of change in the resistant variant phenotype is not known. Cell killing by diphtheria toxin (DT) results from irreversible inhibition of protein synthesis. DT first binds to the cell membrane and then one subunit of the DT protein catalyzes the transfer of ADP-ribose from NAD^+ to a histidine residue of elongation factor-2, thereby inactivating protein synthesis (Pappenheimer, 1977; Van Ness et al., 1980). Most DT resistant mutants contain lesions in the elongation factor-2 structural gene which render the protein synthesis factor resistant to inactivation by DT (Moehring and Moehring, 1979; Gupta and Siminovitch, 1978).

4. Mechanisms of Action of Mutagens

Chemical carcinogens do not have any common structural feature. What chemical carcinogens do have in common is that they give rise to electrophilic reactants that interact covalently with nucleophilic sites in DNA, RNA and protein (Miller and Miller, 1976). Carcinogenic chemicals can be direct acting such as alkylating and acylating agents or they may need to be metabolized to a reactive form, e.g., by a cytochrome P-450 system (Miller, 1970; Weisburger and Williams, 1975). The nucleophilic sites in nucleic acids

that are known to be substituted *in vivo* by carcinogens are the O^6, N^3, N^7 and C^8 positions of guanine, the N^1, N^3 and N^7 positions of adenine, the N^1, N^3 and O^2 positions of cytosine and the N^3 and O^4 positions of thymine (Singer, 1975; Lawley, 1976; Singer, 1976). The nucleophilic atoms in proteins that have been shown to be substituted by carcinogenic electrophiles *in vivo* are the sulfur atoms of methionyl and cysteinyl residues, the ring-nitrogens of histidyl residues and C-3 of tyrosyl residues. The kind and relative proportion of carcinogen induced DNA lesions (adducts) depends on the type of carcinogen. Most chemical mutagens in their ultimate forms are also strong electophilic reactants. Studies have shown that there is a relationship between chemical mutagens and chemical carcinogens (McCann *et al.*, 1975; Miller and Miller, 1971; Montesano, 1976; Stoltz, 1974).

5. DNA Repair Systems

Three basic repair mechanisms are operative in cultured human cells (Cleaver, 1974; Hanawalt, 1978, 1979): photoenzymatic repair, excision repair and postreplication repair. Pyrimidine dimers are the sole lesions acted upon by photoreactivation repair. In this process, dimers are monomerized by a specific enzyme (photolyase) in a two-step reaction. First the photoreactivating enzyme binds to a dimer-containing site, and then, upon absorption of photon energy in the wavelength range 300-600 nm by the enzyme-DNA

complex, the photoproduct is converted back to two normal monomers (Sutherland, 1978).

In the excision process, repair is accomplished by the release of a defective single strand region followed by replacement with undamaged nucleotides, utilizing the complementary intact strand for base pairing instructions. Two different modes of excision repair are known: nucleotide excision repair (Grossman, 1978) and base excision repair (Friedberg *et al.*, 1978).

In the process of nucleotide excision repair, the damaged regions of DNA are enzymatically repaired by the following steps: 1) an incision by an exonuclease is made in the strand of DNA near the point of damage, 2) an endonuclease removes the damaged region, 3) the gap is filled by resynthesis of a patch using the opposite strand as a template with DNA polymerase and 4) the patch is closed by a ligase. In the base excision mode, a modified base such as uracil or 3-methyladenine, is released as a free base by a DNA glycosylase. The site containing the resulting apurinic/apyrimidinic site is then replaced in the same fashion as described above for nucleotide excision repair.

Two basic models of postreplication repair (also termed replicative or bypass repair) have been proposed: (1) upon encountering UV photoproducts, such as dimers, the replication apparatus skips past and reinitiates beyond, leaving large gaps that are eventually closed by *de novo* synthesis (Lehmann, 1974) and 2) the replication machinery

pauses momentarily when reaching lesions and in some fashion eventually circumvents the lesions (Edenberg, 1976). In both models, insertion of the correct nucleotide sequence opposite a lesion-containing site must occur and this can be accomplished by recombinational exchanges between sister or homologous chromatids. Another proposal permits insertion of the correct nucleotide sequence by continuous de novo synthesis (Higgins et al., 1976). Upon reaching a lesion, DNA synthesis proceeds in the strand not containing the lesion but is impeded in the other strand by the presence of the lesion. This newly synthesized single strand could then be displaced and serve as a template for continuation of synthesis in the second strand, thereby circumventing the lesion, using a native template.

B. Hereditary Predisposition to Cancer

Steady progress is being made in elucidating the events leading to cancer. A major question is, what is the role of carcinogen-induced damage to DNA and its inefficient enzymatic repair in the initiation of carcinogenesis? Evidence that such damage to DNA can be an etiologic factor stems from the discovery that in certain rare genetic diseases, affected individuals are predisposed to cancer and their cells in culture exhibit deficient repair of DNA damage.

Xeroderma pigmentosum (XP), ataxia telangiectasia (AT),

and Fanconi's anemia (FA) are associated with a high cancer risk and defective DNA repair. All three diseases are inherited as autosomal recessive traits (Kraemer, 1977; Setlow, 1978). XP patients develop skin cancer, AT patients have a higher than normal frequency of lymphatic cancer and FA patients are prone to leukemia and skin cancer. Paterson and Smith (1979) suggested that many AT strains have depressed levels of repair replication in response to ionizing radiation. FA cell lines are reported to be defective in initiating the repair of interstrand DNA crosslinks caused by physical and chemical carcinogens (Fujiwara et al., 1977). Only the XP syndrome has been identified with a specific molecular defect in DNA repair, that is excision repair (Cleaver and Bootsma, 1975).

1. Xeroderma Pigmentosum Cells Are Defective in DNA Repair

XP patients are extraordinarily susceptible to UV-induced cancer and develop multiple carcinomas of the skin when exposed to sunlight. Cells from most XP patients have been shown to be deficient in DNA excision repair or to have slower rates of postreplication repair of UV-induced thymine dimers (Cleaver, 1969a; Robbins et al., 1974; Cleaver, 1969b; Cleaver, 1974; Burke et al., 1971; Kleijer et al., 1973). Maher and coworkers (1976) investigated the effect of repair on the frequency of mutations in both XP strains and normal cells. When normal skin fibroblasts and

fibroblasts from excision- deficient XP patients were exposed to an equal dose of UV: 1) XP cells were more sensitive to the lethal effects of UV than normal cells and 2) the excision-deficient XP strain showed a higher frequency of mutations per dose of UV which was directly related to the increased cytotoxic effect of UV.

The studies described above were carried out with 254 nm radiation. Since little 254 nm radiation reaches the earth's surface, experiments were also carried out with broad spectrum sunlight (Patton et al., 1984). The results obtained support the hypothesis that the pyrimidine (thymine) dimers are the lesions principally responsible for mutations and cell killing.

The rate of cellular repair is critical because the average number of unexcised lesions remaining in DNA at the time of semi-conservative DNA replication determines the mutagenic effect of the damage. The studies comparing normal human diploid fibroblasts with fibroblasts from XP patients have broadened our understanding of the protective effect of efficient error-free excision repair found in normal human cells. These studies suggest that the cytotoxic and mutagenic effects of exposure to UV result from lesions in DNA which the cells fail to excise and that these mutations are ultimately responsible for the induction of skin cancer in XP patients. Thus there is considerable interest in the possiblity that a genetic predisposition to cancer might be related to a DNA repair defect in cells from

those individuals.

2. Familial Polyposis Coli and Gardner's Syndrome Are Associated With a Genetic Predisposition to Cancer

Certain dominantly inherited disorders are also known to be associated with a genetic predisposition to cancer. Little is known about the fundamental defects in such disorders. Hereditary polyposis (the condition of being affected with polyps) is an uncommon disease clinically characterized by the early appearance (before age 30) of multiple discrete adenomas (benign) of the colon. Frequently, adenocarcinoma (cancerous) will also develop. Adenomatosis coli or familial polyposis coli (FP) is inherited in an autosomal dominant manner. FP is clinically characterized by hundreds of adenomatous polyps in the colon and rectum and inevitably terminates in cancer unless surgically removed (McKusick, 1962). Gardner's syndrome (GS) is also characterized by multiple polyps of the lower digestive tract which are predisposed to malignancy. In addition, GS is associated with the appearance of multiple osteomas (bone tumors), fibromas (connective tissue lesions), sebaceous cysts (skin lesions) and other soft tissue benign tumors (Gardner et al., 1953).

Even though FP develops only in the intestinal mucosa, the gene responsible for FP should be present in all cells of the body. Thus the genetic difference between FP and normal individuals might be expressed in skin fibroblasts in

culture in a similar manner as for XP cells.

3. Abnormalities of FP and GS Skin Fibroblasts in Culture

A study by Heim (1983) showed that no consistent growth differences existed between skin fibroblasts from FP and normal individuals. In contrast, other studies with skin fibroblasts from FP patients have suggested that the predisposition to malignancy appears to be associated with abnormalities in the skin fibroblasts in culture. The abnormalities observed by Kopelovich and his coworkers in culture are: 1) loss of contact inhibited growth in culture (Pfeffer et al., 1976), 2) a decreased serum requirement for growth (Pfeffer et al., 1976), 3) elevated levels of plasminogen activator (Kopelovich, 1977), 4) altered intracellular distribution of actin cables (Kopelovich et al., 1977) and 5) increased susceptibility to viral transformation with Kirsten murine sarcoma virus (Pfeffer et al., 1977). The abnormalities observed are a few of the many properties of transformed cells. Recently, Kopelovich et al., (1979) have shown that FP cells can be neoplastically transformed by treatment with a tumor promoter alone. This would suggest that FP cells exist in an initiated state and only promotion is required to transform the cells.

A study by Hori et al. (198Ø) demonstrated that FP cells exhibit elevated (two fold higher) chromosomal instabilities when exposed to the methylating agent N-methyl-N'-nitroso-N-

nitrosoguanidine (MNNG) as compared to normal skin fibroblasts. The findings of Kopelovich and coworkers (1979, see also Pfeffer et al., 1976; Kopelovich, 1977; Kopelovich et al., 1977; Pfeffer et al., 1977) and Hori et al. (1980) suggest that FP cells are defective in a function that regulates cell condition and FP cells are more susceptible to carcinogens than are normal cells.

Most of the other work with FP cells in culture concerns the cytotoxic response of FP cells to various carcinogens. Cytotoxicity is defined as the inability of a cell to form a colony (reproductive death). Miyaki et al. (1980) reported that cells from FP individuals were two to three times more sensitive than normal cells to the cytotoxic effect of 4-nitroquinoline 1-oxide (4-NQO) and mitomycin C. Miyaki and coworkers (1982) have also reported morphological transformation and chromosomal changes induced in FP fibroblasts by 4-NQO and MNNG but not in untreated FP cells, nor in treated or untreated normal cells. When the susceptibility of FP fibroblasts to UV radiation, methyl methanesulfonate (MMS) or MNNG was examined, Akamatsu and coworkers (1983) found that FP cells were normal in their response . Barfknecht and Little (1982) have reported an abnormal sensitivity of two Gardner's syndrome (GS) cell lines and a FP cell strain to the alkylating agents MMS, ethyl methanesulfonate (EMS), MNNG and 4-NQO. Recently, Little et al., (1980) have shown that GS cells are abnormally sensitive to the lethal effects of UV light,

X-rays and mitomycin C. This is in contrast to the work of Akamatsu et al. (1983), who showed that no FP cells exhibited significant sensitivity to these chemicals with the exception of 4-NQO. Paterson and coworkers (1981) reported that GS cell lines were significantly more sensitive to killing by MNNG than normal fibroblasts. From the totality of the work mentioned above, it appears that FP and GS cells are more sensitive than normal cells to the lethal effects of a variety of carcinogens. This raises the question: Do FP and GS cells have a higher frequency of induced mutations than normal fibroblasts when exposed to carcinogens?

C. Role of Alkylating Agents in Mutagenesis and Carcinogenesis

Studies in experimental animals have implicated methylating agents in chemically induced colon cancer, for example, 1,2-dimethylhydrazine (DMH) (IARC, 1974). Montesano and Bartsch (1976) have shown that the gastrointestinal tract is one of the main target tissues for the carcinogenic action of the model compound MNNG. A direct alkylating agent such as MNNG would be an appropriate model compound to study and it would be of interest to determine if fibroblasts from GS and FP patients were abnormally sensitive to mutations induced by such carcinogens.

1. Lesions Produced in DNA by Alkylating Agents

Figure 1 illustrates the structures of a number of methylating agents. These agents fall into two categories; those that require metabolic activation, such as dimethylnitrosamine (DMN), 1,2-dimethylhydrazine (DMH), and cycasin, and those that do not, such as N-methyl-N-nitrosourea (MNU). Methylating agents that are unstable at physiological pH and do not require activation include: methyl methanesulfonate (MMS), dimethylsulfate (DMS), MNNG, and streptozotocin, in addition to MNU. Figure 2 illustrates that the breakdown of DMN and MNNG results in a common methylating species, monomethylnitrosamine, which is the source of the reactive CH_3^+ ion which can react with DNA as well as RNA and protein. Under physiological conditions, alkylation of DNA can occur at various sites (Figure 3). Alkylation takes place on most of the ring nitrogen atoms of the bases: the 7 position of guanine and adenine and the 3 position of thymine, cytosine, guanine, and adenine. Sites of alkylation on the oxygen atoms are: the 6 position of guanine, the 4 position of thymine and the 2 positions of cytosine and thymine.

The relative proportions of alkylation products formed in DNA by various alkylating agents are shown in Table 1. Of the various adducts formed by methylating agents, the most abundant lesion is 7- methylguanine. Among other adducts produced in DNA in substantial amounts are 3-methyladenine and methylphosphate triesters. The relative

Figure 1. Structures of some simple alkylating carcinogens

1. dimethylnitrosamine
2. N-methyl-N-nitrosourea
3. N-methyl-N'-nitro-N-nitrosoguanidine
4. 1,2-dimethylhydrazine
5. methyl methanesulfonate
6. dimethylsulfate
7. cycasin
8. streptozotocin

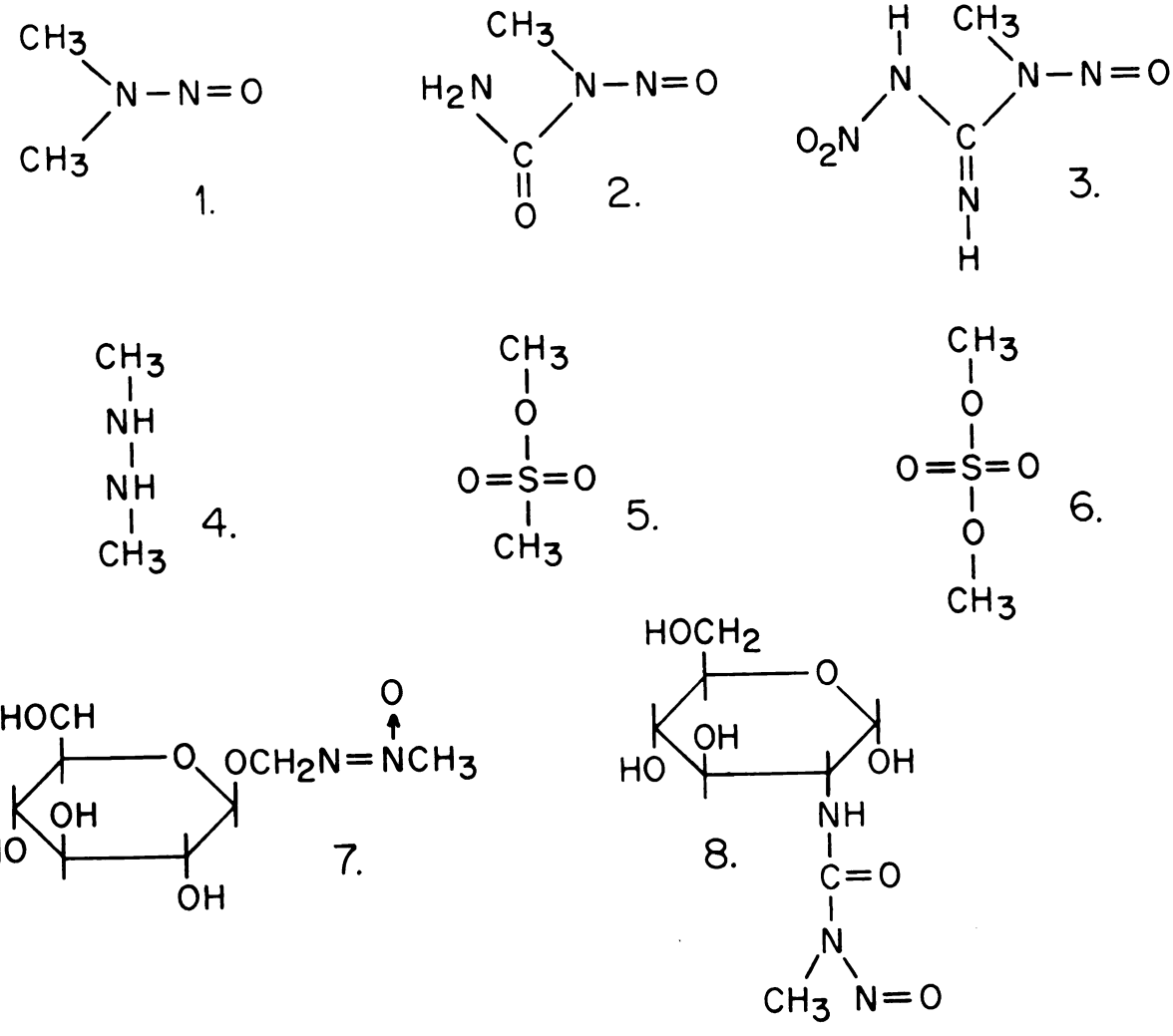

Figure 1

Figure 2. Breakdown of DMN and MNNG. The reaction of DMN requires enzymatic activation but that of MNNG requires only thiol groups. Both generate a carbonium ion which reacts with DNA as indicated.

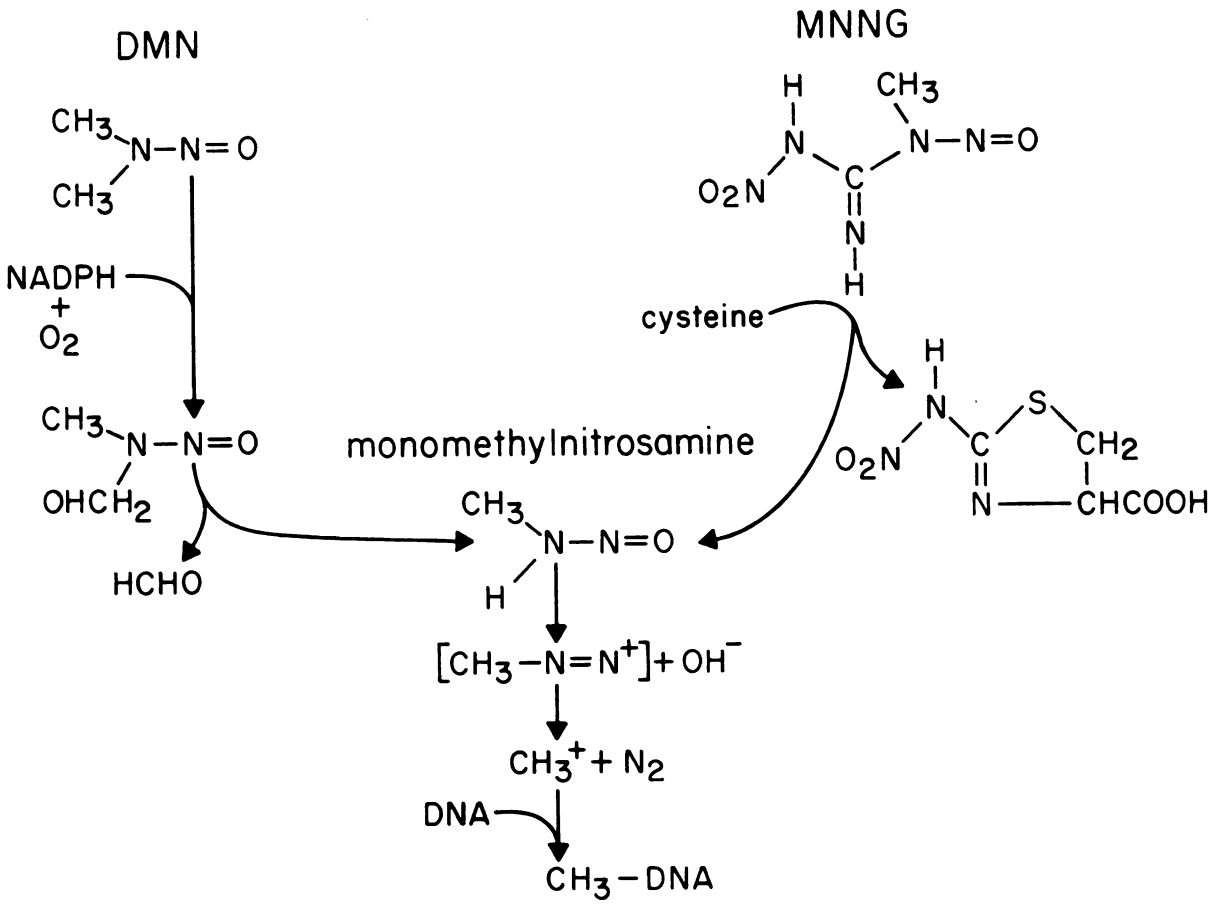
DMN
MNNG
NADPH
+
O2
cysteine
monomethylnitrosamine
HCHO
DNA
CH3-DNA

Figure 2

Figure 3. Sites of alkylation of DNA. From Pegg (1984), Cancer Invest., 2, 223-231.

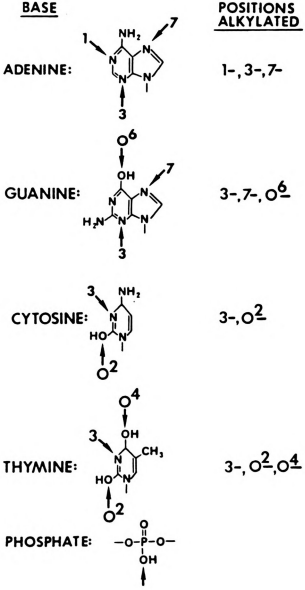
BASE
POSITIONS ALKYLATED
ADENINE:
1-, 3-, 7-
GUANINE:
3-, 7-, O6
CYTOSINE:
3-, O2
THYMINE:
3-, O2, O4
PHOSPHATE:

Figure 3

Table 1. Relative proportions of alkylation products in DNA[a]

Alkylation site	Percent of Total Alkylation				
	DMN[b] MNU (s=0.42)[c] DMH	DEN ENU (s=0.26)	MMS (s=0.83)	EMS (s=0.67)	DMS (s=0.86)
Adenine					
N-1	0.7	0.3	1.2	1.7	1
N-3	8.0	4.0	11.	4.2	16.
N-7	1.5	0.4	1.9	1.9	2.
Cytosine					
N-3	0.5	0.2	ND[d]	0.4	ND
O^2	0.1	3.	ND	0.3	ND
Guanine					
N-3	0.8	0.6	0.7	0.3	1.
N-7	68.	12.	83.	58.	79.
O^6	7.5	8.	0.3	2.	0.2
Thymine					
N-3	0.3	0.8	ND	ND	ND
O^2	0.1	7.	ND	ND	ND
O^4	0.7	2.	ND	ND	ND
Alkyl-phosphates	12.	53.	1.	12.	1

[a]Adapted from Pegg (1983), In: Reviews in Biochemical Toxicology, (Hodgson, Bend, Philpot, eds.), Elsevier Biomedical, pp. 83-133.

[b]DMN, dimethylnitrosamine; MNU, N-methyl-N-nitrosourea; DMH, 1,2-dimethylhydrazine; DEN, diethylnitrosamine; ENU, N-ethyl-N-nitrosourea; MMS, methylmethanesulfonate; EMS, ethylmethanesulfonate; DMS, dimethylsulfate.

[c]Examples of s values from Lawley (1974), Mutation Res., 23, 283.

[d]ND, Not detected.

proportion of alkylation products formed in DNA depends on the alkylating agent. An inverse correlation exists between the Swain-Scott constant, s, for a particular alkylating agent and the proportion of oxygen adducts formed. Agents that have lower s values form a higher percentage of oxygen adducts. MNNG has a low Swain-Scott constant of 0.42, and the ratio of O^6-methylguanine (O^6-MeG) to 7-methylguanine (N7-MeG) in DNA is approximately 0.1. The s value for DMS is 0.86 and the O^6-MeG to N7-MeG ratio is approximately 0.003 (Lawley, 1974). In addition, methylating agents have higher s values than the corresponding ethylating agents.

2. O^6-MeG Involved in the Induction of Tumors in Laboratory Animals

Two decades ago it was believed that 7-alkylguanine was the adduct responsible for the production of liver tumors in rats after exposure to DMN (Magee and Farber, 1962). This conclusion was reached because the adduct 7-alkylguanine was persistent and present in the greatest amount. The same conclusion was reached using the alkylating agents DMS and MNU (Singer 1975). This hypothesis began to be discredited about ten years ago when researchers found that amount or presence of 7-alkylguanine did not correlate with the sites of tumor production. Ethyl methanesulfonate (EMS) was much more carcinogenic in rats than methyl methanesulfonate (MMS) even though 7-ethylguanine was present in smaller amounts than 7-methylguanine (Swann and Magee, 1971). The first

indication that methylating agents could react at sites other than nitrogen was first detected by Loveless (1969) and later Lawley and Thatcher (1970) found O^6-methylguanine to be present in MNNG-treated DNA, but not DMS-treated DNA. Because experimental findings showed that MNNG is a powerful carcinogen whereas DMS is a weak carcinogen, it was postulated that organ specific carcinogenesis correlated with the presence of O^6-alkylguanine.

Experimental studies relating O^6-alkylguanine to carcinogenesis have been provided by measurements of abundance of this lesion in various organs in experimental animals. Alkylating agents that produce little O^6-MeG in the DNA of a particular organ are poor inducers of tumors in that organ. Goth and Rajewsky (1974) demonstrated that in young rats exposed to N-ethyl-N-nitrosourea, O^6-ethylguanine persisted in the target organ (brain). However, in the nontarget organ (liver), this lesion was repaired. A similar finding by Margison and Kleihues (1975) showed the preferential accumulation of O^6-methylguanine in rat brain DNA following exposure to N-methyl-N-nitrosourea. Frei et al. (1978) measured the extent of alkylation of various DNA sites after treatment of mice with radiolabelled ENU and MNU and measured the extent of induction of thymic lymphomas. At doses which yielded an equal carcinogenic response, the only lesion which showed an equal extent of alkylation was O^6-alkylguanine. This correlation between the persistence of O^6-alkylguanine and carcinogenesis has also been

confirmed by other studies dealing with other alkylating agents as well as other target tissues (Pegg, 1977; Montesano, 1981). The findings that O^6-alkylguanine correlates well with carcinogenesis is supported by the work of Loveless (1969) and also Gerchman and Ludlum (1982) who found that this altered base can mispair during replication and thereby lead to mutations, whereas the abundant lesion, 7-methylguanine does not cause misincorporation (see Figure 4).

Although the causal connection between persistence of O^6-alkylguanine in target organs and the subsequent induction of tumors is supported by the above mentioned experiments, there are data that contradict this hypothesis. A controversy exists to date as to whether or not O^6-alkylguanine is necessary for initiation of carcinogenesis by alkylating agents (Pegg, 1984; Singer, 1984). Several experiments have indicated that O^6-alkylguanine persists in the brain of young hamsters, gerbils, Xenopus laevus, and many strains of mice; even though the brain is not the target organ (Buechler et al., 1977; Rogers and Pegg, 1977; Hodgson et al., 1980; Kleihues et al., 1980; Nicoll et al., 1975). Another example is the work of Likhachev and coworkers (1983) where the persistence of O^6-alkylguanine was highest in the nontarget organ, the brain, after exposure to MNU or ENU. However, this was not a well conducted experiment since O^6-MeG was not measured in the target organ.

Figure 4. O^6-MeG mispairing. Upper diagram, cytosine:guanine; center, cytosine:O^6-methylguanine; lower, thymine:O^6-methylguanine. R, represents the position of the deoxyribose moeity of the sugar-phosphate backbone. From Margison and O'Connor (1979), In: Chemical Carcinogens and DNA, Grover, P.L. (ed.), CRC Press, Boca Raton, FL, pp. 112-149.

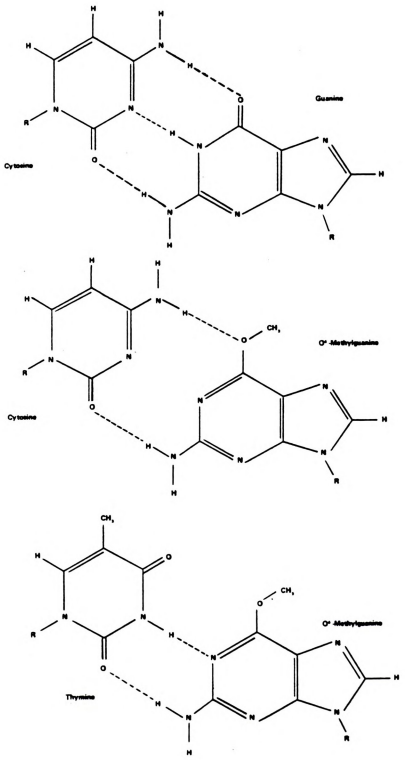
Cytosine
Guanine
Cytosine
O⁶-Methylguanine
Thymine
O⁶-Methylguanine

Figure 4

Other factors must be considered in interpreting the O^6-alkylguanine experiments. These include cell proliferation in an organ, the role of other adducts (e.g., O^2 and O^4-thymine and O^2-cytosine) in carcinogenesis and the variability of the persistence of O^6-alkylguanine in the different cell types of a particular organ. The role of cell division is important in interpreting the experiments that implicate O^6-alkylguanine in carcinogenesis. Methylated bases can lead to tumors only if DNA synthesis occurs while the lesion is present. For example, Kleihues and coworkers (1973) found that after exposure to MNU, all the DNA of the cells in the brain is methylated. However, only the glial cells which divide form tumors.

Furthermore, in adult rats, methylating agents do not form tumors after a single dose, but do produce tumors in neonatal rats where the liver cells are dividing or in cases of partial hepatectomy (Pegg, 1977; Pegg, 1983; Craddock, 1971). Singer's laboratory (1976) demonstrated that ENU reacts readily with pyrimidines in DNA and that the O-alkylpyrimidines are capable of mispairing. Singer and coworkers (1981) showed that in young rats treated with ENU, the target organ (brain) contained O-ethylpyrimidines and these lesions are more persistent than O^6-ethylguanine. Swenberg and colleagues (1984) demonstrated when one is considering repair in the organ as a whole, repair in the cell types that make up that organ must be considered. They separated hepatocytes from nonparenchymal cells and found

that repair of DMH-induced damage in the nonparenchymal cells was poor. The tumors which developed were of nonparenchymal cell origin. Thus, if repair is measured in the whole organ, the result will indicate repair is occurring even though one of the cell types is not repairing at all.

These experiments taken as a whole indicate that O^6-methylguanine is necessary to initiate tumors but other factors determine whether or not the neoplastic process occurs (Pegg, 1984; Singer, 1984).

3. Is O^6-MeG the Mutagenic Lesion in Mammalian Cells in Culture ?

Warren et al. (1979) and Goth-Goldstein (1980) found that after treatment with radiolabelled alkylating agents, Chinese hamster lung (V79) and Chinese hamster ovary (CHO) cell lines were able to excise 3-alkyladenine and 7-alkylguanine residues, but not O^6-alkylguanine residues. Goth-Goldstein (1980) suggested that since O^6-alkylguanine can mispair during DNA replication, a deficiency in the removal of this lesion predicts that the induced mutation frequency will be higher in cells that cannot remove this lesion than in cells that can remove it. However, she only measured the number of adducts remaining after nearly 20 hours and the mutations experiments to test her speculation were not carried out.

Since V79 cells do not excise O^6-MeG from DNA, they are ideal for experiments designed to establish the role of O^6-MeG in the mutagenicity and cytotoxicity of methylating agents. Newbold and coworkers (1980) demonstrated that the mutagenicity of methylating agents in V79 cells is associated with the O^6-MeG adduct. The major lesions 7-MeG, 3-methyladenine (3-MeA) and O^6-MeG were measured and also the induced mutation frequency after exposure to the weak carcinogens DMS and MMS and the potent carcinogen MNU. The mutagenicity, but not the cytotoxicity, of each agent reflected its carcinogenicity and differences in mutation frequency were paralleled by differences in the amount of O^6-MeG. A problem with this study is that only some of the types of DNA lesions were quantitated. Another type of lesion might also correlate with mutations.

Newbold's work is supported by Suter et al. (1980). In V79 cells the levels of DNA alkylation were measured for the adducts: 7-MeG, 3-MeA and O^6-MeG. Also measured was the induced mutation frequency after exposure to MNU or DMS. At equal levels of O^6-alkylation approximately equal numbers of mutants were induced. The survival data did not correlate with O^6-MeG, but from their data, it appears that a correlation with 3-MeA could be made.

Heflich et al. (1982) exposed Chinese hamster ovary cells to tritiated ENU or EMS and measured the induction of TG resistant cells and quantitated the DNA adducts formed. Statistical analysis indicated that mutation induction was

highly correlated with alkylation of guanine at the O^6 position. Sister chromatid exchanges (SCE) were highly correlated with the lesions 3-methyladenine and 3-methylguanine. Work from the same laboratory by Morris et al. (1982) reported that the biological responses of cell killing and SCE are highly correlated. Their interpretation from the two papers is that the group of adducts resulting in mutations at the HPRT locus is not the same set of lesions responsible for inducing cell killing.

In contrast, Fox and Brennard (1980) have shown evidence for the involvement of lesions other than O^6-alkylguanine in mammalian cell mutagenesis. Using a reverse mutation assay (that is, reverting TG resistant cells from HAT medium [hypoxanthine, aminopterin, thymine] sensitivity to HAT medium resistance) and measuring levels of alkylation, they found that EMS, a weak carcinogen, and MNU, a potent carcinogen, both were effective mutagens in the reverse direction. Since EMS produces little of the O^6-alkylation product and MNU produces more of the product (per total amount bound), the amount of O^6-alkylation does not correlate with the mutational response.

The dose response of methylating agents at the HPRT locus in Chinese hamster cells (Couch and Hsie, 1978; Couch et al., 1978; Thielman et al., 1979) was examined and no deviation from linearity was reported. Also the previously mentioned studies by Newbold et al. (1980) and Suter et al. (1980) suggest that the increase in mutation frequency as a

function of dose is linear. A decreased response or threshold at low dose of a mutagenic agent may be connected with changes in the mechanism of repair of the lesions at different dose levels. A threshold implies that the repair is error-free at low doses and that this repair process is saturated at higher doses. However, the above investigators did not pay specific attention to the mutational response at low doses. They measured the response at higher doses and extrapolated to lower doses.

Jenssen and Ramel (1980) investigated the dose response curves for the induction of TG resistant mutants by low doses of the monofunctional alkylating agents EMS, ENU, MMS and MNU in V79 cells. They found that at low doses, the ethylating agents give a linear response, whereas the methylating agents MMS and MNU demonstrate a threshold in the induced mutation response to 6-thioguanine resistance. The threshold suggests a repair mechanism at low doses for methylating agents. This implies that at low doses for ethylating agents the repair mechanism is not as efficient as for methylating agents and/or another repair system is involved in the repair of ethyl adducts.

4. Responses of Human Cells to Alkylating Agents

The persistence of the lesion O^6-MeG has also been studied in human cells. Goth-Goldstein (1977) treated SV40-transformed XP fibroblasts (defective in UV excision repair, complementation group A) and normal SV40-transformed

fibroblasts with MNU and ENU in order to measure the alkylation products O^6-alkylguanine and 7-alkylguanine. She found that normal cells remove O^6-alkylguanine, whereas in XP cells the O^6-alkylguanine is removed to a lesser extent. These results were confirmed by Altamirano-Dimas et al. (1979) who showed that lymphoblastoid cells from a xeroderma patient (complementation group C) are unable to remove O^6-MeG residues in DNA after treatment of the cells with MNNG. However, XP cells are able to remove 3-methyl adenine adducts. They also found that UV excision defective mutants of E. coli, uvr A and uvr B, are able to excise MNNG-induced O^6-methylguanine adducts, indicating that the repair mechanism for these adducts is not the same as the mechanism for UV induced lesions. Therefore it was surprising that XP cells were also unable to remove O^6-MeG.

Sklar and Strauss (1981) examined the kinetics of removal of the methyl group from O^6-MeG in normal and XP-derived lymphoblastoid lines. The removal of the methyl group from O^6-MeG occurred rapidly with a half life of less than one hour. They distinguished between two cell types: Mex^+ (methyl excision proficient), cells that can remove O^6-MeG and Mex^- (methyl excision deficient), cells which are unable to remove the adduct after a low dose of MNNG. The mex^+ and mex^- categories are similar to the mer^+ and mer^- categories of Day et al. (1979; 1980) who reported strains which differ in their ability to reactivate MNNG-treated adenoviruses. Mer^- cells can support the growth of

adenovirus 5 normally, however, when the virus is treated with MNNG, the mer^- cells cannot support its growth. The assay consists of inactivating the adenovirus with MNNG and subsequently assaying for plaque-forming ability, using monolayers of human fibroblasts. Day et al. (1980) reported that mer^- strains: 1) show a greater sensitivity to MNNG induced cytotoxicity, 2) are deficient in removing O^6-MeG from their DNA after MNNG treatment, and 3) have an increased susceptibility to SCE after MNNG treatment.

Medcalf and Lawley (1981) showed that at non-toxic doses of MNU, normal human fibroblasts exhibit rapid removal of O^6-methylguanine ($t_{1/2}$ = 1 hour), but at higher doses, removal is slow ($t_{1/2}$ > 24 hours). They predicted a non-linear dose response for mutation induction in human fibroblasts. A marked non-linear dose response was reported by Penman et al. (1979) for induction of mutations in human lymphoblasts by the methylating agent N-methyl-N-nitrosourethane. However, these authors did not prove that the threshold was due to the more efficient removal of O^6-MeG at low doses. Such a threshold in the induced mutational response of normal human fibroblasts to MNNG was also observed by Jacobs and DeMars (1978).

In contrast to Goth-Goldstein (1977) and Bodell et al. (1979), who observed that following treatment with ENU, SV40 transformed XP fibroblasts removed O^6-alkylguanine from DNA at a slower rate than SV40-transformed normal human fibroblasts, Teo et al. (1983) working with nontransformed

fibroblasts, found similar rates of elimination of the O^6-ethylguanine adduct in two normal and two XP cell lines. The difference between the experimental groups may be due to the SV40 transformation process. It has been reported by Day et al. (1980), Sklar and Strauss (1981) and Shiloh et al. (1983) that transformation of human cells by SV40 or Epstein-Barr virus may be accompanied by a loss of the ability of the transformed cells to remove O^6-methylguanine from their DNA.

D. Role of Methyltransferase in the DNA Repair of Alkylation Damage.

1. Methyltransferase in Bacterial Cells

At the same time that observations dealing with tumor and mutation induction were being published, studies on the alkylation repair mechanism in E. coli bacteria were being performed. These eventually yielded the following insights into the properties of the alkylation repair mechanism in E. coli. Bacterial cells are capable of repairing O^6-MeG by a demethylating mechanism which involves a suicide enzyme. The model emerged that the O^6-methylguanine lesion was repaired by the direct transfer of methyl groups from O^6-methylguanine to protein cysteine residues on a protein acceptor molecule (Olsson and Lindahl, 1980). Karran and coworkers (1979) showed that when DNA which contained the radioactively labelled O^6-methylguanine (methyl group

labelled) substrate was incubated with extracts from MNNG-treated E. coli, the radioactivity was removed from the exogenous DNA. Extracts from uninduced cells (not treated with MNNG) did not remove the radioactive lesions from DNA. However, no free radioactive O^6-methylguanine could be found. Olsson and Lindahl (1980) concluded that following repair, the radioactive material was probably transferred to a protein. This conclusion was based on the discovery of radioactive S-methylcysteine.

Further evidence for the model that the O^6-methylguanine lesion is repaired by the direct transfer of the methyl groups to protein cysteine residues was obtained by Foote and coworkers (1980). By using a substrate radioactively labelled in the purine ring rather than the methyl group, they were able to demonstrate that the model described above regenerated guanine from the mutagenic lesion. Lindahl et al., (1982) and Robins and Cairns (1979) showed that the repair of O^6-methylguanine was limited by the amount of acceptor protein in the reaction, suggesting that repair protein was being consumed in the reaction. Purification of E. coli O^6-methylguanine-DNA methyltransferase to homogeneity by Demple and coworkers (1982) allowed these workers to demonstrate conclusively that the molecule that removes the methyl group becomes inactivated in this process.

2. The Adaptive Response in E. coli

An important aspect of this repair mechanism is that the amount of methyl removed from O^6-MeG is limited by the amount of acceptor protein initially present (suicide enzyme). However, in E. coli the demethylating mechanism for alkylation damage is inducible. This inducibility is termed the adaptive response. This response in E. coli was first demonstrated by Samson and Cairns (1977). They added a subtoxic dose of MNNG to exponentially growing cultures of E. coli. At various times thereafter, samples from the culture were treated with a higher dose of MNNG and the resulting number of mutants and extent of killing were measured. The bacteria became increasingly difficult both to kill and to mutate if they were pretreated with a subtoxic dose of MNNG before the larger dose of MNNG. The resistance to MNNG mutagenesis was prevented by the addition of chloramphenicol, suggesting that protein synthesis is required for attaining resistance to MNNG.

Cairns and coworkers (1981) also showed that the adaptive response was specific for alkylating agents. Resistance to mutation induction was highly efficient, but exhibited saturable kinetics, that is, adapted bacteria could be challenged with low doses of a specific methylating agent without significant induction of mutations, but at higher doses of the alkylating agent, the frequency of mutants induced was the same as in unadapted cells. A good

correlation was found between the presence of the DNA lesion O^6-methylguanine and the frequency of mutations.

Adaptation to killing and to induction of mutations have been found to occur via different pathways since mutants of E. coli have been isolated which possess the adaptive response to mutation induction, but not to killing and vice versa (Evensen and Seeberg, 1982). The adaptive response in E. coli consists in part in the induction of methyltransferase which transfers the methyl group from O^6-MeG in DNA to a protein acceptor. In addition to the MT, the adaptive response involves the induction of a DNA glycosylase which catalyzes the removal of N-methylated purines such as 3-methyladenine (Karran et al., 1982b; Evensen and Seeberg, 1982).

3. Methyltransferase in Mammalian Cells is not Fully Characterized

The loss of O^6-methylguanine from DNA has been demonstrated with cell extracts from rodent liver, kidney, and other mammalian tissues, and a variety of human tissues and cells (Pegg et al., 1983; Bogden et al., 1981; Lemaitre et al., 1982; Pegg and Wiest, 1983; Pegg, 1978; Pegg, 1983b; Pegg et al., 1982; Waldstein et al., 1982a; Waldstein et al., 1982b; Waldstein et al., 1982c). The removal of O^6-methylguanine in mammalian cells is similar to that carried out by the E. coli protein, however, the mammalian system has not been fully characterized. Human and rodent

cells contain an activity with a molecular weight of 20,000 which demethylates O^6-methylguanine (Bogden et al., 1981; Mehta et al., 1981). The reaction involves the transfer of the methyl group from O^6-methylguanine to a cysteine residue of a protein acceptor molecule. For both the E. coli and liver protein, direct evidence for this mechanism is provided by the demonstration that stoichiometric loss of O^6-methylguanine, generation of S-methylcysteine and production of guanine in DNA takes place (Lindahl, 1982; Pegg et al., 1982; Pegg et al., 1983; Demple et al., 1982; Foote et al., 1980; Mitra et al., 1982). Demple and coworkers (1982) have purified the bacterial protein to homogeneity and have shown that the methyl acceptor sites reside in the same protein as that which catalyzes the transfer. This may also be true for the mammalian protein (Foote et al., 1980). The mammalian enzyme has not been purified to homogeneity as of this writing. As in the E. coli suicide enzyme reaction, the protein acceptor becomes inactivated. This is indicated by the stoichiometry between S-alkylcysteine production and O^6-alkylguanine removal by mammalian cell extracts (Pegg et al., 1983; Bogden et al., 1981; Lemaitre et al., 1982; Pegg and Wiest, 1983; Pegg, 1983b; Waldstein et al., 1982c; Mehta, 1981; Craddock, 1982).

4. In Vitro Properties of MT Are in Good Agreement with in Vivo Studies

The in vitro (studies with cell free extracts) properties of methyltransferase are in good agreement with the persistence of O^6-MeG in DNA in vivo. The initial rapid rates of removal in vivo are similar to those seen in vitro with substrates alkylated to the same extent (Scicchitano and Pegg, 1982). Also in vivo, the repair of O^6-methylguanine at high doses becomes saturated which agrees with the in vitro characteristics of methyltransferase. Since the acceptor site is not readily regenerated, the removal reaction proceeds rapidly until all the acceptor sites have been consumed. The number of lesions that can be repaired rapidly is determined by the initial number of protein molecules (Lindahl, 1982; Cairns et al., 1981; Schendel, 1981). This capacity to repair is both species and organ specific. Table 2 shows the content of methyltransferase in different cells and tissues. Rat liver has a high amount of methyltransferase and can remove O^6-methylguanine in vivo, whereas rat brain has a low amount of MT and is unable to remove this lesion.

The content of methyltransferase in cells from different tissues correlates well with the carcinogenesis data from methylating agents described in a previous section. The fact that hamster hepatocytes have a lower amount of methyltransferase than rat hepatocytes correlates well with

Table 2. Content of MT in different cells and tissues[a]

Source		Molecules of MT per Cell
Human	liver	750,000
	brain	30,000
	lymphocytes	14,000-140,000
	HeLa cells	100,000
Rat	liver hepatocytes	60,000
	liver nonparenchymal cells	12,000
	brain	1,500
	kidney	12,000
Hamster	liver	28,000

[a]Adapted from Pegg (1984), Cancer Investigation 2, 223.

the observation that single doses of DMN will cause tumors in hamsters, but not in rats (Montesano, 1981; O'Connor et al., 1982). Rat hepatocytes in culture also have a low rate of DNA synthesis and are found to be more resistant than rat brain or kidney cells to MNU (Pegg, 1977; Kleihues et al., 1982; Lindahl, 1982).

In vivo studies have also indicated that methyltransferase is inducible to a small extent. The activity of methyltransferase was increased when rats were chronically exposed to DMN (Montesano et al., 1980; Pegg et al., 1981; Pegg and Perry, 1981b). Similar increases were produced by the alkylating agents DMH and diethylnitrosamine (DEN) (Swenberg et al., 1982; Pegg and Perry, 1981b). A moderate increase in methyltransferase (7-fold) has been seen in rats following partial hepatectomy or treatment with hepatotoxins (Pegg et al., 1981; Pegg and Perry, 1981b). However, in adapted E. coli a 100-fold increase can be seen.

5. The Adaptive Response in Mammalian Cells

An inducible process for O^6-methylguanine-DNA methyltransferase has not been definitely observed in mammalian cells, although some suggestive observations have been published. Samson and Schwartz (1980) demonstrated that chronic treatment of CHO cells and an SV40-transformed human skin fibroblast line, (using non-toxic levels of MNNG) made the cells resistant to alkylation-induced cell killing and

sister chromatid exchanges. CHO cells were found to have increased resistance to MNNG-induced mutation after adaptation (Samson and Schwartz, 1983; Schwartz and Samson, 1983). However, CHO cells failed to remove O^6-MeG from DNA (Goth-Goldstein, 1980) and lacked either constitutive or inducible MT activity (Foote and Mitra, 1984) and therefore the mechanism for this response in CHO cells may differ from that in E. coli. In Chinese hamster V79 cells, Kaina (1982) reported a moderate resistance to cell killing, a reduction in sensitivity to mutation, and a reduction in the induction of chromosome aberrations after a single pretreatment of the cells with low doses of MNNG or MNU before challenge with a higher dose. Durrant and coworkers (1981) reported an increased survival in Chinese hamster V79 cells following a challenge dose of MNU after previous subtoxic doses of MNU. However, a reduction in induced mutation frequency was not seen. Rat epithelial cell lines exposed to subtoxic doses of MNNG showed a reduction in dose dependence of cell killing when challenged with increasing doses of MNU, as demonstrated by Montesano et al. (1982). Recently, rat hepatoma cells pretreated with alkylating agents showed increased resistance to alkylation-induced cell killing and mutagenesis and an increased repair capacity for O^6-MeG (Laval and Laval, 1984). In contrast, Karran et al. (1982) found that the adaptive response to the cytotoxic effects of MNU is absent in normal human fibroblasts. This may reflect the fact that human cells constitutively have high levels of

methyltransferase (see Table 2).

In addition to the evidence for an adaptive response in human cells (Samson and Schwartz, 1980), Waldstein et al. (1982) found that treatment of mammalian cells with multiple low doses of MNNG resulted in the increase of methyltransferase. However, these results should be viewed with caution since other laboratories have not been able to reproduce these results with the same cell lines (Yarosh et al., 1984; Myrnes et al., 1982; Foote and Mitra, 1984).

E. Studies Dealing with the Repair of Alkylated Bases Other than O^6-MeG

Other enzymes which are involved in the repair of alkylation damage are glycosylases which are able to correct the lesions 3-methyladenine and 7-methylguanine. Glycosylases split the bond between the N-9 position of a purine and the deoxyribose, yielding an apurinic site in DNA. The apurinic sites are then repaired by an excision repair system.

3-Alkyladenine is repaired in DNA by a glycosylase. This has been demonstrated in extracts from human lymphoblasts (Brent, 1979; Singer and Brent, 1981) and placenta (Gallager and Brent, 1982) and in extracts from rodent liver and other tissues (Margison and Pegg, 1981; Cathcart and Goldthwait, 1981; Craddock et al., 1982; Pegg, 1982). Whether the glycosylase is specific for 3-alkyladenine has not been clear, since the enzyme has not been purified to homogeneity

from mammalian extracts. In bacteria 3-methyladenine-DNA glycosylase is important in cell survival. This has been demonstrated through the use of tag mutants which are deficient in the 3-methyladenine-DNA glycosylase. The tag mutants are more sensitive to the killing effect induced by alkylating agents (Mamet-Bratley et al., 1982; Lindahl, 1982).

Enzymatic removal of 7-alkylguanine from DNA has been demonstrated using extracts from: 1) human lymphoblasts (Singer and Brent, 1981), 2) rat liver (Margison and Pegg, 1981; Cathcart and Goldthwait, 1981), and 3) hamster liver (Cathcart and Goldthwait, 1981). In these studies the removal rate was enhanced compared to the rate of spontaneous hydrolysis. 7-methylguanine-DNA glycosylase has not been fully characterized and its substrate specificity is not certain. The removal of 7-methylguanine from DNA is probably not important since in bacteria this adduct does not appear to contribute to mutagenesis and does not affect survival.

Besides O^6-alkylguanine, the alkylation products O^2- and O^4-alkylthymine and O^2-alkylcytosine are also possible miscoding lesions (Singer et al., 1978). The removal of these adducts was studied by Bodell et al., (1979) in human fibroblasts after exposure to ENU. The human fibroblasts used were an XP line and a normal line. They found that both lines had similar ability to remove the alkylpyrimidine products. However, the XP line did not remove

O^6-alkylguanine as well as the normal line and this confirmed the work of Goth-Goldstein (1977).

III. MATERIALS AND METHODS

A. Chemicals

Chemical mutagens were obtained from the following sources: MNNG, Pfaltz and Bauer (Flushing, NY); ENU, MNU and 4-NQO, NCI Carcinogenesis Program. All were stored as solids at -20° C. All chemical mutagens except 4-NQO were dissolved in 100% DMSO (Burdick and Jackson, Muskegon, MI) prior to treatment of the cell cultures. 4-NQO was dissolved in 100% ethanol (Burdick and Jackson, Muskegon, MI). For mutagenicity experiments, 6-thioguanine was obtained from Sigma Chemical Co. (St. Louis, MO) and a 80 mM solution was prepared by dissolving 0.164 g in 2 ml of 1 N NaOH and 98 ml of distilled water. HEPES was also obtained from the Sigma Chemical Co. (St. Louis, MO).

In experiments to deplete MT by exposure to exogenous O^6-MeG, O^6-MeG was a gift from Dr. A.E. Pegg, Pennsylvania State University (Hershey, PA) and was synthesized by the method of Balsiger and Montgomery (1960). O^6-MeG was dissolved by sonication into serum free Eagle's medium. Formaldehyde was obtained from Mallinckcrodt (Detroit, MI). Tritiated thymidine (Amersham, Arlington Heights, IL) and aphidicolin (Sigma, St. Louis, MO) were used in cell

synchrony experiments. A stock solution of aphidicolin was prepared by dissolving in 100% DMSO and storing at -20° C. Crystal violet and methylene blue used for colony staining were obtained from Mallinckcrodt (Detroit, MI). Bisbenzimide # 33258 Fluorochrome, used for determining mycoplasma contamination of cells, was obtained from Calbiochem-Behring (San Diego, CA).

B. Cell Lines

The characteristics and sources of the cell lines used in this study are listed in Table 3. Fibroblasts from foreskin material of normal newborns were initiated in our laboratory as previously described (McCormick and Maher, 1981). The cell lines obtained from the two XP patients, XP12BE and XP12RO, are very deficient in nucleotide excision repair of UV-induced damage (complementation group A) (Robbins et al., 1974; de Weerd-Kastelein et al., 1972). The non-transformed cell lines were between passage 6 and 16 when used (We use the term "passage" to refer to the number of population doublings the cells have undergone following the first subculture).

Table 3. Characteristics of cell lines studied

Designating code	Clinical status	Source of fibroblasts	Age of donor	Cloning efficiency(%)	Lifespan in culture	Supplier
SL66	normal	foreskin	neonate	50-75	finite	--[a]
SL68	normal	foreskin	neonate	49-88	finite	--[a]
SL70	normal	foreskin	neonate	50-75	finite	--[a]
CRL1220	normal	skin biopsy	16 hr	21	finite	ATCC[b]
VE45	normal	skin biopsy	45 yr	14	finite	C.C. Chang[c]
GM0011	normal	skin biopsy	fetus	20-45	finite	IMR[d]
GM637	normal	skin biopsy	18 yr	20-31	unlimited[e]	IMR
XP12BE	XP	skin biopsy	4 yr	20-29	finite	ATCC
XP12ROSV	XP	skin biopsy	--[f]	56-73	unlimited[e]	R. Millette[g]
GM2355	FP	skin biopsy	13 yr	48-65	finite	IMR
GM3314	GS	skin biopsy	48 yr	18-26	finite	IMR
GM3948	GS	skin biopsy	30 yr	20-36	finite	IMR
2938	GS	skin biopsy	--[f]	34-41	finite	F.P. Li[h]
2974	GS	skin biopsy	--[f]	20	finite	F.P. Li

[a] Initiated by authors.
[b] American Type Culture Collection, Rockville, MD.
[c] Michigan State University.
[d] Institute for Medical Research, Camden, NJ.
[e] SV40-virus transformed.
[f] Age unknown.
[g] Wayne State University, Detroit, MI.
[h] Dana-Farber Cancer Institute, Boston, MA.

C. Culture Medium and Conditions

Cells were routinely cultured in a humidified atmosphere (5% CO_2 in air) in Eagle's medium or in modified Ham's F1Ø medium lacking hypoxanthine (Grand Island Biological Co., Grand Island, NY), with additional $NaHCO_3$ to bring the level to 2.2g/liter to provide additional buffering capacity. The medium was supplemented with 1Ø or 15% (vol/vol) fetal bovine serum (Sterile Systems, Logan, UT / KC Biologicals, Lenexa, KA / GIBCO, Grand Island, NY / Biocell, Carson, CA / Flow Laboratories, McLean, VA), and the antibiotics penicillin and streptomycin. This medium was referred to as complete culture medium. For mutant selection, growth medium was supplemented with 4ØuM 6-thioguanine (TG).

D. Storage and Recovery of Cells from Liquid Nitrogen

Cells not immediately needed for experiments were stored in liquid nitrogen for future use. Cells were detached from the culture vessel with trypsin (GIBCO, Grand Island, NY) and resuspended in freezing medium. This medium consisted of Ham's F1Ø or Eagle's medium, supplemented with 2Ø% fetal bovine serum, antibiotics and 1Ø% DMSO (anhydrous; stored over molecular sieve). The cell suspension in freezing medium was transferred to a freezing vial and placed at -8Ø^{o} C in an insulated styrofoam container for 2 hours. This

procedure allowed the cells to undergo freezing at approximately one degree C per minute. Vials were then placed in a liquid nitrogen freezer where the cells can be stored indefinitely. In order to obtain the greatest recovery and viability of cells during the thawing process, the frozen cells were warmed in a 37^{o} C water bath as quickly as possible and then pipetted directly into a culture flask which contained at least 25 ml of complete culture medium. 25 ml of media was required for thawing to dilute the concentration of DMSO present in the freezing medium. The cells were allowed to attach for 6-18 hours and then fresh complete culture medium was added.

E. Testing for Mycoplasma

Cells were allowed to attach to coverslips in 35 mm diameter dishes under sterile conditions for at least 6 hours. The coverslips with attached cells were then rinsed in phosphate buffered saline (PBS) and fixed in 25% PBS/75% methanol solution for 15 minutes. The fixative was then removed and a solution of 0.001 % (w/v) of Hoechst dye 33258 in absolute ethanol was added and the cells were stained for 10 minutes before being removed from the dye and air dryed. The coverslips were mounted on glass slides with 50% buffered glycerol, pH 5.5 and the cells were examined for mycoplasma contamination with a fluorescence microscope.

F. Cell and Colony Counts

After using 0.25% trypsin (Grand Island Biological Company, Grand Island, NY) to detach cells from the tissue culture vessel (Corning Glass Works, Corning, NY), the cells were suspended in medium and then stored on ice for up to one hour before plating. Counting the cells was performed by using a hemacytometer or using an electronic counter (Coulter Count, Hialeah, FL). Serial dilutions were carried out to obtain cells at various concentrations. Colonies were visually scored at 14 days after seeding for cytotoxicity and induced mutation measurements. The colonies were stained before counting and only colonies of 50 cells or greater were counted. The plates containing the colonies were rinsed in saline and stained with 1% crystal violet (in 70% ethanol) or stained in (0.2 %) methylene blue after being fixed in methanol.

G. Cytotoxicity and Cloning Efficiency Determinations

Sensitivities of cell lines to various chemical mutagens were determined as described by McCormick and Maher (1981) and Maher et al. (1979). Cytotoxicity or survival was determined from the loss of colony-forming ability by an in situ assay in which the cells were treated at cloning densities. Cells were plated into 60-mm or 100-mm diameter dishes at several different densities (50-1000 cells per

dish; 4-6 dishes per density) to obtain at least one set in which the number of colonies ranged from 20-80 after chemical treatment. Eighty colonies per dish are easily countable for such cells as XP12ROSV which develop dense, small diameter colonies. Cell density is important, since cells plated at more than 10,000 cells per 21-cm^2 show an increased cloning ability compared to those plated at less than 10,000. Therefore, cells plated at high densities tend to give an artificially increased survival. Feeder layers of X-irradiated cells were not used.

The protocol for determining the cytotoxic effects of chemical carcinogens is diagrammed in Figure 5. Cells in exponential growth were trypsinized and plated at appropriate densities for determining survival 6-12 hours before treatment with MNNG, ENU or 4-NQO. Complete culture medium was replaced with serum free medium, buffered with 15mM Hepes, pH 7.4. MNNG was freshly dissolved in anhydrous dimethylsulfoxide and immediately delivered into the serum free medium in the dishes by micropipette. ENU was also dissolved in DMSO. 4-NQO was dissolved in 95% ethanol. The final concentration of dimethylsulfoxide did not exceed 1% and the final concentration of ethanol did not exceed 0.45%. After 1 hour incubation, the medium containing the chemical mutagen was removed and replaced with complete culture medium. Cells were fed one week after treatment and stained at 2 weeks.

Figure 5. Protocol for determining the cytotoxic and mutagenic effects of chemical carcinogens

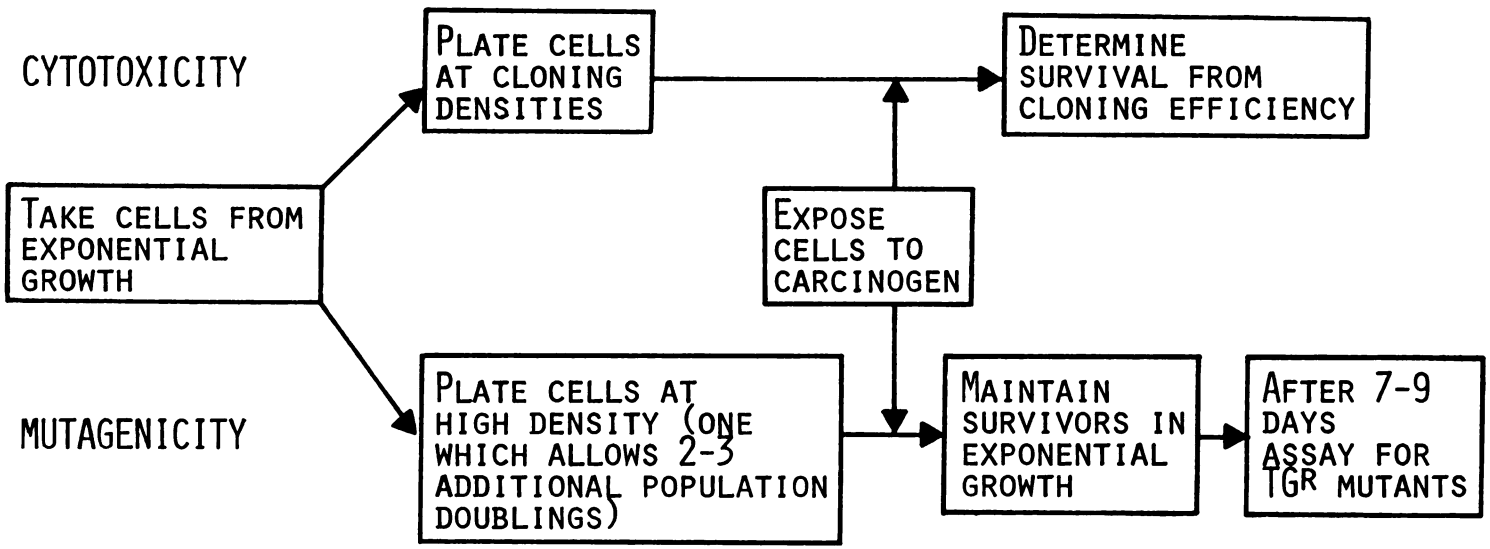
CYTOTOXICITY
PLATE CELLS AT CLONING DENSITIES
DETERMINE SURVIVAL FROM CLONING EFFICIENCY
TAKE CELLS FROM EXPONENTIAL GROWTH
EXPOSE CELLS TO CARCINOGEN
MUTAGENICITY
PLATE CELLS AT HIGH DENSITY (ONE WHICH ALLOWS 2-3 ADDITIONAL POPULATION DOUBLINGS)
MAINTAIN SURVIVORS IN EXPONENTIAL GROWTH
AFTER 7-9 DAYS ASSAY FOR TGR MUTANTS

Figure 5

In a replating cytotoxicity assay, cells were treated at the same density at which cells were treated for mutation experiments (see below). After the treatment period of 1 hour, the medium was removed and the cells were detached from the culture dish by trysinization, resuspended in culture medium and plated into dishes at cloning densities (50-1000 cells per dish; 4-6 dishes per density).

Cell survival was determined by dividing the cloning efficiency of the treated cells by the cloning efficiency of the control cells and expressing this value as percent.

H. Mutagenicity Assay

The general procedures used for assaying the induction of 6-thioguanine resistance in human cells have been published (McCormick and Maher, 1981; Maher et al., 1979) and the protocol for determining the mutagenic effects of chemical carcinogens is diagrammed in Figure 5. Cells in exponential growth for three days were trypsinized and plated into dishes at the desired densities (0.1 - 1.0 x 10^6 cells per 150-mm diameter dish). Sufficient numbers of target cells were treated for each exposure to insure 1 to 2 x 10^6 surviving cells at the beginning of the expression period. Cells were allowed 6-12 hours to attach and were then exposed to mutagen as described above. To maintain the treated and untreated populations in exponential growth during the expression period, the cells were trypsinized

once before they reached confluence and pooled, and 1-2 x 10^6 cells were plated at a lower density to continue exponential growth. The number of population doublings was monitored by electronic cell counting daily or at selected times during the expression period. At least four to six doublings over a period of 6 to 9 days are required before the cell has diluted out the residual number of HPRT enzyme molecules present in the cell before the mutational event occurred (Maher et al., 1979).

At the end of 7-10 days of expression, the cells were pooled (to obtain a representative population of cells) and 1-2 x 10^6 cells were plated in selection medium into 100-mm diameter dishes at 400-500 cells per cm^2 (40-92 dishes per determination). The cells were plated at such a low density in order to minimize the loss of mutants from metabolic cooperation. Human cells have been demonstrated to be able to transfer the phosphorylated nucleoside of 6-thioguanine (Corsaro and Midgeon, 1977) and this passage from $HPRT^+$ to $HPRT^-$ cells results in cell death for the $HPRT^-$ mutant. The cloning efficiency of the cells at the time of selection was also assayed for each treatment in non-selective medium and used to correct the observed mutant frequencies. The total number of mutants divided by the cloning efficiency and the total number of cells plated gives the induced mutation frequency per 10^6 clonable cells.

Another way of determining the frequency of mutation is by using the Poisson distribution function. One determines

the probability of a mutational event occurring per experimental culture dish (x) by measuring the fraction of culture dishes containing no clones, P(Ø), and applying the formula $P(Ø) = \exp(-x)$.

I. Reconstruction Studies

To determine the efficiency of TG resistant/$HPRT^-$ cells in the presence of $HPRT^+$ cells, reconstruction studies were performed. A known number of $HPRT^-$ cells were seeded into dishes containing $HPRT^+$ cells and exposed to selective medium. The cloning efficiency of the $HPRT^-$ cells in the presence of $HPRT^+$ cells, divided by their cloning efficiency alone, determines the efficiency of recovery of resistant colonies.

J. Determination of O^6-Methylguanine-DNA Methyltransferase Activity in Cell Extracts

Methyltransferase activity was determined by measuring the decrease of radioactive labeled O^6-methylguanine from DNA folowing the procedure of Pegg et al. (1983). Cells ($2 - 5 \times 10^7$) were suspended in complete medium, centrifuged, washed with buffer containing 50mM Tris-HCl (pH 7.5), 1mM dithiothreitol and Ø.1mM EDTA and recentrifuged. Cell pellets were stored at -80^oC until assayed. Frozen cell samples were thawed and additional buffer was added to

bring the volume to 1.5-3.Ø ml. The sample was sonicated for 2 periods of 3Ø seconds each, separated by a 1 minute interval and centrifuged to remove cell debris from the supernatant containing the cell extract. Additional buffer was added to the pellet and the material was sonicated and centrifuged again. Both supernatants were pooled and various amounts of this cell extract were combined with Ø.5 ml of an assay mixture and the volume brought to 2 ml with the buffer.

The assay mixture contained 66mM Tris-HCl, pH 8.3, 5mM dithiothreitol, Ø.1mM EDTA, 1.3mg of unlabeled calf thymus DNA, and a calf thymus DNA substrate containing labeled methyl nucleosides prepared by reaction with tritiated N-methyl-N-nitrosourea as described (Pegg and Balog, 1979). The 3Ø min reaction at 37^{o}C was stopped by the addition of perchloric acid at a final concentration of Ø.25M.

To insure complete precipitation of DNA, rat liver protein was added during the acid precipitation step. The precipitated DNA was centrifuged and hydrolyzed by heating in Ø.75ml of Ø.1 N HCl at 7Ø^{o}C for 3Ø minutes. The hydrolysate was centrifuged and the supernatant was saved. The pellet was resuspended and hydrolyzed again. The hydrolysate was centrifuged and the supernatant was combined with the earlier supernatant. The DNA bases present in this supernatant were separated by high pressure liquid chromatography. Fractions were collected and the amount of radioactivity present in the O^{6}-methylguanine peak was

determined. Results were expressed as fmol O^6-methylguanine removed per mg protein. The protein content in the cell extracts was determined by the method of Bradford et al. (1976) using bovine serum albumin as the standard.

Briefly, the protein determination method involved the binding of Coomassie Brilliant Blue G-250 to protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm and the increase in absorption at 595 nm is monitored. To 0.1 ml of protein solution was added 0.1 ml of Tris buffer. Five ml of protein reagent was then added. The reaction was complete in 2 minutes and the absorbance was read at 595 nm.

K. Assay for Ability of MT to Remove Methyl from O^4-Methylthymine

The ability of human MT to remove methyl from the O^4 position of thymine was assayed using a radioactive labeled methylated poly(dT)·poly(dA) substrate prepared as described (Dolan et al., 1984). Briefly, 25 units of poly (dT) were reacted with 0.1 mCi of [^{3}H]-methylnitrosourea in 0.05 M Tris, pH 8.0 at 37°C for 30 minutes. The alkylated poly(dT) was dialyzed extensively against 0.05 M Tris, pH 8.0 to remove remaining unbound activity. The alkylated poly(dT) was incubated with 25 units of poly(dA) in this same buffer at room temperature for 2 hours. Cell extracts were prepared as described in the section immediately above

and incubated at 37°C for 90 minutes with the methylated poly(dT)·poly(dA) substrate. The number of O^4-methylthymine bases remaining in the substrate was determined by HPLC as described (Dolan et al., 1984) and compared with the number of 3-methylthymine bases remaining.

L. Achieving Cell Synchrony With Aphidicolin

The protocol for synchronizing cells at the G_1/S border of the cell cycle is diagrammed in Figure 6. Cells were seeded at 10^4 per cm^2, grown to confluence, fed each day for 5 days with complete medium, and then starved for 3-4 days. The cells were then trypsinized, suspended in complete medium containing 0.2 ug/ml of aphidicolin, counted by electronic cell counting, and plated at 1.5 x 10^6 cells per 150-mm dish or 0.6 x 10^6 cells per 100-mm dish. The cells were incubated in aphidicolin for 24 hours to bring them to the G_1/S border (Grossman et al., 1985).

M. Measuring Incorporation of Tritiated Thymidine

Progression through S was determined by measuring the amount of radiolabelled thymidine incorporated during a 15 minute pulse. Incorporation was measured every half hour for 8 hours. At the indicated time, the growth medium was exchanged for Eagle's medium containing ^{3}H-TdR (5 uCi/ml, 60 mCi/mmole) and the cells were incubated for 15 minutes.

Figure 6. Protocol for synchronization of human fibroblasts at the G_1/S border by aphidicolin

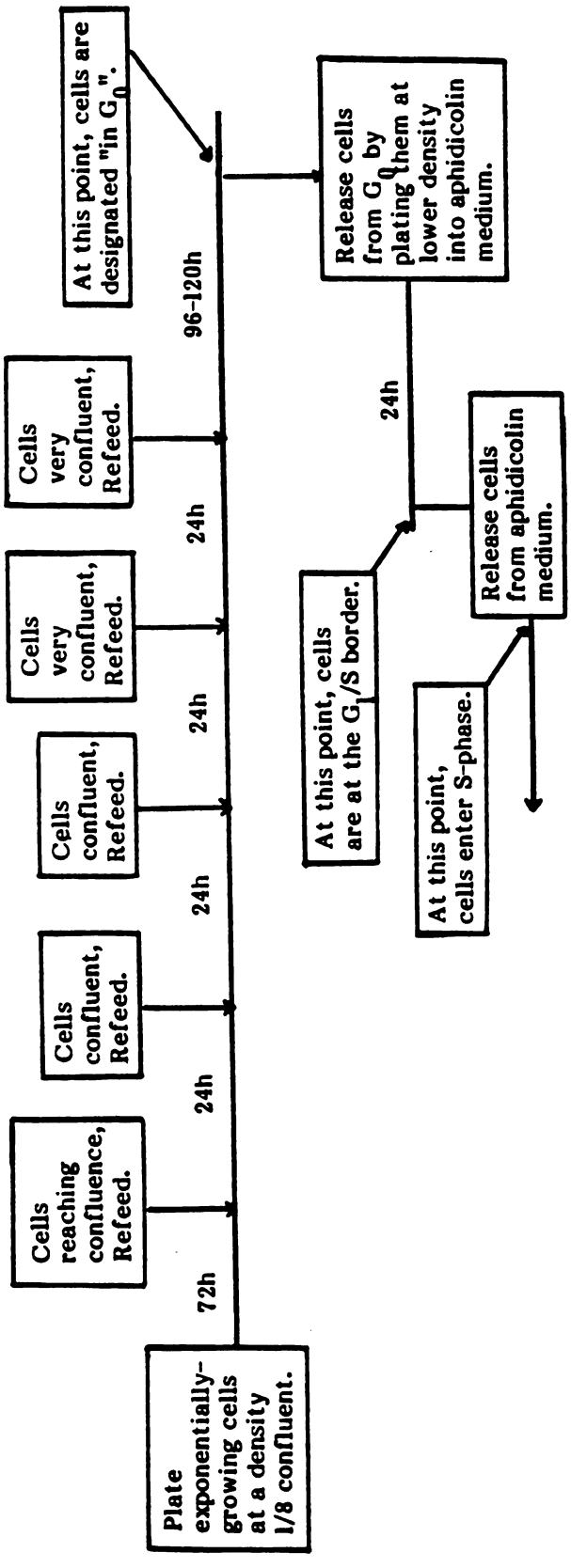
Plate exponentially-growing cells at a density 1/8 confluent.
72h
Cells reaching confluence, Refeed.
24h
Cells confluent, Refeed.
24h
Cells confluent, Refeed.
24h
Cells very confluent, Refeed.
24h
Cells very confluent, Refeed.
96-120h
At this point, cells are designated "in G_0".
Release cells from G_0 by plating them at lower density into aphidicolin medium.
24h
Release cells from aphidicolin medium.
At this point, cells are at the G_1/S border.
At this point, cells enter S-phase.

Figure 6

After the 15 minute pulse, cells were removed from the dishes with trypsin, and transferred onto glass fiber filters (Whatman, GF/C). The filters with cells were then washed twice with ice cold PBS and ice cold trichloroacetic acid (10%) and then washed once with ice cold ethanol (95%). The filter was dried under a sunlamp for 5-10 minutes. The radioactivity was measured by placing the glass fiber filter into a toluene-based scintillant and reading the counts per minute with a Beckman liquid-scintillation counter.

IV. RESULTS

A. Sensitivity of Cells to the Killing Effect of MNNG

As mentioned in the literature review section, several groups of investigators had reported that fibroblasts derived from patients with familial polyposis (FP) or Gardner's syndrome (GS) are abnormally sensitive to a variety of DNA-damaging agents. These results suggest that cells from such patients might be deficient in DNA repair. If so, these cells might also be more sensitive than normal cells to the mutagenic effect of these same agents. Before beginning a study comparing the frequency of mutants induced in normal, FP and GS cells by carcinogens, a comparison of their sensitivity to cell killing was carried out. N-methyl-N-nitro-N-nitrosoguanidine (MNNG) was selected as the carcinogen of interest because N-nitroso compounds have been implicated in colon cancer.

Figure 7A compares the survival curves of an FP cell line and four GS cell lines with that of normal fibroblasts, SL68 (neonatal) and GM0011 (fetal), exposed to MNNG at cloning densities. The data were obtained from a series of cytotoxicity studies, but SL68 cells were always included in each experiment for comparison. Therefore, the survival

Figure 7. Comparison of cytotoxicity (A) and mutagenicity (B) induced by MNNG in human fibroblasts. Cells in exponential growth were treated with MNNG for 1 h at a density of not more than 8 x 10^5 cells per 150-mm dish for mutagenicity determination. The background mutation frequencies per 10^6 clonable cells were: 3 and 8 for normal cells (SL68); 13 and 0 for normal cells (GM0011); 0, 7 and 11 for FP cells (GM2355); 0 and 0 for GS cells (GM3948); 45 for GS cells (2938); and 20 and 53 for GS cells (GM3314). The mutagenicity data have been corrected for these backgrounds. The cloning efficiencies of these cell lines are listed in Table 3. Bars representing the standard error of the mean are included for the cytotoxicity data. Symbols without error bars represent individual determinations of survival or determinations for which the symbol drawn is greater than the error bar. Lines were fitted by eye.

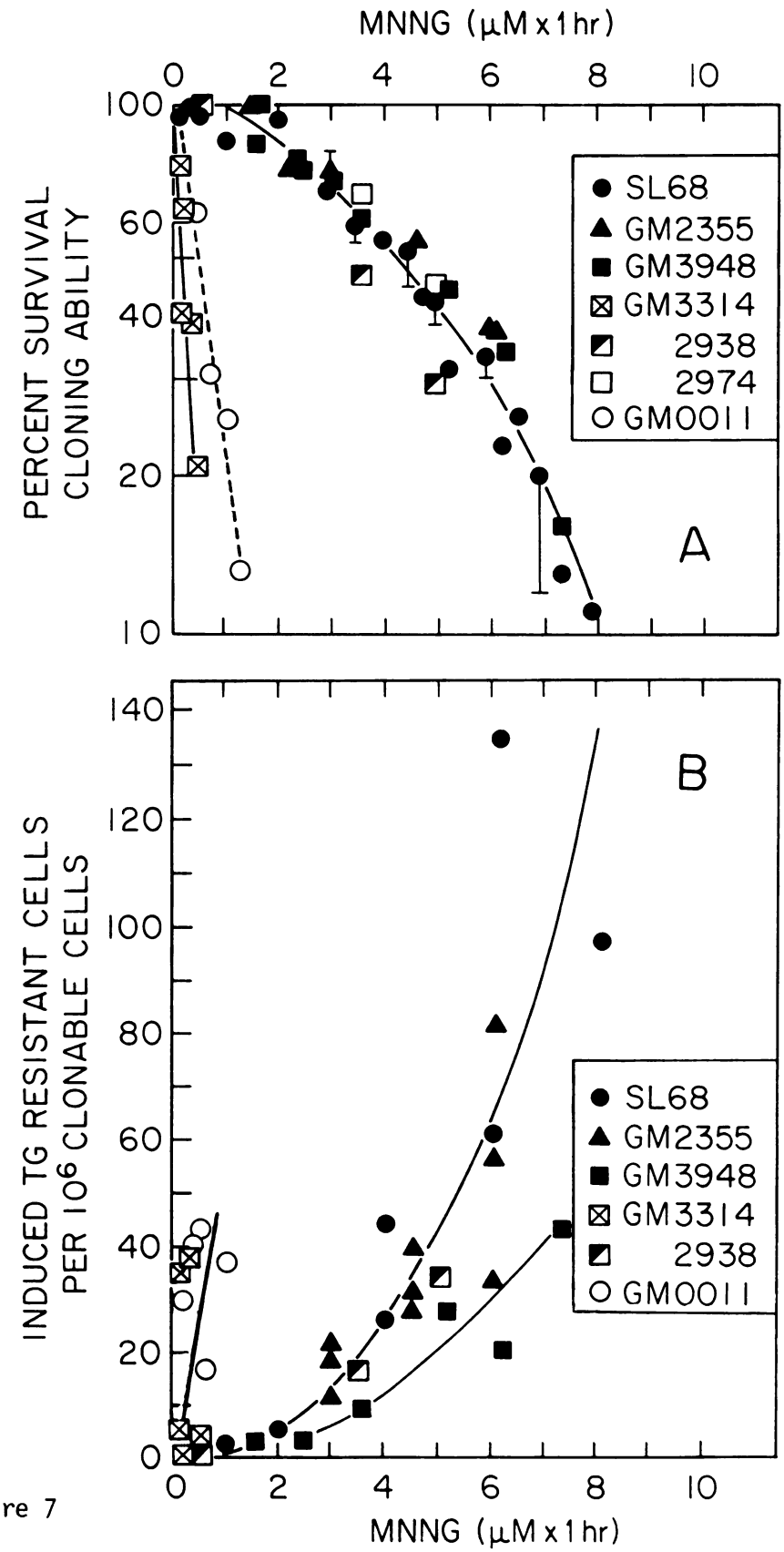
MNNG (μM x 1 hr)
0
2
4
6
8
10
100
60
40
20
10
PERCENT SURVIVAL
CLONING ABILITY
SL68
GM2355
GM3948
GM3314
2938
2974
GM0011
A
140
120
100
80
60
40
20
0
INDUCED TG RESISTANT CELLS
PER 10^6 CLONABLE CELLS
B
SL68
GM2355
GM3948
GM3314
2938
GM0011
MNNG (μM x 1 hr)

Figure 7

data for SL68 cells exposed to particular doses of MNNG in many different experiments were combined and averaged. The error bars shown in Figure 7A give the standard error of the mean for SL68 cells.

Since our normal strain, SL68, is derived from neonatal fibroblasts and the FP and GS cell lines are from patients that range in age from 11 to 45 years old, it was important to investigate the survival data for age matched normal cells. Also, survival experiments for other neonatal cells were carried out to find the range of cytotoxicity induced by MNNG in normal fibroblasts. The MNNG survival data obtained with a series of neonatal foreskin-derived cells and skin fibroblasts from a 16-year-old (CRL1220) and a 45-year-old person (VE45) were similar to that shown for SL68 cells (Table 4).

The data in Figure 7A show that the FP cells and three GS cell lines had normal sensitivity to MNNG cytotoxicity, but GM3314 and GM0011 cells were extremely sensitive. GM3314 fibroblasts are from a patient with Gardner's syndrome. GM0011 cells are from an apparently normal fetus (8 weeks old).

To determine whether the sensitivity to MNNG of the fetal line GM0011 was a general characteristic of fetal skin fibroblasts, we measured the response of three other fetal fibroblast lines (WI-38, 57372 and GM1380) to the cytotoxicity of MNNG. The results are presented in Table 5. All three cell lines showed a normal response, similar

Table 4. Cytotoxicity of MNNG in normal human fibroblasts

MNNG (uM)[a]	% SURVIVAL SL68	VE45	CRL1220
Ø	1ØØ	1ØØ	1ØØ
3.5	55	5Ø	52
5	33	22	29
7	12	7	9

[a]Cells were exposed for 1 hour.

Table 5. Cytotoxicity of MNNG in normal neonatal and fetal cell lines

MNNG (uM)	% SURVIVAL				
	normal neonatal	normal fetal			
	SL68	WI-38	GM1380	57372	GM0011
0	100	100	100	100	100
0.5	93	71	80	82	55
1	85	65	78	71	33
5	44	16	27	32	2

to that of the normal neonatal cell line SL68.

GM0011 cells were recently reported by Middlestadt et al. (1984) to exhibit increasing sensitivity to MNNG with increasing passage number in culture. The GM0011 cells shown in Figure 7A were treated at passage 8. They showed slightly higher survival at passage 6 (D_0 = 1.5uM) and slightly lower survival at passage 14 (D_0 = 0.5uM) (data not shown). However, the difference was not significant enough to warrant further investigation.

B. Sensitivity of Cells to the Mutagenic Effect of MNNG

Figure 7B gives the mutagenicity results obtained with an FP cell line, four GS cell lines and the normal fibroblast cell line SL68. Each mutation experiment was accompanied by a cytotoxicity experiment and these corresponding data are included in Figure 7A along with the results of experiments comparing survival only. GM3314 and GM0011, the two cell lines that were abnormally sensitive to the cytotoxic effect of MNNG, showed a dose-dependent increase in TG resistant cells at very low doses, i.e., < 1.5uM. All the other cell lines showed little or no increase in mutant frequency at these levels of MNNG. Only after treatment with MNNG doses of 2uM or greater did they exhibit an increase in mutant frequency. Note that the increase was not linear. No significant increase in mutant frequency occurred at an MNNG dose of 2uM. Between 3uM and

4uM, the increase was linear, but then the frequency increased rapidly. GM3948 cells, taken from a Gardner's syndrome patient, appeared to be slightly more resistant to the mutagenic effect of MNNG than were the rest of the cell lines with normal resistance. However, much more data would be needed to prove that this difference was statistically significant.

C. Comparing Human Cell Lines for Their Levels of O^6-Methylguanine-DNA Methyltransferase Activity

It is known that human cells contain O^6-methylguanine-DNA methyltransferase (Grafstrom et al., 1984). As explained in the literature review, this repair protein functions not as an enzyme, but as a methyl protein acceptor molecule. Since human cells have a finite number of such proteins, the supply may well be exhausted by very large doses of methylating agents. If this protein acceptor molecule caused rapid removal of the majority of the potentially mutagenic lesions induced by MNNG at low doses, but was unable to do so in cells exposed to higher doses (Medcalf and Lawley, 1981), this would explain the observed "threshold" on the mutation curve for the majority of cell lines shown in Figure 7B. Such a hypothesis predicts that GM3314 cells and GM0011 cells are deficient in this repair mechanism, i.e., have a much lower level of MT activity than the other cell lines tested.

The level of MT activity (ability to remove methyl groups from the O^6 position of guanine) in the cell lines shown in Figure 7B and in two additional skin fibroblast cell lines prepared from foreskins of normal neonates (SL66 and SL7Ø) was determined by measuring the decrease of radioactively labelled O^6-methylguanine from a DNA substrate using procedures previously described (Pegg et al., 1983; Pegg and Balog, 1979). For such studies, approximately $1Ø^7$ cells of each cell were prepared, harvested in the appropriate buffer (see section III - Materials and Methods), stored at $-8Ø^oC$ and transported to the laboratory of Dr. Anthony Pegg where the analysis was conducted. The analyses shown in Table 6 were conducted by myself under the direction of Dr. Pegg during my stay at Pennsylvania State University, Hershey, PA. Later analyses were carried out by Dr. Pegg and his associate, Dr. Eileen Dolan.

For comparative purposes, three additional cell lines were also examined. These included two SV4Ø virus-transformed cell strains (XP12ROSV and GM637) known to differ in ability to support survival of MNNG-treated adenovirus and classified respectively as methyl repair deficient (mer^-) and methyl repair proficient (mer^+) (Day et al., 198Ø). The third cell line, designated XP12BE, is a non-transformed XP cell line, previously shown to be extremely deficient in excision repair of DNA damage induced by ultraviolet radiation (Robbins et al., 1974) or by carcinogens that form multi-ringed DNA adducts (Heflich et

al., 1980; Yang et al., 1980), but to exhibit normal sensitivity to the killing action of MNNG (Simon et al., 1981).

Table 6 shows the results of the MT activity assay for each of these cell lines. Each value listed is the mean of three determinations. The GM3948 cells, which exhibited the lowest frequency of MNNG-induced mutations (Figure 7B) showed one of the highest levels of MT activity. GM3314 and GM0011, the two sensitive strains, showed much lower or virtually non-detectable levels of MT, as did the mer^- XP12ROSV cell line. Although the data in Table 6 suggest that the level of MT activity in GM0011 declined with increased passage number, one cannot be certain of this because the levels observed were so low. (A value of 8 fmol methyl removed per mg protein is close to or below the detection limit of the assay.) Of the 8 cell lines with measureable levels of MT activity, the GM637 cells, exhibited the lowest values. The FP cell line (GM2355) and three GS cell lines (GM3948, GM3314 and GM2938) showed levels of MT activity similar to that of the three normal foreskin-derived cell lines.

Table 6. Comparison of the levels of O^6-methylguanine-DNA methyltransferase in cell extracts of various human fibroblast cell lines

Cell line	O^6-methylguanine-DNA methyltransferase activity (fmol O^6-MeG removed per mg protein)	
	Individual experiments	Mean
XP12BE	193[a], 229	211
GM3948	148, 267	208
SL70	202	
SL66	152	
GM2355	126, 159	142
SL68	112, 135	123
2938	103, 108	105
GM637	121, 85, 83	96
GM0011 p. 6[b]	16	
GM0011 p. 13	<10	
GM3314	10, 6	8
XP12ROSV	12, 3	7

[a] Each experiment used different preparations of cells and the MT activity of the cell extracts was measured at 2 or 3 protein concentrations and the average value is given in this column. The next column gives the mean value from the individual experiments.

[b] The cells were supplied as passage 3 and allowed to replicate 8-fold.

D. Ability of MT to Remove Methyl from O^4-Methylthymine

It has recently been shown (McCarthy et al., 1983; McCarthy et al., 1984; Ahmmed and Laval, 1984) that the methyltransferase of E. coli can remove methyl groups from the O^4 position of thymine, as well as from the O^6 position of guanine. Because O^4-methylthymine has been shown to miscode in vitro (Loveless ,1969), it was important to see if human MT could remove this lesion. Therefore, XP12BE cells, the cell line with the highest level of MT activity (see Table 6), were grown to large numbers, prepared and shipped to the laboratory of Dr. Pegg to be tested for the ability of the extracts to remove methyl groups from the O^4 position of thymine.

The assay consists of testing the ability of human cell extracts to remove methyl from the O^4 position of thymine using a radioactively labelled poly(dT)·poly(dA) substrate. At the same time, the cell extract was tested for the removal of 3-methylthymine, O^6-methylguanine and 7-methylguanine. The results are shown in Table 7. The XP12BE cell extract had normal activity against O^6-methylguanine but was completely inactive against O^4-methylthymine.

Table 7. Specificity of human O^6-methylguanine-DNA methyltransferase

Extract[a] added	Methylated bases remaining in substrate (pmol)			
	O^4-methylthymine	3-methylthymine	O^6-methylguanine	7-methylguanine
None	Ø.36	Ø.51	Ø.54	5.6
1.35 units of alkyltransferase from human fibroblasts XP12BE	Ø.39	Ø.56	Ø.Ø4	>4.3

[a]The extract was incubated with the appropriate DNA substrate (either methylated poly(dT)·poly(dA) for analysis of methylated thymines or methylated calf thymus DNA for methylated guanines) and the effect on specific methylated bases was determined by HPLC. One unit of O^6-methylguanine-DNA methyltransferase activity corresponds to the ability to remove 1 pmol of methyl groups from O^6-methylguanine in DNA.

E. Comparing the MNNG Sensitivity of These Other Three Cell Lines

Once the level of MT activity in cell lines XP12ROSV, GM637, and XP12BE was known, these same cell lines were compared to the others for their response to the cytotoxic and mutagenic effect of MNNG. The results are shown in Figure 8. The data for the various strains shown in Figure 7 have been included in Figure 8 for comparison. As predicted from their level of MT activity shown in Table 6, XP12BE cells showed a normal response to MNNG; GM637 cells were more sensitive than normal; and XP12ROSV cells were extremely sensitive to the cytotoxic and mutagenic action of MNNG. As was seen for GM3948 cells, the response of the XP12BE cells, which had a high level of MT activity, to the mutagenic effect of MNNG appeared to be somewhat lower than that of normal cells.

For comparative purposes, Table 8 presents the data from two representative experiments for each cell line except GM3314. The latter cell line had background mutant frequencies that ranged from 20-500 mutants per 10^6 clonable cells. In only two experiments did we find the background frequencies to be low: 20 and 53. It will be seen that a dose-dependent increase in the frequency of TG resistant cells was obtained in GM0011 and XP12ROSV cells at "threshold doses", doses that did not cause a significant increase in normal cells or in GS cell lines that were shown

Figure 8. Comparison of cytotoxicity (A) and mutagenicity (B) induced by MNNG in XP12BE cells and two SV40-transformed cell lines. Data from Figure 6 are shown for comparative purposes. The background mutation frequencies per 10^6 clonable cells were: 2 for XP12BE cells (CRL1223); 2 and 18 for GM637 cells; and 0, 1 and 0 for XP12ROSV cells. The cloning efficiencies of these cell lines are listed in Table 3. Lines shown were fitted by eye.

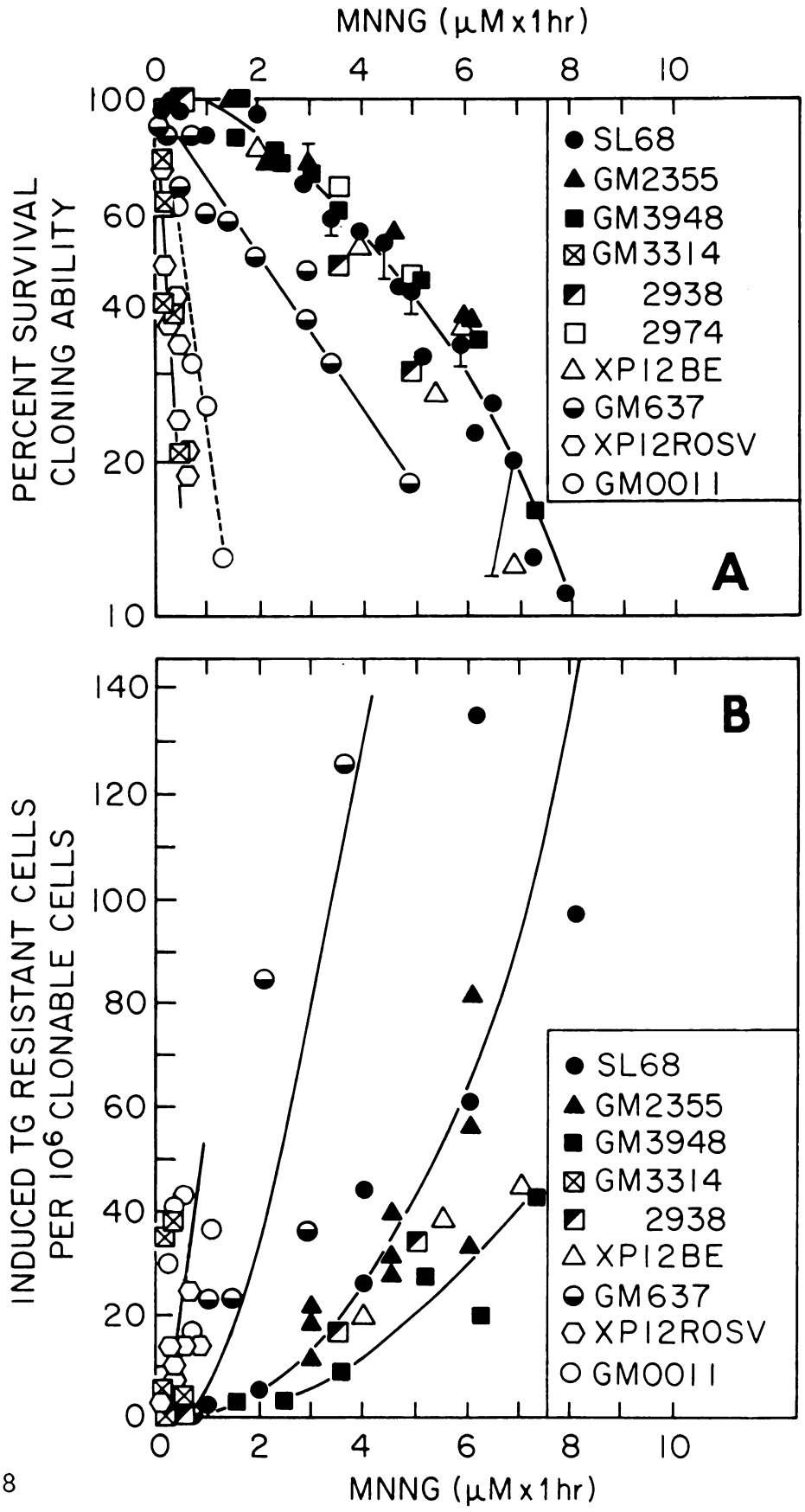
MNNG (μM x 1hr)
0
2
4
6
8
10
100
60
40
20
10
PERCENT SURVIVAL
CLONING ABILITY
SL68
GM2355
GM3948
GM3314
2938
2974
XP12BE
GM637
XP12ROSV
GM0011
A
140
120
100
80
60
40
20
0
INDUCED TG RESISTANT CELLS
PER 10^6 CLONABLE CELLS
B
MNNG (μM x 1hr)

Figure 8

Table 8. Representative mutagenicity data of MNNG in various cell lines

Target cells	MNNG concentration (uM x 1 h)	Original surviving fraction	Cells selected ($\times 10^6$)	Thioguanine resistant colonies	Cloning efficiency at selection	Mutants per 10^6 clonable cells
SL68	0	1.00	1.19	5	0.50	8
	1	0.86	1.19	10	0.65	13
expt.	2	0.95	1.19	10	0.59	14
24	4	0.51	1.19	35	0.56	52
	6	0.26	1.19	87	0.51	143
expt.	0	1.00	1.32	2	0.53	3
21	4	0.56	1.32	16	0.42	29
	6	0.27	1.32	26	0.31	64
	8	0.11	1.29	28	0.22	100
GM3948	0	1.00	1.76	0	0.53	0
	4.5	0.46	1.76	18	0.38	27
expt.	5.5	0.35	1.76	12	0.36	19
54	6.5	0.16	1.76	21	0.28	43
	0	1.00	1.72	0	0.18	0
expt.	1.6	0.85	1.76	1	0.17	3
22	2.5	0.76	1.69	1	0.19	3
	3.6	0.62	1.63	2	0.14	9
GM2355	0	1.00	1.32	7	0.47	11
	3	0.68	1.32	14	0.49	22
expt.	4.5	0.48	1.32	22	0.40	42
19	6.0	0.32	1.32	46	0.38	92
	0	1.00	1.32	5	0.54	7
expt.	3	0.71	1.32	13	0.40	25
18	4.5	0.34	1.32	23	0.38	46
	6.0	0.36	1.32	17	0.32	40

Table 8. (continued)

Target cells	MNNG concentration (uM x 1 h)	Original surviving fraction	Cells selected ($\times 10^6$)	Thioguanine resistant colonies	Cloning efficiency at selection	Mutants per 10^6 clonable cells
GM637	0	1.00	1.76	2	0.28	4
	1.0	0.55	1.76	15	0.31	27
expt.	2.0	0.44	1.76	39	0.25	89
38	3.5	0.31	1.76	59	0.26	130
	0	1.00	1.41	18	0.32	40
expt.	0.5	0.69	1.41	16	0.28	41
49	1.5	0.59	1.41	25	0.28	62
	3.0	0.50	1.39	29	0.28	75
XP12ROSV	0	1.00	2.05	1	0.38	1
	0.25	0.42	2.11	8	0.42	9
expt.	0.40	0.34	2.06	11	0.40	13
51	0.60	0.19	2.11	19	0.36	25
	0	1.00	1.74	0	0.52	0
expt.	0.1	0.78	1.74	3	0.53	3
37	0.2	0.53	1.74	6	0.48	7
	0.3	0.37	1.69	11	0.47	14
GM0011	0	1.00	0.68	2	0.23	13
expt.	0.65	0.31	1.34	10	0.25	30
yy	1.00	0.26	1.66	23	0.28	49
	0	1.00	1.74	0	0.40	0
expt.	0.3	0.84	1.76	23	0.45	29
62	0.6	0.84	1.74	33	0.46	41
	1.0	0.63	1.94	30	0.36	44
GM3314	0	1.00	1.74	6	0.065	53
	0.125	0.77	1.76	7	0.068	58
expt.	0.25	0.40	1.76	8	0.050	90
32	0.50	0.24	1.76	4	0.040	57

to have normal levels of MT activity.

These SV40-transformed cells differ from non-transformed fibroblasts in that they are less tightly attached to the surface of the culture vessel. Therefore, in determining the mutation frequencies with these cell lines, as well as with the others, the frequencies were calculated not only from the observed number of mutant colonies as shown in Table 8, but also using the Poisson distribution function. That is, the chance of a TG resistant colony per dish was calculated from the number of dishes containing no colonies. One determines the probability of a mutational event occurring per experimental culture dish (x) by measuring the fraction of culture dishes containing no clones, P(Ø), and applying the formula $P(Ø) = \exp(-x)$. Use of this method eliminates the possibility that the frequencies observed were artificially increased by the formation of satellite colonies. No evidence of such increase was found. The results were the same using either method, indicating that formation of satellites was not a problem.

F. Sensitivity of Another SV40 Transformed Cell Line to the Cytotoxic Action of MNNG

Day and coworkers (1980) reported that the SV40 transformed cell line XP12RO that they used called XPT703 was intermediate in sensitivity to MNNG. Our results (Table 9) indicate that XP12ROSV was extremely sensitive to MNNG. The assay of Day et al. (1980) was inactivation of adenovirus but this assay usually gives similar results to a survival curve. Therefore, we examined the line from Dr. Day and measured the cytotoxic response of XP12ROSV and XPT703 to MNNG under the same experimental conditions. The results showed that indeed XPT703 was not as sensitive as XP12ROSV.

G. Measuring Effect of MT Depletion on Mutations and Cell Survival

1. Inhibition of MT by Formaldehyde

Pegg and coworkers (1983) have found that in mammalian cell extracts, methyltransferase is specific for the removal of the lesion O^6-MeG. If O^6-MeG is the lesion responsible for cytotoxicity and mutagenicity in human cells, then if one could inhibit the action of the DNA repair protein or decrease the number of molecules and then challenge such cells with MNNG, it should be possible to detect a decreased

Table 9. The cytotoxicity of MNNG in two viral transformed XP cell lines

MNNG dose(uM)[a]	% SURVIVAL XP12ROSV40	XP12T703
experiment 64		
0	100	100
0.25	30	48
0.50	35	81
2.0	17	65
3.0	8	50
experiment 59		
0	100	100
0.25	36	65
0.40	17	54
0.60	11	46
2.00	NP[b]	40
4.00	NP	31

[a]Cells were exposed for 1 hour.

[b]Not performed.

survival and an increased mutation frequency. Use of such procedures should give an indication of the biological role of the O^6-MeG lesion in DNA.

Preliminary observations by Grafstrom et al. (1983) indicated that MT activity in human cells could be inhibited by exposing cells to formaldehyde. Therefore SL68 cells plated at cloning densities were exposed to concentrations of formaldehyde of Ø, 25, 5Ø, 75 and 1ØØ uM in serum-free medium for 5 hours to determine the cytotoxicity of formaldehyde in preparation for its use in subsequent survival and mutation experiments with the alkylating agents MNNG and ENU. THe results are shown in Table 1Ø.

To determine the effect of low doses of formaldehyde on these two parameters, cells were plated at cloning densities to determine cell survival in 1ØØ-mm dishes and at Ø.4 x $1Ø^6$ cells per 15Ø-mm dishes to determine induced mutation frequencies. The cells were allowed to attach for 6 hours before addition of 6ØuM formaldehyde (8Ø% survival) and either 1.75 mM ENU or Ø.5uM MNNG for 1 hour. This solution was then removed and formaldehyde (6ØuM) in serum-free media was added for an additional four hours before complete medium was added. Table 11 shows the results. No increase in mutation frequency was seen. However, the level of MT activity in the cells pre-treated with formaldehyde was not measured in this experiment. Therefore the negative result may reflect the fact that the formaldehyde concentration used did not inhibit MT significantly. Further

Table 10. In situ cytotoxicity of formaldehyde (HCHO) in normal cells (SL68)

HCHO (uM)[a]	% SURVIVAL
0	100
25	95
50	87
75	76
100	41

[a]Cells exposed for 5 hours.

Table 11. Effect of formaldehyde (HCHO) on the cytotoxic and mutagenic response of SL68 cells to alkylating agents

Dose HCHO	Dose ENU	Dose MNNG	Percent SURVIVAL	Mutants ($\times 10^6$)
Ø	Ø	Ø	100	Ø
60uM	Ø	Ø	72	Ø
Ø	0.75mM	Ø	93	69
Ø	1.75mM	Ø	58	96
60uM	1.75mM	Ø	27[a]	102
Ø	Ø	0.5uM	77	Ø
Ø	Ø	5.0uM	50	54
60uM	Ø	0.5uM	80[b]	Ø

[a]This corresponds to 38% of its own control.

[b]This corresponds to 110% of its own control.

experimentation using formaldehyde to reduce the MT activity in human cells was not pursued because sometime later Dr. Peter Karran reported (personal communication) that addition of the free base O^6-methylguanine to the medium caused a decrease in the level of MT activity in cells. Therefore, experiments to reduce MT activity by exposure of cells to exogenous O^6-MeG were carried out. This was the preferred method since an increase in mutations with formaldehyde plus an alkylating agent could be due to the fact that formaldehyde causes strand breaks and this could be a synergistic effect (Grafstrom et al., 1984).

2. Depletion of MT by Exposure to Exogenous O^6-MeG

Experiments were conducted in normal human cells (SL68) to determine whether or not the level of methyltransferase activity could be reduced by exposure to exogenous O^6-MeG in the culture medium. As shown in Table 12, when exogenous O^6-methylguanine was present in the culture medium (Ø.4 or Ø.8 mM) of SL68 cells (8 x $1Ø^6$ cells per 15Ø-mm dish) for 15 or 24 hours, there was a substantial loss of MT activity in extracts prepared from the cells (3Ø-7Ø x $1Ø^6$). The average reduction in MT activity was 65%. Increasing the dose of exogenous O^6-MeG from Ø.4 mM to Ø.8 mM did not decrease the activity substantially.

It should be noted that cells with depleted levels of MT activity could regenerate MT after the removal of the O^6-MeG medium (Table 13). In MT depleted cells, the MT activity

Table 12. Effect of exposure to exogenous O^6-MeG for 15 h or 24 h on the level of MT activity in human cell extracts

Experiment number	Control population: MT activity (fmol removed per mg protein)[a]	O^6-MeG-exposed population: Dose O^6-MeG (mM)	MT activity (fmol removed) per mg protein)	Activity remaining (% of control)
I	272	0.4	108	40%
II	210	0.4	80	38%
III	480	0.4[b]	200	42%
		0.8[b]	150	31%
IV	317	0.4	66	21%

[a]Each value is the mean of 3 determinations.

[b]15 h exposure.

Table 13. Regeneration of MT activity in cell extracts after depletion by exposure to exogenous O^6-MeG

Experimental conditions	MT activity (fmol removed per mg protein)	Percent of control
No exposure to O^6-MeG	317	100%
24 h of exogenous O^6-MeG (0.4mM)	66	21%
24 h after removal of exogenous O^6-MeG	258	80%
48 h after removal of exogenous O^6-MeG	298	94%

regenerated to 81% of its initial value in 24 hours and to 94% at 48 hours.

The ability of the free base, O^6-MeG, to act as a substrate for MT provides an opportunity to specifically manipulate MT levels. This permits one to determine the effect of MT on the cytotoxic and/or mutagenic effect of alkylating agents. Cells were plated at a density of $0.5\text{-}1.0 \times 10^6$ cells per 100-mm dish for determining cell survival and induced mutations. The cells were allowed to attach for 6 hours before addition of the O^6-MeG medium. Fifteen or 24 hours later, the O^6-MeG medium was removed and the MT-depleted populations and their corresponding non-depleted control populations were challenged with various doses of MNNG and assayed for cytotoxicity and mutagenicity. As shown in Figure 9, at every dose of MNNG the frequency of TG resistant cells was higher in the MT-depleted cells (triangles) than in the corresponding non-depleted populations (circles). Data from a representative mutagenicity experiment are shown in Table 14. The non-depleted populations showed no increase in mutant frequency at doses of MNNG below 2.5uM, a linear increase at intermediate doses of MNNG, but then the frequency increased rapidly. In contrast, in the depleted populations, a dose of 2uM MNNG was sufficient to induce mutations and the frequencies induced by 3.5uM MNNG were similar to those induced by 4.5uM in the populations with normal levels of MT activity. From the insert in Figure 9,

Figure 9. Mutagenicity induced by MNNG in populations of human fibroblasts with reduced levels of MT activity or with normal levels. Data derived using cells with decreased MT activity caused by 15 or 24 h exposure to Ø.4mM exogenous O^6-MeG are shown as triangles, that from cells with normal levels of MT are shown as circles. Cells were treated with MNNG for 1 h at a density of ~1 x $1Ø^6$ cells per 15Ø-mm diameter dish and selected for TG resistance (4ØuM) after an expression period of 7 d. The induced frequencies have been corrected for the background frequencies in the populations not treated with MNNG and for the cloning efficiencies of the cells at time of selection. Data from individual experiments are distinguished by the shading of the symbols used, e.g., Exp. II, ◐, ◭ ; Exp. IV, ◑, ◭.

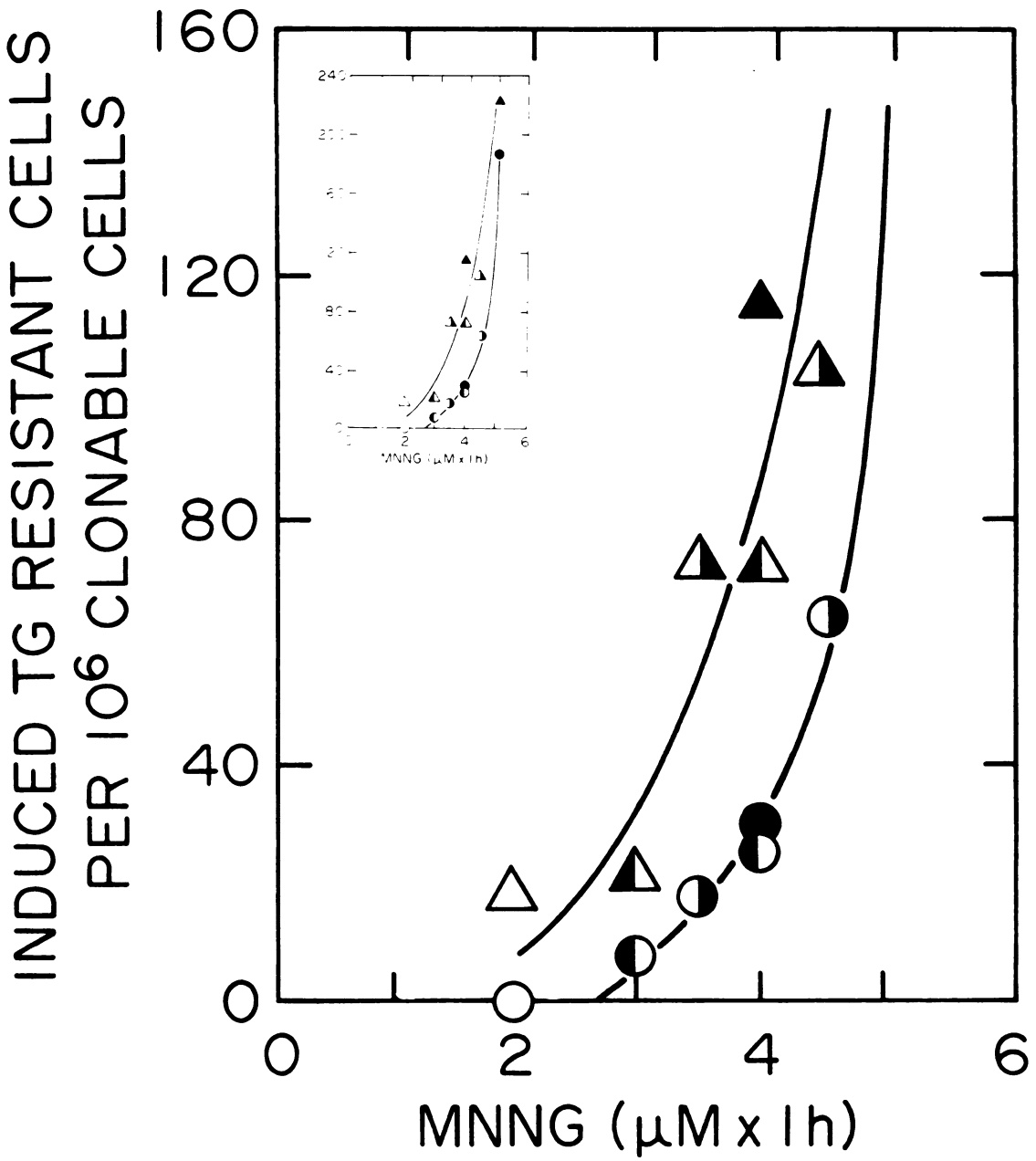
INDUCED TG RESISTANT CELLS
PER 10⁶ CLONABLE CELLS
160
120
80
40
0
0
2
4
6
MNNG (μM x 1h)

Figure 9

Table 14. Effect of depletion of MT activity on the cytotoxicity and mutagenicity of MNNG in diploid human fibroblasts

MT activity in target cells (fmol removed per mg protein)	Dose MNNG (uM)	Cloning efficiency of target cells	Survival target cells (percent of control)	Total cells selected ($\times 10^{-6}$)	Thioguanine resistant colonies observed	Cloning efficiency at time of selection	Mutants per 10^6 clonable cells
317	0	0.88	100	1	1	0.64	1
	3.5	0.52	59	1	18	0.84	19
	4.5	0.39	44	1	38	0.58	65
66[a]	0	0.85	100	1	1	0.62	2
(21% of	3.5	0.55	65	1	46	0.53	75
control)	4.5	0.47	55	1	80	0.66	107

[a]Cells exposed to 0.4mM exogenous O^6-MeG.

it can be seen that at high doses of MNNG (5uM), the difference in the mutagenic response of the two populations (non-depleted and depleted) was less pronounced, as expected, if the number of potentially mutagenic lesions induced by high doses of MNNG far exceeds the repair capacity of either population.

Each mutation experiment was accompanied by a replating cytotoxicity experiment and these corresponding data are shown in Table 15. The data indicate that populations with decreased levels of MT activity, caused by 15 or 24 hours of exposure to exogenous O^6-MeG, were not significantly more sensitive to the killing action of MNNG than non-depleted populations.

H. Effect of the Cell Cycle on MNNG Induced Cytotoxicity and Mutagenicity

1. Use of Aphidicolin to Synchronize Human Fibroblasts

Recent studies by Grossman et al. (1985) in this laboratory demonstrated that it is possible to synchronize human cells using a G_1/S block. The block is aphidicolin, which is known to inhibit DNA polymerase alpha (Pedrali-Noy et al., 1980). In preparation for those studies, experiments were carried out designed to determine the length of the S-phase in synchronized human cells following release from a G_1/S block. The cells had been synchronized by being grown to confluence and starved for mitogens (G_0

Table 15. Effect of depletion of MT activity on the cytotoxicity of MNNG

Experiment number	MT activity remaining (% of control)	Dose MNNG (uM)	Cloning efficiency of target populations (non-depleted)	Cloning efficiency of target populations (MT-depleted)	Percent survival of target populations (non-depleted)	Percent survival of target populations (MT depleted)
I	40%	0	0.70	0.72	100	100
		2			97	100
II	38%	0	0.88	0.84	100	100
		3			83	62
		4			68	65
III	42%	0	0.80	0.79	100	100
		4			68	70
		5			53	63
IV	21%	0	0.88	0.85	100	100
		3.5			55	65
		4.5			47	55

state), then plated at a lower density and incubated for 24 hours in medium containing aphidicolin (Grossman et al., 1985).

The onset and length of the S phase was determined by measuring the extent of incorporation of radiolabelled thymidine during 15 minute pulses every hour for 16 hours. The incorporation data in Figure 10 shows that upon release from the aphidicolin block the S phase starts immediately and is 8 hours in length.

2. Effect of Cell Cycle on Mutagenesis by MNNG

The effect of the cell cycle on the frequency of mutations induced by MNNG in normal SL68 cells is shown in Figure 11. Cells were treated with MNNG, 1.5 to 2.5 hours into the onset of the S phase (solid circles) at a density of 1×10^6 cells per 150-mm dish. The dashed line represent data (taken from Figure 7) for SL68 cells that were in exponential (asynchronous) growth when treated with MNNG. When synchronous cells were treated with MNNG (3, 4 and 5uM) during the S phase, the induced mutation frequency was approximately 3 times higher than if asynchronous cells were treated with MNNG.

3. Effect of Cell Cycle on the Cytotoxicity of MNNG

The effect of cell cycle on the survival of SL68 cells after treatment with MNNG is shown in Figure 12. The dashed line represents data (taken from Figure 7) for SL68 cells

Figure 10. Incorporation of ^{3}H-TdR during S phase in cells synchronized by release from aphidicolin. At the times post-release, cells were pulse labeled for 15 min with ^{3}H-TdR and analyzed for incorporation of label into acid insoluble material as described in Materials and Methods, section III.

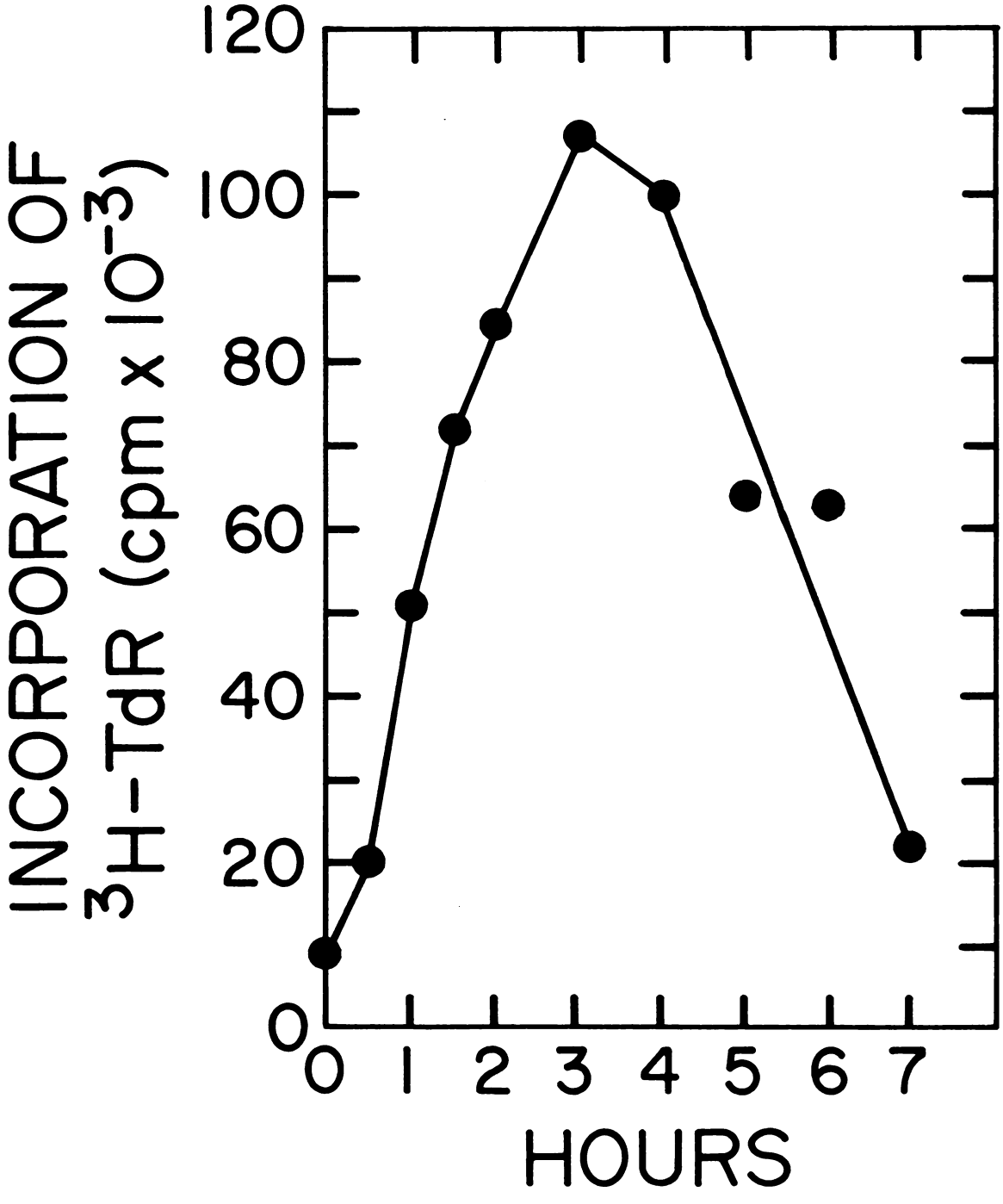
INCORPORATION OF
^{3}H-TdR (cpm x 10^{-3})
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7
HOURS

Figure 10

Figure 11. Mutagenicity of MNNG in SL68 cells at S and with time for repair. The background mutation frequencies per 10^6 clonable cells were: 4 and 12 for experiments in which cells were treated at S; Ø and 57 for experiments in which cells were treated 24 h to S. The asynchronous curve has been taken from Figure 6. The mutagenicity data have been corrected for these backgrounds.

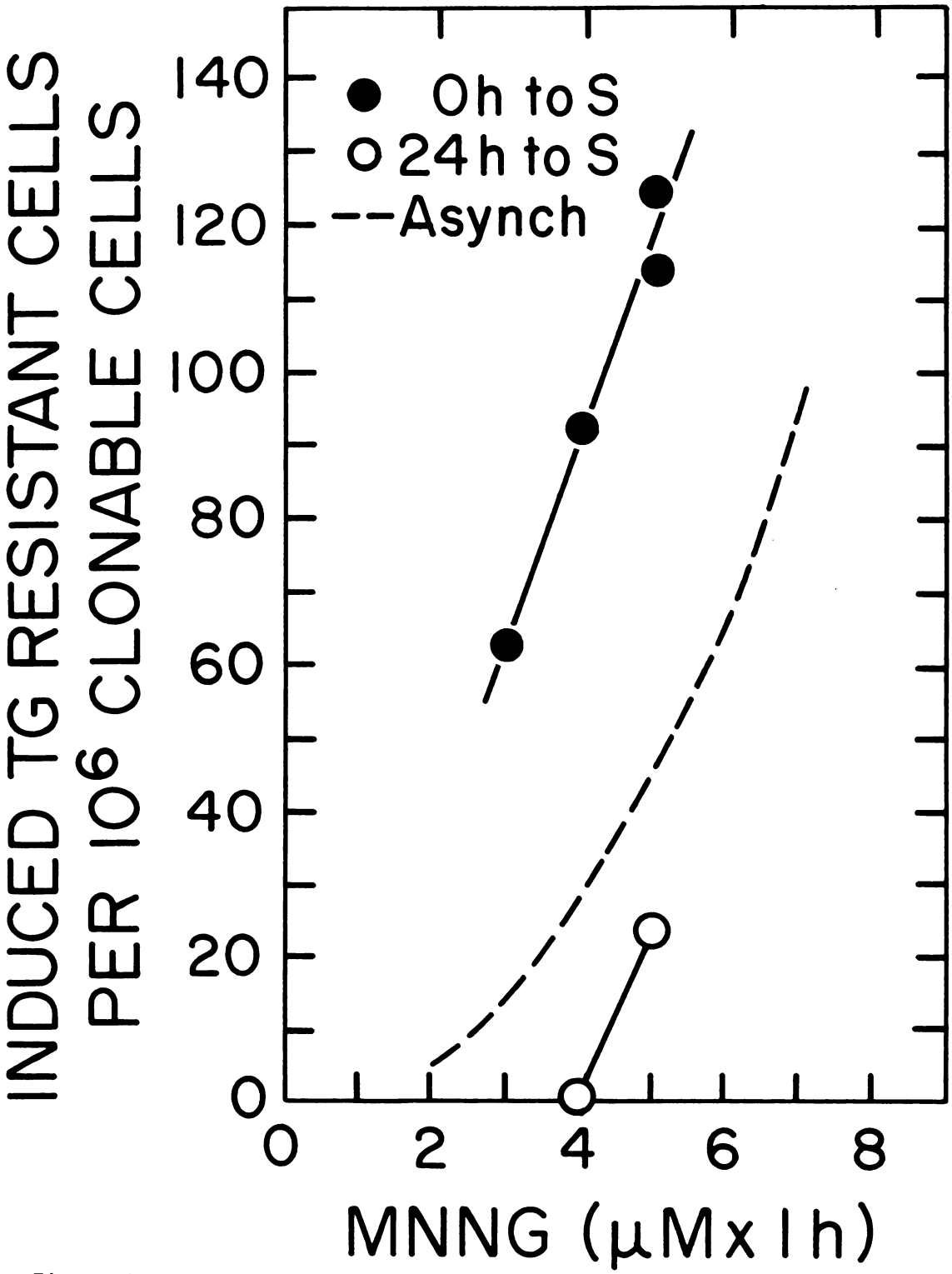
INDUCED TG RESISTANT CELLS
PER 10⁶ CLONABLE CELLS
140
120
100
80
60
40
20
0
0
2
4
6
8
MNNG (μM x 1h)
Oh to S
24h to S
Asynch

Figure 11

Figure 12. Cytotoxicity of SL68 cells treated at S

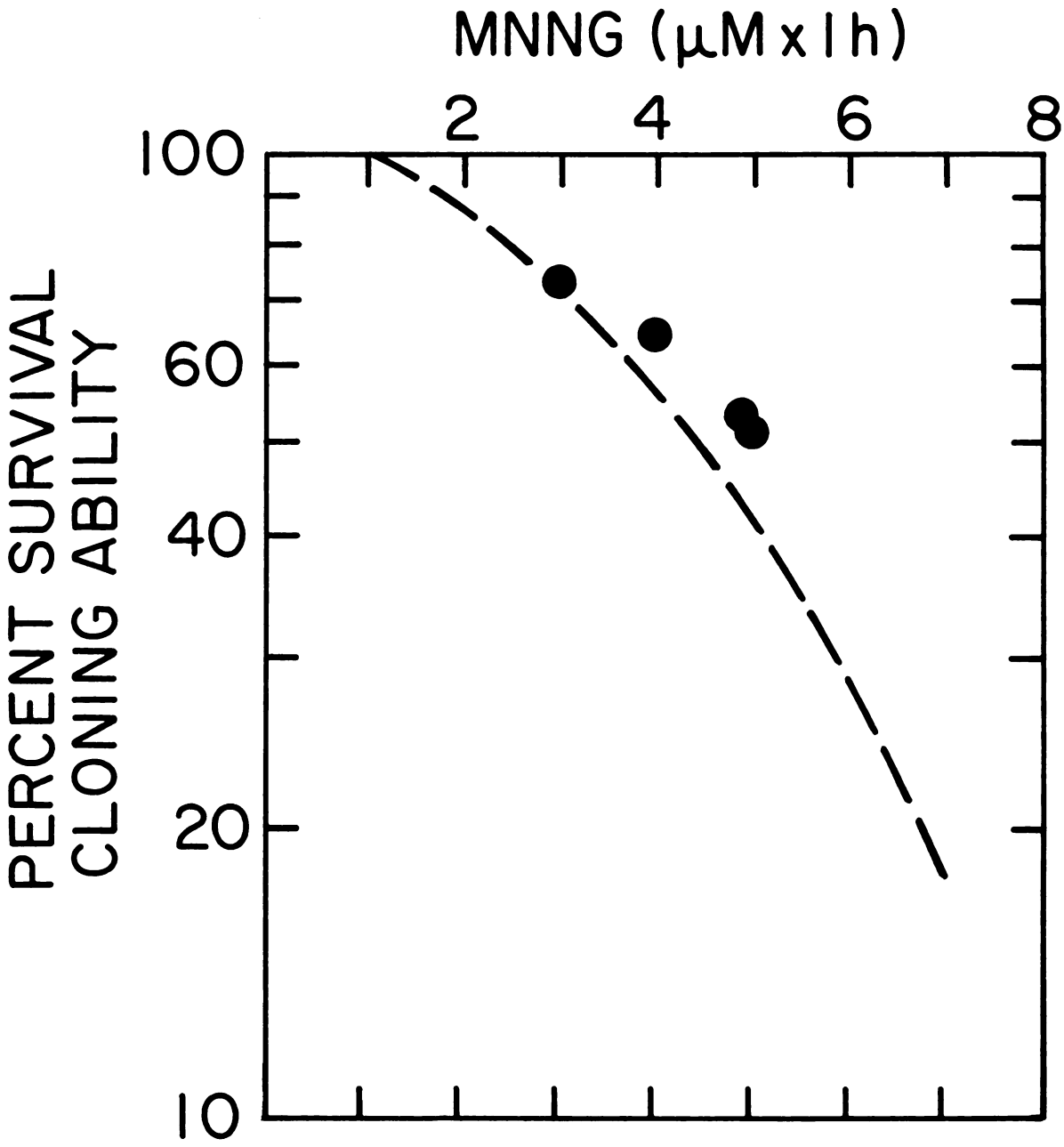
MNNG (μM x 1h)
2
4
6
8
100
60
40
20
10
PERCENT SURVIVAL
CLONING ABILITY

Figure 12

that were asynchronous when treated with MNNG. When synchronous cells (0.2 x 10^6 cells per 100-mm dish, 24 hr. aphidicolin) were treated with MNNG (3, 4 and 5uM) at 1.5-2.5 hours into the onset of the S phase (solid circles) and then replated at cloning densities, the survival was found to be no different from that of asynchronous cells.

I. Effect of Holding Cells at the G_1/S Border Post-treatment on the Mutagenicity and Cytotoxicity of MNNG

Liquid holding or recovery experiments are designed to measure the repair of DNA lesions that occur when the cells are held in a non-dividing state. Otherwise, if the cells are dividing, the lesion can become fixed in the S phase (Konze-Thomas et al., 1982; Yang et al., 1982). With the inhibitor of DNA synthesis, aphidicolin, it is possible to hold cells in a non-replicating state. If cells in aphidicolin are treated with MNNG and held at the G_1/S border to allow lesions to be repaired before fixation, then the mutation frequency should decrease because of repair of the O^6-MeG lesion.

1. Measuring the Cytotoxic Effect of Incubation of Cells in Aphidicolin

Before attempting to determine whether holding cells in aphidicolin affected their response to MNNG, experiment were initiated to determine if aphidicolin itself was cytotoxic.

Cells (0.5 x 10^6 per 100-mm dish) were synchronized with aphidicolin and held in aphidicolin for an additional 0, 8, 16 or 24 hours before they were seeded at cloning densities. The results in Figure 13 clearly demonstrate that holding cells in aphidicolin medium decreases the cloning efficiency of the cells at 0 uM MNNG. The cloning efficiencies were 0.80 (100%), 0.76 (84%), 0.63 (79%) and 0.48 (61%) for cells held in aphidicolin medium for 0, 8, 16 and 24 hours.

2. Measuring the Effect of Holding Cells in Aphidicolin on Their Response to MNNG

To determine whether cells have the ability to remove potentially cytotoxic or mutagenic lesions induced by MNNG, when given time to repair between treatment and entry into S, cells were released from confluence and plated at 1.5 x 10^6 cells per 150-mm dish (mutagenicity) and 0.6 x 10^6 cells per 100-mm dish (cytotoxicity) in medium containing aphidicolin. The cells were then held in aphidicolin medium for 0, 8, 16 or 24 hours after treatment with MNNG (0, 3, 4 or 5 uM). Cells were held in aphidicolin medium for 0 and 24 hours for the mutagenicity determinations and 0, 8, 16 and 24 hours for the survival determinations. Figure 13 demonstrates that the survival decreases when holding cells in aphidicolin for 16 or 24 hours after treatment with MNNG. The survival data for an 8 hour holding period in aphidicolin medium was not significantly different than the data for 0 hours. These results indicate that holding cells

Figure 13. The cytotoxic effect of holding human cells (SL68) at the G_1/S border in aphidicolin medium and the effect of holding on their cytotoxic response to MNNG. Cells were held in aphidicolin for Ø (●), 8 (◆), 16 (▲), or 24 (■) hours after treatment with several concentrations of MNNG. The cloning efficiency for the control was 80%.

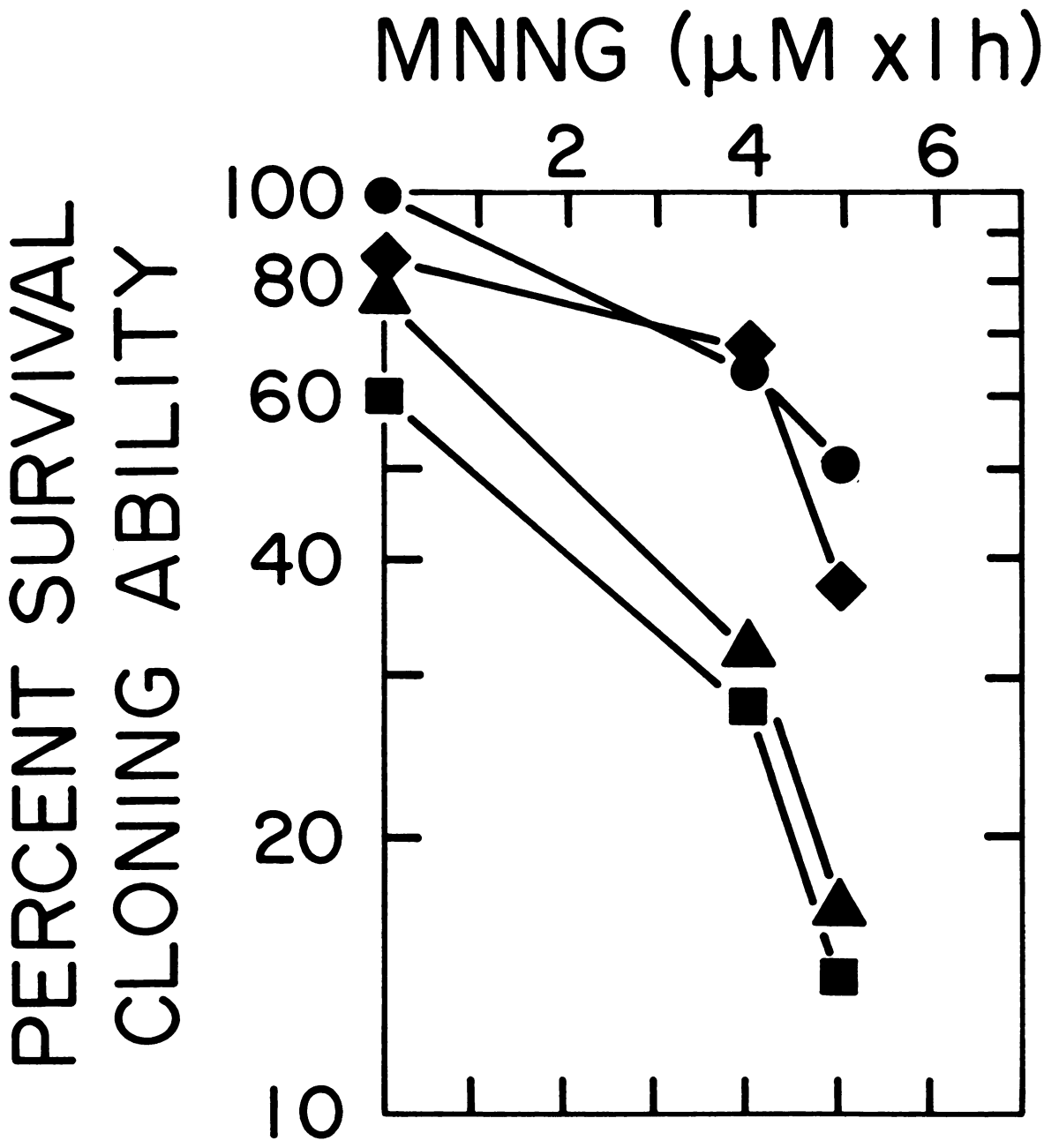
MNNG (μM x1h)
2
4
6
100
80
60
40
20
10
PERCENT SURVIVAL
CLONING ABILITY

Figure 13

in aphidicolin medium for longer than 16 hours is toxic to the cells and also that holding in aphidicolin does not cause any significant change in survival. The mutagenicity data are illustrated in Figure 11. For Ø hours holding in aphidicolin medium, the data are represented by the solid circles (Ø hours to S) and holding for 24 hours is represented by the the open circles (24 hours to S). The induced mutation frequency decreased with holding in aphidicolin medium. This preliminary result indicates that the potentially mutagenic adduct is removed when time is allowed for repair.

V. DISCUSSION

A. O^6-MeG Is a Cytotoxic and a Mutagenic Adduct

Simple alkylating agents, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), can react with all nucleophilic sites in DNA (Singer and Kusmierek, 1982). The lesions O^6-methylguanine, O^2-methylthymine, O^4-methylthymine and O^2-methylcytosine are capable of miscoding *in vitro* and have been proposed as potentially mutagenic (Singer and Kusmierek, 1982). Studies with CHO cells (Heflich *et al.*, 1982) and V79 cells (Newbold *et al.*, 1980) show that O^6-alkylation of guanine in DNA is strongly correlated with mutation induction, but not cell killing. However, in the present comparative study using a series of human fibroblast cell lines, the data suggest that O^6-MeG is a potentially cytotoxic and mutagenic adduct.

1. Correlation Between MT Activity and Resistance of Human Cells to the Mutagenic and Cytotoxic Effects of MNNG

The data in Tables 6 and 7 and Figure 8 indicate that the ability of human cells to remove methyl groups from the O^6 position of guanine is highly correlated with resistance

to both the cytotoxic and mutagenic effect of MNNG, suggesting that O^6-methylguanine is a potentially cytotoxic lesion as well as a potentially mutagenic lesion. The three cell lines virtually devoid of MT activity were extremely sensitive to the cytotoxic effect of MNNG and exhibited mutation induction at doses which are non-mutagenic for cells with measurable levels of MT. A cell line with an intermediate level of MT activity showed intermediate sensitivity to the cytotoxicity and mutagenicity of MNNG. The five cell lines with normal levels of MT showed a distinct shoulder on their survival curves and a corresponding threshold on their mutation induction curves.

However, Waldstein et al. (1982c) reported that cells that were deficient and proficient in the reactivation of MNNG-treated adenovirus (mer^- and mer^+) had the same levels of MT activity. However, these investigators used only a single, relatively low amount of protein in their assay on crude cell extracts. They determined MT activity from the number of radioactive counts transferred to an acid precipitable fraction. It is difficult to detect consistent differences between mer^+ and mer^- cells under these experimental conditions. Furthermore, studies with Chinese hamster cell lines with unlimited lifespans, i.e., CHO cells (Heflich et al., 1982) and V79 cells (Newbold et al., 198Ø) showed that O^6-alkylation of guanine in DNA was strongly correlated with mutation induction, but not with

cytotoxicity. Further work will be needed to see if these differences reflect the species difference in the target cells.

2. MT Is Specific for the Removal of O^6-MeG

Besides O^6-MeG, another miscoding adduct formed in DNA by methylating agents is O^4-methylthymine. Recently, three laboratories have reported preliminary data indicating that E. coli contains a MT protein which acts on O^4-methylthymine (McCarthy et al., 1983; McCarthy et al., 1984; Ahmmed and Laval, 1984). Therefore, in this study it was important to determine whether MT in human cells was specific for the removal of O^6-MeG.

The experiments in this study indicate that the cell extract with the highest activity of MT (XP12BE) was active on methyl groups at the O^6 position of guanine but did not remove methyl groups from O^4-methylthymine, and this indicates that human cell methyltransferase differs from that of E. coli (McCarthy et al., 1983, McCarthy et al., 1984, Ahmmed and Laval, 1984). This argues that O^6-MeG is the principal mutagenic and cytotoxic lesion induced in human cells by MNNG. We did not test the XP12BE cell extract for ability to remove methyl groups from phosphate triesters, i.e., lesions repaired by the MT protein of E. coli (McCarthy et al., 1983).

Recently it has been shown by Dolan et al. (1984) that partially purified preparations of O^6-alkylguanine-DNA

alkyltransferase from rat liver cannot repair O^4-methylthymine in a methylated poly(dT)·poly(dA) substrate. In contrast, Becker and Montesano (1985) found that rat liver MT repairs O^6-MeG as well as O^4-methylthymine residues in DNA. These workers used double-stranded poly [d(A-T)·d(A-T)] which was alkylated with tritiated MNU as their substrate. Recently, Richardson et al. (1985) investigated the accumulation and removal of O^4-methylthymine and O^4-ethylthymine in liver DNA from rats exposed to 1,2-dimethylhydrazine or diethylnitrosoamine using a sensitive radioimmune assay to detect these adducts. Their study showed that rat liver efficiently removes O^4-methylthymine in vivo. The contradiction between Dolan et al. (1984) and these investigators might be due to repair of O^4-methylthymine in rat liver by enzymes other than MT and/or the inability of rat liver MT to act on the poly(dA)·poly(dT) substrate. However, recently Pegg has used a DNA substrate and there still was no removal of methyl from O^4-methylthymine (personal communication).

In addition the persistence of O^4-ethylthymine (Muller and Rajewsky, 1983; Swenberg et al., 1984) and of alkylphosphate triesters (Margison and O'Connor, 1979) in rat tissues under conditions when O^6-alkylguanine is repaired rapidly, suggests that mammalian cell methyltransferases are specific for O^6-alkylguanine.

3. MT Is in Limited Supply and Can, Therefore, Become Depleted by Low Doses

The fact that there was a shoulder on the survival curve and a threshold on the mutation curve for the cells with high levels of MT, but none on the curves of the three cell lines that lack the repair system is further evidence that O^6-methylguanine might be a principal cytotoxic and mutagenic lesion. Such a threshold was also found for normal human fibroblasts by Jacobs and Demars (1978). This result is expected if at low doses of MNNG, MT-proficient cells rapidly remove the methyl groups from the O^6 position of guanine before these can exert their potentially harmful effect, but at higher doses, the number of O^6-MeG lesions far exceeds the number of repair molecules, so that killing and mutation induction occurs. Our biochemical data support this hypothesis.

From the MT activity observed, we estimate that in the human cell fibroblasts that were investigated in this study, the number of molecules of MT per cell, range from less than 1200 for a MT deficient line (XP12ROSV) to 39,000 for a MT proficient cell line (XP12BE). The MT activity we observed was comparable to the work of Harris et al. (1983) and Grafstrom et al. (1984), who observed similar activities in other human cell lines. In lymphoid cell lines, Harris et al. (1983) found that MT proficient cell lines contained as much as 25,000 molecules of MT per cell. Similarly, Grafstrom et al. (1984), reported that epithelial cells and

fibroblasts contained respectively 26,000 and 29,000 molecules of MT per cell.

In keeping with our data, Medcalf and Lawley (1981) showed that at non-toxic or only slightly toxic doses of N-methyl-N-nitrosourea, normal human fibroblasts exhibit rapid removal of O^6-methylguanine (half life of 1 h), but at doses 3- to 4-times higher, removal is extremely slow (half-life > 24 h). A similar rapid rate of removal at low doses of MNNG by a human lymphoblastoid cell line was reported by Sklar and Strauss (1981).

4. The Frequency of MNNG-Induced Mutants Is Higher in Cells Which Are Specifically Depleted of MT Activity

The data in this study suggest that O^6-methylguanine is the principal cytotoxic and mutagenic lesion induced in human cells by MNNG. However, it is always possible that other DNA repair systems are also operating on the MNNG-induced lesions and these systems influenced the results. Therefore, the MT activity in normal cells was specifically decreased by exposure to exogenous O^6-MeG and the effect on cytotoxicity and mutagenicity of MNNG was determined. The results indicated that at low doses, the frequency of TG resistant cells was significantly higher in MT-depleted cells than in the corresponding non-depleted populations. This is the result expected if O^6-MeG is the principal mutagenic lesion. The fact that at high doses of

MNNG (5uM), the differences in the mutagenic response of the two populations (non-depleted and depleted) was less pronounced also supports the hypothesis that O^6-MeG is the principal mutagenic lesion. This is because at a dose of 5uM, even the cells with normal levels of MT activity give evidence that the number of potentially mutagenic lesions induced far exceeds the repair capacity.

Our results in Table 12 indicating that MT activity of cultured human cells can be greatly reduced by exposure to exogenous O^6-MeG substrate in the medium, agree with Dolan et al. (1985) who demonstrated that when HeLa cells are exposed to exogenous O^6-MeG for 8-24 hours, an 80% reduction in MT activity is observed, indicating that MT activity of cultured human cells can be greatly reduced by exposure to exogenous O^6-MeG substrate in the medium. They found, as we did, that increasing the exogenous O^6-MeG concentration above 0.2mM or increasing the exposure time beyond four hours did not further deplete the MT activity in the cells.

Our work indicates that the regeneration in normal human diploid fibroblasts is 24 hours or less. Dolan et al. (1985) also found that the loss of MT activity brought about by exogenous O^6-MeG is reversible on removing the substrate, but at least 48 hours are required for complete restoration of the activity in HeLa cells. These data are in keeping with those of Yarosh and coworkers (1984) who found that when human tumor cell lines are treated with MNNG, MT is depleted, but regeneration of the activity is complete in 48

hours. In contrast, Karran and Williams (1985) recently showed that the restoration of MT activity in human lymphoid cells is complete in 4 hours. These differences in regeneration times are probably due to the different cell lines used. Dolan et al. (1985) also observed that the loss of MT activity is not prevented by the addition of inhibitors of nucleic acid or protein synthesis, suggesting that protein synthesis or the incorporation of O^6-MeG into nucleic acids is not required for the loss of activity.

5. Survival Is Not Affected When MT Activity Is Depleted

Studies comparing the survival of human cell lines constitutively deficient in MT activity with those containing normal levels indicate that O^6-methylguanine is involved in the cytotoxicity of MNNG (Domoradzki et al., 1984; Scudiero et al., 1984; Harris et al., 1983). Yet, our experiments indicate that normal populations with depleted-MT activity were not significantly more sensitive to the killing action of MNNG than non-depleted populations. A possible explanation for this lack of increased sensitivity is that, in contrast to mutation induction by MNNG which is essentially complete within a short period of time following exposure (Figure 11) cell death (loss of ability to form a colony) reflects a process that is not completed until much later and regeneration of MT protein in these human fibroblasts occurred rapidly enough to remove the potentially cytotoxic lesions before their effect was

made permanent ("fixed"). Evidence in support of this hypothesis was obtained by measuring the regeneration time of MT activity in depleted populations. Regeneration was found to be rapid and occurred within 24 hours. Therefore, the lesions were probably removed before survival could be affected more than in the control populations.

6. Aphidicolin Synchrony to Study Repair of O^6-MeG

Aphidicolin, a tetracyclic diterpenoid mycotoxin, specifically inhibits DNA polymerase alpha but not polymerase beta or gamma. The inhibition of polymerase alpha *in vitro* correlates positively with the inhibition of DNA replication *in vivo* (Sugino and Nakayama, 1980). An additional role for polymerase alpha cannot be excluded (Miller and Chinault, 1982; Ciarrocchi *et al.*, 1982; Dresler *et al.*, 1982). However, there is controversy concerning the effect of aphidicolin on DNA repair synthesis (Seki *et al.*, 1982; Giulotto *et al.*, 1981; Pedrali-Noy and Spadari, 1980).

It is possible to synchronize cells using aphidicolin because polymerase alpha, involved in DNA replication, is blocked. We adapted this method to synchronize diploid human fibroblasts and measured the frequency of MNNG-induced mutants (resistance to TG) at the S phase of the cell cycle when mutations are "fixed" and at the G_1/S border when sufficient time has been allowed for DNA repair. In theory, a synchronous population of normal fibroblasts (normal levels of MT) treated with MNNG, just as the gene for TG

resistance is to be replicated, should have virtually no time to repair and the frequency of mutations induced per dose should approximate that observed in a MT deficient strain. When SL68 cells (MT proficient) were treated with MNNG, at the S phase, the frequency of MNNG-induced mutations increased per dose and approximated the frequency seen in a MT deficient strain. Actually, the induced mutation frequency for the MT deficient strain may be two-fold higher than the theoretical yield because mutagenic lesions may well persist in these cells through successive rounds of DNA replication and continue to generate mutations.

When SL68 cells were treated at the G_1/S border and allowed 24 hours to repair their DNA before entering the S phase, the frequency of MNNG-induced mutations per dose decreased to a level below that normally seen with SL68 cells. The mutagenicity results indicate that in cells capable of removing lesions, the time available for repair between treatment and the S phase is the important factor in determining the ultimate mutagenicity of MNNG treatment in human cells.

In contrast to the mutation data in which DNA synthesis appears to be the critical event which converts DNA damage into mutations, our cytotoxicity data indicate that no single cell cycle-related event, such as DNA synthesis, is critical in translating DNA damage into cell death. The cytotoxicity data in cells treated at S or at the G_1/S

border and allowed 24 hours to repair before being allowed to begin cycling are similar. Yet during that time, the potentially mutagenic lesions, presumably O^6-MeG, were removed by the cells. If O^6-MeG is also a principal potentially cytotoxic lesion, these lesions were removed during that period in aphidicolin. The fact that the survival of the cells held at the G_1/S border was not higher than that of cells not held in aphidicolin is consistent with previous findings of Konze-Thomas et al. (1982) and Yang et al. (1982).

It has been suggested by Konze-Thomas et al. (1982) that the amount of time available for repair before cell death is regulated by the cells' need for critical cellular proteins and their respective mRNAs, so that if the DNA template for transcription of these mRNAs is still blocked by lesions at the time the cell has need for them, reproductive death (i.e., inability to form a colony) is the result. Although the kinds of proteins being synthesized by cells that are being held at the G_1/S border undoubtedly differ from those being synthesized by cells in early S, the overall average need for transcription and translation by the two populations may well be comparable. If so, this could explain why the lethal effect of MNNG was comparable in cells treated at the onset of S phase or at the G_1/S border followed by time post-treatment before cell cycling.

When synchronous cells are treated with MNNG, a 300% increase occurs in the induced mutation frequency when

compared to MNNG treated asynchronous cells. In contrast, the difference in induced mutation frequency for UV treated synchronous and asynchronous cells (Konze-Thomas et al.,1982; Maher et al., 1979) is not as dramatic, only 33%. The large difference between MNNG treated synchronous and asynchronous populations can be attributed to several factors. In the asynchronous population only a small fraction of the cells are progressing through the S phase and rapid repair of O^6-MeG is occurring. Therefore, the induced mutation frequency is low. In the synchronous population, the induced mutation frequency is near the theoretical yield probably because the mutations are rapidly put in since the cells are treated when the HPRT gene is being replicated and direct miscoding during replication is very likely taking place. Also in the synchronous population, MNNG doesn't interfere as much with DNA replication as UV does (Grossman, Maher and McCormick; unpublished studies). Therefore, in the MNNG-treated synchronous cells, replication proceeds on the template still containing the O^6-MeG lesions and miscoding can take place. All of the above mentioned factors work to increase the frequency of TG resistant mutants.

B. Sensitivity to MNNG Is Not a Common Feature of FP and GS

Persons with familial polyposis coli and Gardner's syndrome develop multiple polyps of the lower digestive

tract which are predisposed to malignancy. A number of investigators have reported that fibroblasts derived from skin biopsies of such FP and GS patients are abnormal in their response to alkylating agents (Hori et al., 1980; Miyaki et al., 1982; Paterson et al., 1981; Barfknecht and Little, 1982). Because methylating agents have been implicated in colon carcinogenesis (IARC, 1974), we determined whether fibroblasts from FP and GS patients were abnormally sensitive to mutations induced by such carcinogens. A positive result would support the hypothesis that mutations occurring in the colon epithelial cells of these patients at an abnormally high frequency are a contributing factor in the disease. The results of this study indicate that the predisposition to colon cancer of FP and GS patients is not necessarily correlated with an increased sensitivity of their fibroblasts to mutations induced by methylating carcinogens. The study also indicates that lack of MT is not an essential feature of FP and GS.

1. Lack of MT Is Not an Essential Feature of FP and GS

Abnormal increased sensitivity to the mutagenic effects of carcinogens has been shown to be characteristic of skin fibroblasts from XP patients, both classic (Robbins et al., 1974; Maher et al., 1977; Yang et al., 1980; Arlett and Harcourt, 1983) and variant (Maher et al., 1976; Myrh et al., 1979; Patton et al., 1984) and more recently of

fibroblasts from persons with hereditary cutaneous malignant melanoma (Howell et al., 1984). However, the present study shows that this is not necessarily the case for FP or GS patients. Skin fibroblasts from only one of the individuals predisposed to colon cancer (GM3314) showed a low level of MT activity and hypersensitivity to MNNG. The cells of an affected daughter of this individual (GM3948) had a high level of MT activity and showed a normal response to killing by MNNG and a somewhat lower than normal response to mutation induction. Middlestadt et al. (1985) recently demonstrated that cells from five affected individuals in a GS family each have a different level of MT activity, ranging from 10% to 100% of normal. This argues that the lack of methyltransferase in fibroblasts is not an essential feature of Gardner's syndrome or familial polyposis coli. Similarly, the finding that fibroblasts from an apparently normal fetus (GM0011) are deficient in MT and hypersensitive to MNNG suggests that these characteristics are not restricted to cells from persons with a genetically inherited predisposition to cancer.

2. Comparison of Our Cytotoxicity Data with That of Others

Our survival curve data for normal cells agree with that of Paterson et al. (1981) who reported that exposure for 1 hour to 7.3uM MNNG in serum-free medium lowered the colony-forming ability of normal cells to 10% of the

untreated control (D_{10}). In our experiment D_{10} = 8uM. However, in contrast to our results, they reported a D_{10} for GM3948 as 2.8uM and for GM3314 as 2.1uM. Our data indicate a D_{10} of 8uM for GM3948 and extrapolation of our data for GM3314 gives a D_{10} of 0.6uM. Our results with FP cell line GM2355 agree with the MNNG-induced cytotoxicity data recently reported by Barfknecht and Little (1982). However, they reported that GS strain 2938 was more sensitive than normal cells to MNNG. The difference in results might be accounted for by the low cloning efficiencies in their experiments (1-9%).

3. Possible Mechanism for Hereditary Colon Cancer

The underlying mechanism responsible for the increased susceptibility to colon cancer is unknown. However, Lipkin et al. (1983) has provided insight into this process. The cellular proliferative zone in the mucosa of the large intestine of humans is located in approximately the basal (lower) three-fourths of the colonic crypts. Epithelial cells migrate toward the gut lumen and are extruded from the mucosal surface between the crypts. In humans, about 10% to 20% of all cells undergo DNA synthesis in the lower three-fourths of the crypt column. In the upper portion of the crypt, cell proliferation decreases, more cells differentiate and proliferation stops as cells near the surface (Lipkin et al., 1970). In individuals with a hereditary predisposition to colon cancer, such as FP, an

early abnormal characteristic of colonic epithelial cells is cell replication in the upper as well as the lower portions of the crypts. The adenomatous polyps apparently form as a direct result of this abnormal cell replication pattern.

Cell cycle studies in rat epithelial cells (Tong et al., 1980) indicate that the frequency of mutations induced by MNNG is higher in cells treated in early S phase than in early G_1 or in a non-proliferating state. This is supported by the cell cycle studies described in this dissertation. This S-phase sensitivity was also found in human cells exposed to UV radiation (Konze-Thomas et al., 1982) or a reactive metabolite of benzo(a)pyrene (Maher et al., 1976). These data suggest that the colonic epithelial cells of FP patients should be at a greater risk of being mutated by endogenous or exogenous mutagens than the comparable epithelial cells from normal individuals, even if their rate of DNA repair is normal. These mutant cells can expand to form new clones and undergo additional mutagen-induced events. This could account for the increased sensitivity of FP patients to colon cancer, but does not rule out many other explanations.

APPENDIX

APPENDIX

CLONING THE GENE FOR METHYLTRANSFERASE

INTRODUCTION

A direct approach to determine whether O^6-MeG is the DNA adduct responsible for both the mutagenic and cytotoxic response to MNNG would be to transfer the MT repair gene into a methyltransferase deficient cell line and to determine what effect this has on survival and induced mutations after treatment with MNNG.

Although it is known that the response of human cells to mutagens is determined by the proficiency of DNA repair systems, there exists little detailed knowledge of the nature of these systems or the genes that control them. The characterization and isolation of DNA repair genes by recombinant DNA technology is a new approach to the elucidation of repair systems. Rubin *et al.*, (1983) and Westerveld *et al.*, (1984) identified a human DNA repair gene following DNA mediated gene transfer of DNA from human cells proficient in DNA repair into Chinese hamster ovary mutant cells that, like XP cells, are sensitive to a variety of DNA damaging agents and are defective in excision repair. These

investigators co-transfected the repair deficient (sensitive to mitomycin-C) recipient cells (Chinese hamster ovary cells) with human HeLa cell DNA and the plasmid pSV2-GPT containing the dominant ecogpt gene from the bacterium E. coli. After calcium phosphate mediated gene transfer, cells were initially selected for the expression of the ecogpt gene (selectable marker) in growth medium containing mycophenolic acid. Selection of the ecogpt gene provides a means of pre-selecting the fraction of transfection competent recipient cells. The cells were then selected for repair proficiency in medium containing mitomycin-C. Secondary transfections yielded transformants that were proficient in repairing DNA damage, contained the ecogpt gene and contained human DNA sequences.

In a similar study, Westerveld and coworkers (1984) identified a human DNA repair gene that complemented the repair defect in a Chinese hamster ovary mutant cell line. To demonstrate the successful integration of human DNA, both studies made use of the fact that the human genome contains a family of highly repetitive sequences (Alu sequences). They were able to distinguish human DNA against a background of CHO DNA by the hybridization technique of Southern (1975). Westerveld and coworkers (1984) were also able to identify the DNA repair gene. They constructed a cosmid recombinant library in E. coli from the DNA of the repair proficient transformant and screened the resulting library by colony filter hybridization using a ecogpt probe which

was the selectable marker in their system.

We reasoned that using an approach similar to the experiments described above, it should be possible to transfer the MT gene from a MT repair proficient cell to a MT repair deficient cell. In the DNA mediated gene transfer method, the transformants would be co-selected for resistance to an alkylating agent, such as MNNG, and for resistance to neomycin because the selectable marker gene neo would be utilized in a co-transfection.

The cell line XP12ROSV was chosen as the recipient of DNA from a MT-proficient cell line (rat liver cells) because it is deficient in MT, because it has an infinite lifespan, and because it is extremely sensitive to MNNG. A two species system (human, rodent) such as the one used by Rubin et al. (1983) was employed, so that after transfection, it would be possible to distinguish the DNA sequences from the transfected DNA from those of the recipient cells by Southern blot analysis of the DNA (Southern, 1975).

Figure A-1 illustrates the protocol to be used. Rat liver DNA and pSV2-neo DNA (a selectable marker) are co-transfected into XP12ROSV cells by a calcium phosphate ($CaPO_4$) precipitation procedure (Graham and van der Erb, 1973). The cells are then selected for resistance to MNNG and geneticin (which selects for expression of the neo gene) and the resistant colonies further analyzed for MT activity, for the presence of rat DNA sequences, for continued resistance to MNNG and for the frequency of MNNG induced

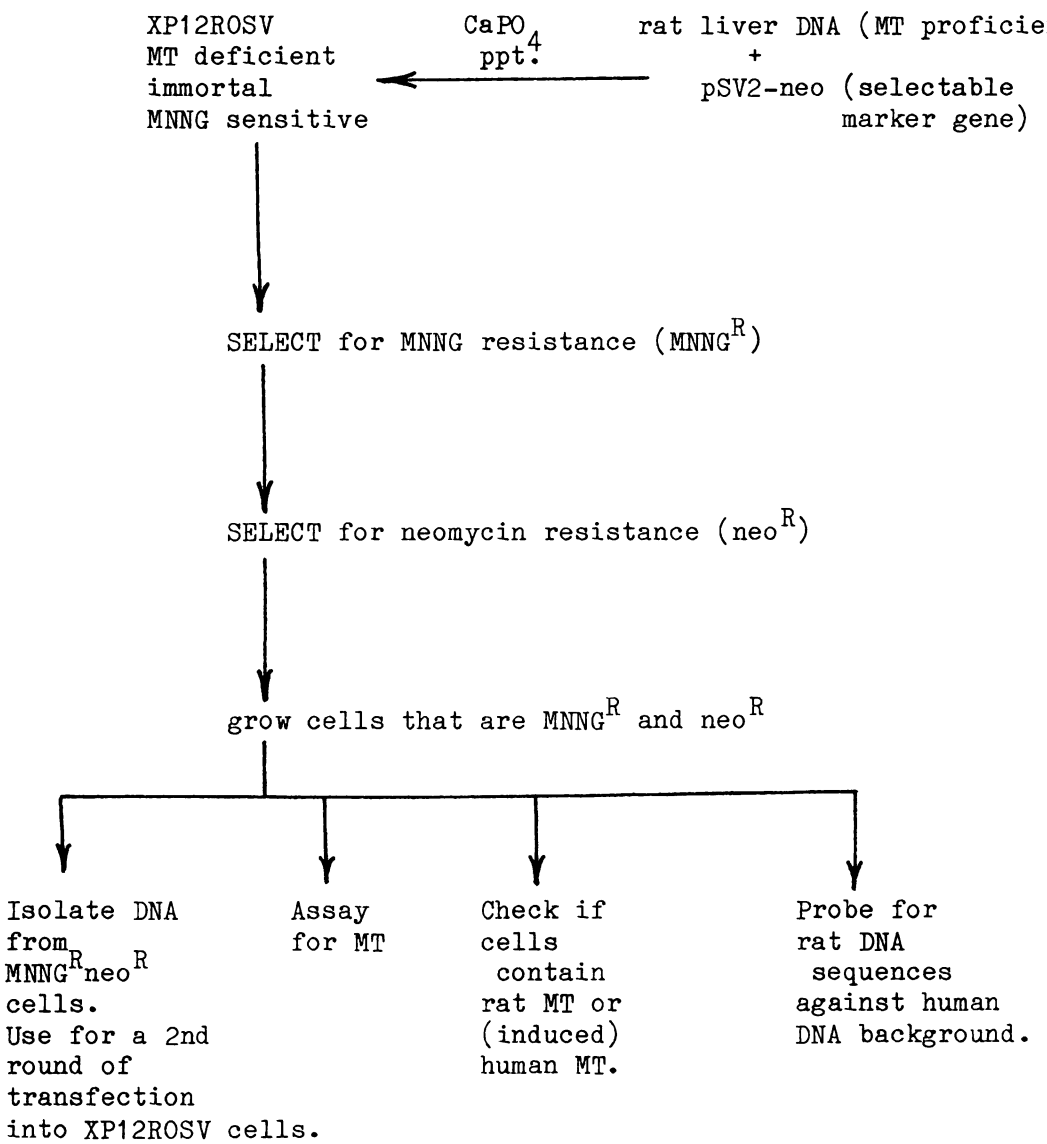

Figure A-1. Scheme of MT Cloning Experiment

mutations. To isolate the gene, a series of secondary transformations need to be performed in order to isolate cells that are free of most nonessential transforming DNA sequences.

MATERIALS AND METHODS

Cells and Culture Media

XP12ROSV cells were used and the source of these cells is given in Section II along with the culture conditions.

Isolation of Rat Liver DNA

Nuclei from rat liver cells were isolated initially and then DNA was isolated from the nuclei. Rat liver was homogenized in a Waring blender using a lysing solution which consisted of 2% Titron X-100, 2mM $CaCl_2$, 2mM $MgSO_4$, 30 mM KCl, 100 mM NaCl and 50 mM Tris-HCl, pH 7.4. The tissue was homogenized until it was microscopically observed that the nuclei were free of the cell debris. The sample wa centrifuged for 5 minutes (30 xg). The pellet was re-suspended in 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 100 mM NaCl. Protease K was added to a final concentration of 100 ug/ml and sodium dodecyl sulfate to a final concentration of 0.5%. The sample was incubated at 50^{o}C for 6-12 hours. The DNA was extracted with Kirby phenol-chloroform solution until the interface was clear. T_1RNase was added at 25

units/ml and incubated at 37°C for 30 minutes. DNA extraction was repeated in Kirby phenol-chloroform. The DNA was ethanol precipitated, pelleted and re-suspended in buffer consisting of 10 ml EDTA, 100 mM Tris-HCl, and 50 mM NaCl.

Isolation of Plasmid pSV2-neo DNA

Plasmid DNA was isolated by an alkaline extraction procedure according to the method of Birnboim and Doly (1979).

DNA Transfection

XP12ROSV monolayers were transfected with rat liver and pSV2-neo plasmid DNA as a co-precipitate by the method of Sutherland and Bennett (1984). Cells were plated into 150-mm diameter tissue culture dishes at a density of 0.8 x 10^6 cells per dish. Twenty-four hours later, the culture medium was removed and the cells were washed twice with serum-free medium. The cells were overlayed with 5 ml of polyethyleneglycol (PEG) solution prepared by adding 20 ml of serum-free medium to 10 g of melted PEG 6000 (Sigma, St. Louis, MO), which was cooled to 37°C before use. After 2 minutes, the PEG solution was removed and the dishes were washed twice with serum free medium. Growth medium was added (30 ml), plus 0.3 ml of a DNA $CaPO_4$ co-precipitate. This co-precipitate was made by mixing plasmid DNA (13 ug/ml) and rat liver DNA (256 ug/ml) in a total of 27 ml

with 0.5 M $CaCl_2$ (27 ml) and 2 x Hepes buffered saline (54 ml; 280 mM NaCl; 10 mM KCl; 1.4 mM Na_2HPO_4; 40 mM Hepes, pH 7.05) at a ratio of 1:1:2. The medium was removed 18 to 24 h later and the cultures were washed twice with serum free medium and refed with fresh growth medium. Seven days following transfection, the cells were harvested and seeded in 100-mm diameter dishes and selected for resistance to MNNG and neomycin.

Selection for Resistance to Geneticin

For selection with Geneticin (Grand Island Biological Co., Grand Island, NY), the cells were seeded at 4 x 10^5 cells per 100-mm diameter dish. After allowing 12 to 18 hours for cell attachment, geneticin, dissolved in Hepes pH 7.4, was added to a final concentration of 200 ug/ml of active compound. The cells were fed with growth medium plus geneticin after one week. At two weeks, the transfection frequency was determined by staining several dishes in 1% crystal violet and counting the number of colonies.

Selection for Resistance to MNNG

Cells were seeded at 4 x 10^5 cells per 100-mm dish and treated with 2 uM MNNG for one hour. If selecting for MNNG resistant and neo-resistant colonies, then following MNNG treatment, the cells were exposed *in situ* to geneticin.

RESULTS

Initially, two DNA-$CaPO_4$ co-precipitation techniques were compared in order to determine which technique would give a higher transfection frequency. Normal cells (SL68) and also MT deficient cells (XP12ROSV) were transfected with different concentrations of the plasmid pSV2-neo by the suspension method of Chu and Sharp (1981) or the monolayer method of Sutherland and Bennett (1984). The results in Table A-1 indicate that for XP12ROSV, the monolayer method gave the highest yield of transfectants; 318 per 10^6 cells selected per 10 ug of pSV2-neo. This frequency is comparable to the one obtained by Spivak et al. (1984).

Co-transfection of XP12ROSV (MT deficient) cells with pSV2-neo and rat liver DNA (MT proficient) by the technique described in the Materials and Methods section of this appendix, resulted in 474 neo resistant colonies per 0.4 x 10^6 cells per dish. Selection in geneticin and MNNG resulted in 53 neo resistant, MNNG resistant clones per 0.4 x 10^6 cells per dish.

A total of 250 dishes were used because the frequency of transformants was expected to be low based on the results of such a transfection protocol by Rubin et al. (1983). The high frequency we obtained could be explained if the XP12ROSV cells were not as sensitive to MNNG as was expected. Extrapolation from the survival data shown in

Table A-1. Comparison of two DNA-$CaPO_4$ co-precipitation techniques[a] in SL68 cells (normal) and XP12ROSV (MT deficient) cells

Cells	Method	No. of cells selected	ug pSV2-neo	No. of cells transfected	total clones
XP12ROSV	suspension[b]	1×10^6	10	1×10^6	38
XP12ROSV	suspension	1×10^6	5	1×10^6	34
XP12ROSV	monolayer[c]	1×10^6	10	1×10^6	318
XP12ROSV	monolayer	1×10^6	5	1×10^6	206
SL68	suspension	1×10^6	10	1×10^6	7
SL68	suspension	1×10^6	5	1×10^6	1
SL68	monolayer	1×10^6	10	1×10^6	4
SL68	monolayer	1×10^6	5	1×10^6	5

[a] Transfection with pSV2-neo.

[b] Chu and Sharp (1981).

[c] Described in Methods and Materials section of this appendix.

Figure 8 of the Results section indicates that at a dose of 2uM of MNNG, only 0.01% of the cells should survive. However, only doses of up to 0.7uM had been tested previously and the latter dose gave 10% survival. Therefore, the survival curve of XP12ROSV was determined at higher doses of MNNG.

Figure A-2 illustrates the survival curve for XP12ROSV at higher doses of MNNG. Instead of an exponential decline, the survival curve showed two components, which is indicative of a mixed population of cells. These results suggested that in the transfection protocol, we had selected for MNNG resistant cells but the latter had not necessarily attained resistance by acquiring the rat MT gene. There was a resistant sub-population of cells in the XP12ROSV cell line even before transfection.

Before trying to repeat the cloning experiment, it was necessary to isolate a pure population of MNNG sensitive cells such that a dose of 2uM would yield a 0.01% survival. Therefore, the XP12ROSV cells were seeded into two 96 well microtiter dishes at a density of 1 cell or less per well and allowed to form colonies. Per microtiter dish, 30 clones were obtained. These clones were replicated and their cells were then tested for resistance to MNNG (2uM) by exposing the developing clones at the 200-1000 cell stage. Several of the clones that appeared to be extra sensitive to MNNG were chosen for further study of their survival after MNNG treatment. The <u>in situ</u> cytotoxicity results of such a

Figure A-2. In situ cytotoxicity of MNNG in the human transformed cell line XP12ROSV and in its derivatives. The cloning efficiencies were 77% for XP12ROSV; 48% for clone B-9; 40% for clone B-21 and 40% for clone A-22.

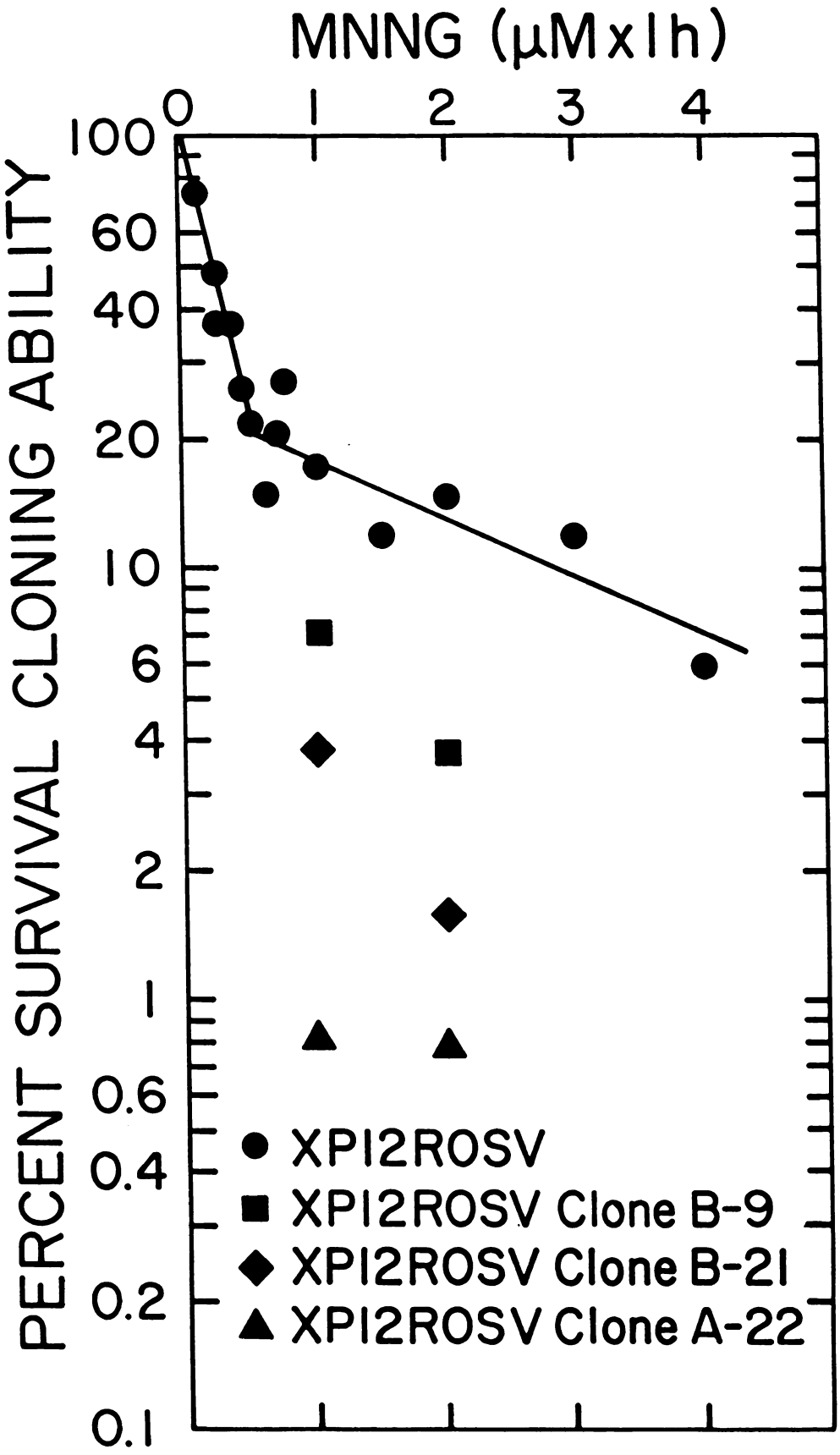
MNNG (μMx1h)
0
1
2
3
4
PERCENT SURVIVAL CLONING ABILITY
100
60
40
20
10
6
4
2
1
0.6
0.4
0.2
0.1
XP12ROSV
XP12ROSV Clone B-9
XP12ROSV Clone B-21
XP12ROSV Clone A-22

Figure A-2

study are shown in Figure A-2. Clone XP12ROSV, A-22, was found to be the most sensitive.

DISCUSSION

Use of the clonal line of XP12ROSV, A-22, should make the cloning experiment outlined above feasible. By the method of Rubin *et al.* (1983), the rat MT gene would be isolated but this gene might serve as a probe for identifying the human MT gene.

An alternative and more recent method of isolating the human MT gene would be to use the human cosmid library of Lau and Kan (1983, 1984). Their system utilizes a cosmid vector that can shuttle cloned sequences between bacterial and mammalian cells. They constructed a complete human cosmid library. DNA from the total library (containing thymidine kinase) was transfected into mouse L cells (thymidine kinase deficient) by the calcium phosphate method. Transformants (thymidine kinase proficient) were then selected for resistance to HAT medium. As the cosmid vector contains the cohesive ends of the bacteriophage lambda, they were able to retrieve human DNA sequences. Total DNA from the transformants was packaged *in vitro* with lysogenic bacterial extracts and used to infect *E. coli*. The sequence of interest (thymidine kinase gene) was then found in one of the resulting cosmids.

Human cells that lack the MT gene could be transfected with cosmid DNA carrying the neo gene. Cells that integrate cosmid DNA would be selected for their ability to grow in geneticin. Cells that have also integrated a MT gene would be selected for their ability to grow in MNNG. Cells from MNNG and neo resistant colonies could then be isolated, propagated and the MT gene rescued from the chromosome by the technique of lambda packaging (Hohn, 1979). Such studies are to be carried out in the near future in the Carcinogeneis laboratory.

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